Maternal Metabolic Health and Female Fertility: a bovine model to study the effect of elevated non-esterified fatty acid concentrations on oocyte and embryo physiology

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Maternal Metabolic Health and Female Fertility:
a bovine model to study the effect of elevated non-esterified fatty acid concentrations on oocyte and embryo physiology

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by

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Laboratory for Veterinary Physiology and Biochemistry
# Table of Contents

List of abbreviations.  

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maternal metabolic health, pathways to subfertility. General Introduction.</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>The impact of elevated non-esterified fatty acid concentrations on female fertility: focus on the early stages of life. Literature Review.</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>Aims of the study.</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology.</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>Oocyte developmental failure in response to elevated non-esterified fatty acid concentrations: mechanistic insights.</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>The interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations.</td>
<td>123</td>
</tr>
<tr>
<td>7</td>
<td>Elevated concentrations of non-esterified fatty acids during bovine embryo culture compromise pre-implantation embryo development and influence gene transcription.</td>
<td>155</td>
</tr>
<tr>
<td>8</td>
<td>General Discussion.</td>
<td>185</td>
</tr>
<tr>
<td>9</td>
<td>Conclusions.</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>Summary.</td>
<td>229</td>
</tr>
<tr>
<td></td>
<td>Samenvatting.</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments - Dankwoord.</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>Curriculum Vitae.</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>Bibliography.</td>
<td>249</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
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<tr>
<td>β-MA</td>
<td>β-Mercaptoacetate</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>COC</td>
<td>Cumulus Oocyte Complex</td>
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<td>CT</td>
<td>Cycle Threshold</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>ECD</td>
<td>Electrochemical Detection</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGA</td>
<td>Embryonic Genome Activation</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
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<td>GSH</td>
<td>Glutathione</td>
<td></td>
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<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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<tr>
<td>HSL</td>
<td>Hormone Sensitive Lipase</td>
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<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
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<tr>
<td>IVF</td>
<td>In Vitro Fertilisation</td>
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<td>IVM</td>
<td>In Vitro Maturation</td>
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<tr>
<td>IVC</td>
<td>In Vitro Culture</td>
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<tr>
<td>JC-1</td>
<td>5,5',6,6'-tetrachloro-1,1',3,3'-tetrachlorobenzimidazolocarbocyanine iodide</td>
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<tr>
<td>LA</td>
<td>Linoleic Acid</td>
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<tr>
<td>LDHA</td>
<td>Lactate Dehydrogenase</td>
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<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
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<tr>
<td>mEGF</td>
<td>Murine Epidermal Growth Factor</td>
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<tr>
<td>n-3</td>
<td>Omega-3</td>
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<tr>
<td>NADPH</td>
<td>Reduced Nicotinamide Adenine Dinucleotide Phosphate</td>
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<tr>
<td>NEB</td>
<td>Negative Energy Balance</td>
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<td>NEFA</td>
<td>Non-Esterified Fatty Acid</td>
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<tr>
<td>NRF2</td>
<td>Nuclear Factor-Like 2</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Oleic Acid</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic Acid</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>p.i.</td>
<td>Post Insemination</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C Pathway</td>
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<td>PUFA</td>
<td>Poly-unsaturated Fatty Acids</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
<td></td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
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<td>REDOX</td>
<td>Reduction-Oxidation</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>SA</td>
<td>Stearic Acid</td>
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</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SOF</td>
<td>Synthetic Oviductal Fluid</td>
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<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
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<tr>
<td>TCM</td>
<td>Tissue Culture Medium</td>
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<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
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<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
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</tbody>
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CHAPTER 1

Maternal metabolic health, pathways to subfertility. General Introduction.

1. “Fatness and flabbiness are to blame. People of such constitution cannot be prolific. The womb is unable to receive the semen and menstruates infrequently and little” (Hippocrates, 410 B.C.).

In our Western world, this historical quote remains highly relevant. The rising incidence of metabolic disturbances highlights effects on reproduction, the biology of which remains partially undefined. A normal ‘metabolic health’ status of the mother is not only a prerequisite to ensure female fertility, but rather a ‘conditio sine qua non’ to safeguard successful ovulation, conception and embryo development. All mediators affecting the metabolic health status of the mother, for example over- and under-nutrition, unbalanced diets, lifestyle factors (such as smoking, alcohol abuse, or sedentary behavior), need to be finely controlled in order to guarantee fertility (Figure 1.1).

Figure 1.1. Major factors influencing the metabolic health status of a mother.
2. The impact of metabolic disorders on female fertility.
The observation that maternal metabolic disorders affect female fertility has been described in human reproductive research. Obesity and type II diabetes in the mother are risk factors for polycystic ovarian syndrome, menstruation problems and anovulation (Hartz et al., 1979; Lake et al., 1997). Furthermore, obese women take longer to conceive, even if cycling regularly (Rich-Edwards et al., 1994; Gesink et al., 2007). Several metabolic disorders compromise the success rates of in vitro fertilisation procedures (Lashen et al., 1999; Wang et al., 2000; Fedorcsak et al., 2001; Koloszar et al., 2002). For example, human clinical centers document on a reduced oocyte developmental competence in vitro and higher abortion rates during early pregnancy in obese patients (Zaadstra et al., 1993; Fedorcsak et al., 2000). In this context, oocytes collected from obese women undergoing assisted reproductive treatments have a significantly lower chance to develop into a top quality embryo in vitro and to establish pregnancy (Pasquali et al., 2003; Metwally et al., 2007).

Also in animals, the link between metabolic disorders and subfertility has been recognized. One obvious example is the metabolic health status of dairy cattle. High producing cows are not as fertile as non-lactating cows due to a conflict between metabolic and reproductive needs (Beam and Butler, 1997; Rhodes et al., 2003; Leroy et al., 2008). Following parturition, nutritional requirements increase rapidly with excessive milk production, resulting in a metabolic status of negative energy balance (NEB). NEB triggers mobilization of adipose tissue and is strongly associated with an increased length of the post-partum anovulatory period (Butler et al., 2005). Up to 50% of modern dairy cows display abnormal oestrous cycles post-partum, with particular reference to reduced oestrous expression or even anoestrus, cyst formation and delayed first ovulation (Opsomer et al., 1998). In addition, disappointing conception rates (Bousquet et al., 2004; Santos et al., 2009) and increasingly high incidences of early embryonic mortality (Dunne et al., 1999; Bilodeau-Goeseels and Kastelic 2003; Mann et al., 2006) are indicative of problems at the oocyte and/or early embryonic level in these metabolically stressed dairy cows.

Not only catabolic conditions impact on fertility in animals, but also ‘over-nutrition’ related metabolic conditions impact on oocyte quality and
embryo development, both in vivo (Mantovani et al., 1993, McEvoy et al., 1995, Negrao et al., 1997) and in vitro (Papadopoulos et al., 2001). For example, maternal obesity in mice results in reduced fertility rates, an effect induced around the period of conception (Igosheva et al., 2010; Bermejo-Alvarez et al., 2012). Type II diabetes, which is another metabolic disorder linked to lipolytic conditions and closely linked with obesity, seems to predispose to subfertility as well. For example, oocytes recovered from diabetic mice tend to be meiotically incompetent (Diamond et al., 1989) and total cell numbers of blastocysts are reduced in diabetic rats (Lea et al., 1996; Pampfer et al., 1997).

Periconceptional data, both available from human and animal studies, emphasize that a distorted ‘maternal metabolic health status’ can influence fertility, even in females that ovulate normally. The role of the maternal metabolic status in relation to reproduction is more complex than once thought. Not only the physiological processes of normal follicular growth and ovulation are under pressure. The increasing incidence of embryonic and fetal losses suggests that the quality of the female gamete at the moment of ovulation, around conception and/or early post-conception, may be jeopardized (Leroy et al., 2012).

Because women are not born with an infinite number of oocytes, the impact of the maternal environment on the ovary is particularly consequential. Negative environmental exposures may affect the developmental competence of the oocyte and/or early embryo. Besides the emotional impact of this subfertility issue in mothers suffering metabolic disorders, there is the economical and public impact as well. The reduced probability of successful spontaneous conception requests intensive and expensive fertility treatments leading to higher costs for the patient and social security. For example, Koning et al. (2010) documented that obesity leads to an additional cost of 1500 euro per artificial treatment. Also in the modern dairy industry, subfertility is threatening the sustainability of the sector. Only an optimal reproductive performance at the herd level guarantees an acceptable ecological foot print of milk production (Garnsworthy, 2004). A worrisome decrease in the reproductive
performance of dairy cows affects all countries benefiting from high yielding dairy herds (for review see: Leroy and de Kruif, 2006). This is a world-wide issue in the modern dairy industry, as fertility influences average daily milk production, the cow’s lifetime milk production, number of calves born per year, the generational interval and ultimately the farmer’s income (Leroy and de Kruif, 2006). In this (economical) context, it is important to consider the dramatic incidence of pregnancy loss in high yielding dairy cows as mentioned above. The majority of the pregnancy disruptions occurs during the first 2 months of pregnancy (Santos et al., 2004; Diskin and Morris, 2008). There is an initial period with a high risk on pregnancy loss between day 14 and 20 after fertilisation when the elongating embryo is secreting interferon-tau for maternal recognition of pregnancy (Thatcher et al., 1995; Berg et al., 2010). A second major period of pregnancy loss occurs between 20 days after fertilization up until day 60 of gestation. This latter period is perhaps more costly because the dam has already invested more time in the pregnancy (Santos et al., 2004; Diskin and Morris, 2008).

3. How do these fertility problems originate in the first place? A quick overview on the mechanisms involved.
Evidence supporting a link between maternal metabolic health and female fertility, with particular focus on oocyte and embryo physiology, may be considered at different levels (overview in Figure 1.2) and will be briefly discussed below.
Figure 1.2. Presentation of the major pathways through which an abnormal metabolic health status of the mother can impact on fertility.
A. Central pathways affecting the hypothalamo-pituitary-ovarian crosstalk.
Maternal metabolic health influences the central mechanisms governing gonadotropin release (Schneider et al., 2004). Cyclic reproductive function is controlled by the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is produced by the hypothalamus, triggering the production of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, which in turn regulate oestradiol and progesterone production in the ovary (shown in Figure 1.2).

For the hormonal control of the terminal stages of folliculogenesis and the determination of ovulation rate, the secretion of FSH is important and controlled primarily by negative feedback at the pituitary level through a synergistic interaction between inhibin and oestradiol (Scaramuzzi et al., 2011). After follicle selection, the biggest follicle able to produce the highest concentrations of oestradiol will become dominant, while other follicles thus become atretic. Acquiring dominance is an interplay between changes in the bio-availability of IGF I and IGF II and the increasing dependence of LH (Roche, 1996). In the absence of elevated progesterone concentrations, the follicle will reach the pre-ovulatory phase and will ovulate after the LH surge (Cupp et al., 1995). Gonadotropins induce the final maturation of the preovulatory follicle, resulting in increased secretion of oestradiol. These increased oestradiol levels, in combination with the relative absence of progesterone, acts on receptors in the brain to trigger the release of LH (Gordon, 2003). The preovulatory LH surge is responsible for a series of events that results in the ovulation of the oocyte that is present in the dominant follicle (Gordon, 2003).

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 cyclicity. In several species, metabolic imbalances, for example as a consequence of under-nutrition, impact on maternal fertility through suppression of GnRH secretion, decreased oestradiol levels and inhibition or delay of the preovulatory LH surge (Boland et al., 2001). It is known that lactating cows, compared with non-lactating heifers, have dominant follicles that are less estrogenic. These follicles require a prolonged growing phase to increase their diameters in
order to trigger an adequate LH pulse frequency and surge (Lopez et al., 2004; Sartori et al., 2004). The fact that NEB cows are hypoglycemic might aggravate this, as low glucose levels inhibit LH pulsatility at the central level in sheep, cattle and humans (Jorritsma et al., 2005). In this context, positive correlations between insulin concentrations and LH pulsatility have been reported (Webb et al., 1999; Lucy, 2003). A more ‘in depth’ description of the specific metabolic mediators influencing the central hypothalamo-pituitary-ovarian axis is provided in the recent review of Scaramuzzi et al. (2011).

### B. Peripheral pathways that influence follicle health.

Metabolic changes in the mother’s blood can directly impact on folliculogenesis, suggesting that specific metabolic-sensing mechanisms exist in the follicle. An ‘in time’ resumption of ovarian activity and cyclicity should lead to the selection and growth of a healthy follicle, enclosing a competent oocyte, and ultimately leading to oestrus and ovulation (Van Soom et al., 2006). The growth and maturation of the preovulatory follicle proceed in distinct phases, including recruitment, selection and dominance. Any inaccuracy in this sequence of events may lead to reproductive failure as presented in Figure 1.2. Such errors may relate to metabolic status. Britt (1992) hypothesized that the health and steroidogenic capacity of a follicle are determined by the specific biochemical environment during the long period of follicular growth prior to ovulation. In cows, this period is calculated to be 3 months. Given this theory, the search continues for signals that may be critical for integrating reproductive and metabolic function (for an overview: Purcell and Moley, 2011; Leroy et al., 2012). For example, high bodyweight and adiposity lead to increased circulating leptin. Leptin inhibits oestradiol secretion and stimulates folliculogenesis (Scaramuzzi et al., 2011). Also insulin and IGF-I have been proposed as important metabolic mediators regulating follicle health (Gutierrez et al., 1997; Mackey et al., 1999; Bossis et al., 2000; Butler et al., 2004). Adequate insulin and IGF-I concentrations are beneficial for follicle growth (Landau et al., 2000; Armstrong et al., 2002), due to dose-dependent stimulatory effects on the proliferation and steroid synthesis of bovine granulosa and theca cells (Spicer and Echternkamp, 1995; Gutierrez et al., 1997). Other obvious molecules
(such as TNFα) have been intensively discussed as metabolic clues linking metabolic disorders and subfertility (Mu et al., 2001; Spicer et al., 2001; Kendall et al., 2004).

C. Peripheral pathways influencing oocyte and early embryo quality.
Besides the well-described impact of metabolic status on the endocrine axis and follicle health, recent reports provide evidence for a reduced oocyte quality as major factor causing subfertility in females suffering metabolic disorders. Why is the oocyte so sensitive? It is widely recognized that the oocyte dictates several critical steps in meiosis, fertilisation and early cleavage of the embryo. Molecular and genetic abnormalities initiated during oogenesis might thereby impact on the subsequent activation of the embryonic genome, manifested later in embryo development. O’Callaghan and Boland (1999) were the first to suggest that subfertility in females suffering metabolic disorders, with particular reference to NEB in dairy cows, is rather a problem of direct metabolic actions on the oocyte and embryonic stages instead of problems only at central and/or follicular level. At present, there is more and more evidence to assume that serum metabolites, reflected in the oocyte’s and embryonic micro-environment (Leroy et al., 2005; Robker et al., 2009; Valckx et al., 2012) can affect the developmental capacity of the oocyte, subsequent development and even embryonic physiology (Figure 1.2). Several ‘candidate’ metabolic factors directly impacting at the oocyte and early embryo level have been already described. One of them is glucose, i.e. an indispensable molecule for proper oocyte maturation and the expansion of the surrounding cumulus investment (Down and Utecht, 1999; Cetica et al., 2002; Bilodeau-Goeseels, 2006; Sutton-McDowall et al., 2010). Addition of glucose to the in vitro maturation medium improves cumulus expansion, nuclear maturation, embryo cleavage and blastocyst development (Krisher and Bavister, 1998; Sutton-McDowall et al., 2004). In this context, NEB cows suffer hypoglycemic conditions that are reflected in the micro-environment of the preovulatory oocyte, which can compromise the oocyte’s developmental capacity (Leroy et al., 2006). In type II diabetes or obese patients, the opposite is true: hyperglycemic insults, reflected in the follicular fluid, may have long-term negative effects on oocyte development through
delayed nuclear maturation (Jungheim et al., 2010; Sutton-McDowall et al., 2010). Also direct toxic effects of elevated ammonia and urea concentrations on oocyte quality can be expected (Sinclair et al., 2000; De Wit et al., 2001; Leroy et al., 2004). Effects of crude protein overfeeding on oocyte and embryo quality have been reviewed before (Leroy et al., 2008). Furthermore, exposure of pre-implantation embryos to dietary induced hyperlipidemic conditions results in a distorted embryo development and reduced embryo quality (Leroy et al., 2010). Interestingly, metabolic conditions to support optimal follicle growth are not necessarily conditions that safeguard proper oocyte and/or embryo quality. Garnsworthy et al. (2009) recommended a post-partum diet in two phases. Follicular growth in the first weeks post-partum is supported by an insulinogenic diet. By contrast, immediately before breeding, cows are given a diet enriched with saturated fat supplements to level off insulin concentrations and to prevent overstimulation of the oocyte.

4. Focus on non-esterified fatty acids as key link between maternal metabolic disorders and fertility issues.

Scientific attention shifted towards elevated concentrations of ‘non-esterified fatty acids’ (NEFAs) as potential metabolic clue linking lipolytic disorders to disappointing fertility outcome. Several research groups from all over the world focused on these NEFAs and designated them as key cytotoxic molecules for several cell types, such as pancreatic β-cells, Leydig cells, monocytes and hepatocytes. The latter reports in somatic cell lines prompted reproductive biologists to believe that NEFAs might also directly impact at the ovarian and oviductal level. This assumption has been substantiated by the finding that, both in human and animals, increased NEFA concentrations in the mother’s blood are reflected in reproductive tract fluids (Leroy et al., 2004; 2005; 2011; Robker et al., 2009; Jungheim et al., 2011; Valckx et al., 2012): the micro-environment in which oocyte maturation, fertilisation and embryo development occur. Kruip and Kemp (1999) were ‘pioneers’ in considering direct toxic effects of high NEFA concentrations at the level of the ovary. Recently, Ribeiro et al. (2011) reported that dairy cows, with elevated serum NEFA concentrations, regularly have a delayed resumption of ovarian cyclicity.
and experience problems to become pregnant from the first insemination compared to non-lactating cows. Pregnancy rates for cows with elevated serum NEFAs are lower compared to cows with physiological serum NEFA levels (Ospina et al., 2010). Also Sinclair et al. (2010) and Garcia-Garcia (2011) pointed to high NEFA concentrations as causative factor for disappointing reproductive performance in dairy cows. However, our understanding of the biological significance and functions of NEFAs at ovarian and oviductal level on early stages of life is very limited. The hypothesis that elevated NEFA concentrations during the time-window of oocyte maturation and embryo culture can also perturb subsequent embryo development and/or physiology, needs to be explored. Impaired oocyte developmental competence may affect the developmental potential and/or phenotype of the embryo, which may distort the process of implantation and result in subsequent embryonic mortality. Studies evaluating potential ‘carry-over’ effects from NEFA-exposed oocytes through further stages of development are absent. Furthermore, information on direct consequences of NEFA exposure during early embryo development is limited, though particularly relevant, when taking into account the high rates of embryonic loss in metabolically compromised females. Data from detailed mechanistic studies are not available so far, though important as the level and nature of NEFAs may be the keys in determining the mechanisms for disappointing reproductive performance in females suffering lipolytic-related metabolic disorders. As such, there is a great need of a valid animal model to investigate the effect of metabolic stress, with particular focus on elevated NEFA concentrations, around the moment of conception. The support for a bovine in vitro reproduction model (oocyte and pre-implantation embryo), complementary to the more traditional rodent model, has steadily grown the last decade as there are substantial similarities between human and bovine ovarian function and oocyte characteristics (Campbell et al., 2003; Adjaye et al., 2007). Bovine and human gametes are remarkably similar with respect to microtubule formation during fertilisation, the timing of genome activation, metabolic requirements, interactions with the culture medium and duration of pre-implantation development (Navara et al., 1995; Anderiesz et al., 2000; Ménézo et al., 2000).
List of References


CHAPTER 2

The impact of elevated non-esterified fatty acid concentrations on female fertility: focus on the early stages of life. Literature Review.

1. Metabolic status versus fertility: focus on elevated NEFAs.
Reproductive performance is increasingly considered as a ‘barometer’ of general well-being of the animal (Walsh et al., 2011). A distorted metabolic health status affects fertility, even in females ovulating normally. Not only ovarian activity and ovulation are under pressure, but also the quality of the female gamete around the moment of ovulation seems to be jeopardized (O’Callaghan and Boland, 1999; Pasquali et al., 2003; Leroy et al., 2008; 2012). When alterations in serum metabolites are reflected in the oocyte’s and embryonic micro-environment (Leroy et al., 2005; Robker et al., 2009; Valckx et al., 2012), these metabolic changes can affect follicle health, oocyte development and even subsequent embryo physiology. The search continues for signals that may be critical for the early developmental stages in life (for an overview: Purcell and Moley, 2011; Leroy et al., 2012). In this context, elevated concentrations of non-esterified fatty acids (NEFAs), mainly break-down metabolites from endogenous lipid stores, might be involved in the reduced fertility rates observed in females suffering lipolysis-related metabolic problems. Kruip and Kemp (1999) were the first suggest that elevated NEFA concentrations might impact directly at the ovarian level. Recently, several studies emphasize the relevance of this assumption. Nevertheless, the need remains to further elucidate the direct impact of high serum NEFA concentrations on follicle cell viability, oocyte development and embryo physiology. As such, in this chapter, we will outline most information available and, by doing this, we will evaluate to what extent elevated serum NEFA concentrations are a potential threat around the period of conception.
2. Fatty acids that are not esterified: what’s in a name?

Non-esterified fatty acids are defined as fatty acids that are not bound to any lipid fractions present in biological fluids (e.g. triglycerides, phospholipids, cholesterol). Based on chemical structure (Figure 2.1), we distinguish saturated and unsaturated fatty acids. For example, palmitic acid (PA, C16:0) and stearic acid (SA, C18:0) are both saturated fatty acids. Unsaturated fatty acids are available under different conditions. There are mono-unsaturated fatty acids, such as oleic acid (OA, C18:1), and poly-unsaturated fatty acids (PUFA).

![Figure 2.1. Molecular structure of fatty acids.](image)

A. NEFAs originating from lipolysis of adipose tissue.

The main source for NEFAs in the blood is the adipose tissue. Hormone sensitive lipase (HSL), the key enzyme regulating lipolysis, will hydrolyse stored triacylglycerols (TAGs) from adipocytes into glycerol and NEFAs (Figure 2.2). Subsequently, in the blood, NEFAs will bind to albumin, a protein that facilitates NEFA transport (Salway, 1999). The activity of the HSL enzyme is regulated by numerous factors and hormones (Figure 2.2): catecholamines stimulate lipolysis through β-adrenergic receptors, which prompts HSL activity in adipocytes, whereas insulin inhibits this process (Gil-Hernandez, 2002). Also testosterone decreases HSL action (Wang et al., 2008). Conversely, oestradiol suppresses adipose growth by stimulating HSL enzyme activity (Homma et al., 2000; Mayes et al., 2004). Furthermore, growth hormone stimulates HSL activity as well and TNFα
promotes lipolysis by decreasing perilipin formation, a protein that forms the protective coat of lipid droplets. A lower amount of perilipin, in the lipid droplet coat, makes the droplets more susceptible to HSL degradation (Moller, 2000; Arner, 2005).

**Figure 2.2.** The major NEFA source: lipolytic actions in the adipose tissue.

Both conditions of ‘overweight’ and ‘losing weight’ are associated with lipolytic actions and such metabolic conditions are recognized to impact on reproductive outcome. How can we explain that the latter metabolic disorders, caused by utterly opposite lifestyles, are both characterized by elevated NEFA concentrations in the blood?

**Losing weight and negative energy balance (NEB).** Lipolysis is preferentially activated over lipogenesis when energy is needed, and this is especially true during exercise and fasting (Albright and Stern, 1998; Boden and Shulman, 2002; Jaworski et al., 2007). During fasting, the net flow of NEFAs is outward from adipocytes into the circulation, where they are bound by albumin to be oxidized as fuel by liver, muscle, and other cells while preserving glucose for utilization by neurons and erythrocytes (Frayn, 2002; Rosen and Spiegelman, 2006). The demand for NEFAs is particularly high during periods of metabolic stress, as for example during high milk production (Rukkwamsuk et al., 1999). This is the result of low insulin concentrations and increased levels of stress associated
catecholamines (for mechanism see Figure 2.2), subsequently up-regulating the degree of lipolysis and down-regulating the rate of re-esterification of NEFAs in the adipose tissue (Vernon, 2002).

**Obesity and insulin resistance.** In obesity, high levels of circulating NEFAs originate from the diet and from insulin-resistant adipocytes undergoing uncontrolled lipolysis. In addition, levels of NEFAs are elevated in obesity partly because of the corresponding increase in TNFα, which stimulates lipolysis (Arner, 2005) (for mechanism: see Figure 2.2). NEFA metabolism in obesity has been the subject of many studies over the last 50 years: there are a number of excellent reviews in this field (Frayn et al., 2003; Bartness et al., 2005; Bohler et al., 2010). Under conditions of increased caloric intake, adipocytes store more TAGs, but as adipocytes grow, their capacity to limit lipolysis in response to insulin drops. A NEFA increase leads to lipid deposition in skeletal muscle and liver and significantly contributes to the induction of insulin resistance (Bergman and Ader, 2000; Borst, 2000; Wajchenberg, 2000). Insulin resistance is a condition in which cells fail to respond to the normal actions of the hormone insulin. In this context, the failure of insulin to suppress HSL, additionally stimulates the release of NEFAs from lipolytic active visceral fat (Aguilera et al., 2008). NEFAs are re-esterified in the liver, leading to steatosis, and the resultant inflammation further increases insulin resistance and hyperinsulinemia (Svedberg et al., 1990; 1991). NEFAs also suppress insulin secretion from pancreatic β-cells (Pankow et al., 2004; Boden, 2008; Lai et al., 2008), which results in the conclusion that elevated serum NEFA concentrations are associated with the development of type II diabetes (Boden, 2008; Lai et al., 2008). A more in depth description of the mechanistic links between elevated NEFA concentration and the onset of insulin resistance will be provided below, e.g. in the section describing NEFA toxicity.

**Other pathological conditions.** Alcohol abuse (Laposata and Lange, 1986) and cold stress (Brustovetsky et al., 1990) are stimuli for up-regulation of lipolysis and thus accompanied by increased serum NEFA concentrations.
B. NEFAs provided by the diet.
While adiposity may contribute to altered serum NEFA concentrations, diet can also be important. After a meal, dietary lipids are transported in lymph to the circulation by chylomicrons, where they acquire a series of proteins that allow for interaction with lipoprotein lipase (LPL), an enzyme regulating fatty acid trafficking (Lafontan, 2008). LPL is stimulated by insulin and hydrolyzes TAGs, forming NEFAs and glycerol (Williams and Larsen, 2003; Arner and Langin, 2007). Whether the source of fatty acids (originating from lipolytic or dietary actions) and form (esterified in cholesteroles, TAGs or phospholipids, or present as NEFA) is important, from a cell biology point of view, remains a matter for debate, but will not be discussed in this thesis. We will predominantly focus on the effects of increased NEFA concentrations originating from up-regulated lipolysis. Apparently, subcutaneous fat is less disposed to lipolytic processes compared to visceral fat (Bohler et al., 2010), which implies that the NEFA profile in the blood of a mother, suffering lipolytic disorders, predominantly mirrors the profile present in the adipocytes located abdominally, mainly containing saturated (PA and SA) and mono-unsaturated (OA) fatty acids (Hostens et al., 2012). As shown in Table 2.1, circulating NEFA concentrations and profiles in the cow during physiological conditions, but also during an episode of up-regulated lipolysis (Meyer et al., 1983), are very similar to NEFA concentrations detected in women with a normal metabolic health, and in women suffering lipolysis-linked disorders (respectively). This offers a unique basis for (prospective) interspecies speculations (Reaven et al., 1998; Valckx et al., 2012).

Table 2.1. Basal and lipolytic NEFA concentrations in bovine and human serum.

<table>
<thead>
<tr>
<th></th>
<th>Bovine serum</th>
<th>Human serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHYSIOLOGICAL</td>
<td>200 μM (Meyer, 1983)</td>
<td>200 - 600 μM (Wolever et al., 1995; Valckx et al., 2012)</td>
</tr>
<tr>
<td>NEFA CONCENTRATIONS</td>
<td>(Meyer, 1983)</td>
<td></td>
</tr>
<tr>
<td>PATHOLOGICAL</td>
<td>400 - 1200 μM (Meyer, 1983)</td>
<td>400 - 2000 μM (Reaven et al., 1988; Valckx et al., 2012)</td>
</tr>
<tr>
<td>NEFA CONCENTRATIONS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFA PROFILE</td>
<td>21% oleic, 17% palmitic, and 27% stearic acid</td>
<td>38% oleic, 24% palmitic, 21% linoleic, and 10% stearic acid</td>
</tr>
<tr>
<td>(under physiological conditions)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. The cytotoxic effects of non-esterified fatty acids.

3.1. Functional role of NEFAs.

NEFAs play important physiological roles as sources of metabolic energy, as substrates for cell membrane biogenesis, and as precursors for many inter- and intracellular signaling molecules such as prostaglandins, leukotrienes, thromboxanes, platelet-activating factors, and others (Stolba et al., 1993; Grimminger et al., 1997; McArthur et al., 1999). In the blood, the majority of NEFAs is associated with albumin, a carrier protein (McArthur et al., 1999), that is known to have both high and low lipophilic binding sites (Chung et al., 1995; Richieri and Kleinfelt, 1995). The quantity of NEFAs bound to albumin is determined by the degree of saturation and the length of the carbon chain of the fatty acids involved (Spector, 1975; Demant et al., 2002; Ulloth et al., 2003). The cell has multiple overlapping mechanisms to assure adequate uptake and directed intracellular movement of NEFAs required for maintenance of physiological functions (McArthur et al., 1999). Weisiger et al. (1981) postulated that albumin is directly involved in the uptake process of the NEFAs. Albumin:NEFA complexes may bind to the cell’s albumin receptor and thereby deliver NEFAs directly to the cell membrane, bypassing the aqueous dissociation of albumin:NEFA to unbound NEFAs (McArthur et al., 1999). Besides this contribution of albumin to the cellular uptake process, albumin serves as a reserve for the serum NEFA pool and even fulfills a protective function (Tabernero et al., 2002). In contrast to extracellular total NEFA concentrations, which may vary as much as 13-fold, intracellular fatty acid levels are maintained relatively constant due to multiple pathways (described in Figure 2.3) whose net effect is to rapidly internalize NEFAs, esterify the fatty acids, and shunt them towards other pathways (McArthur et al., 1999).

3.2. Effect of NEFAs when present in excess.

NEFAs become toxic for several cell types when exceeding a certain level. Elevated NEFA concentrations have been causatively linked with reduced immune cell function, impaired β-cell insulin secretion and hampered insulin sensitivity leading to glucose intolerance both in human and animals (Petersen and Shulman, 2006). For example, NEFA toxicity has been reported in pancreatic β-cells (Shimabukuro et al., 1998), granulosa
cells (Mu et al., 2001; Vanholder et al., 2005), Leydig cells (Lu et al., 2003), nerve growth factor differentiated cells (Ulloth et al., 2003) and hepatocytes (Wu and Cederbaum, 2000). The cytotoxic potential of NEFAs depends on the number of double bonds in the carbon chain (Begin et al., 1988), with saturated NEFAs being more toxic and unsaturated NEFAs being relatively harmless (Cnop et al., 2001; Maedler et al., 2001; Listenberger et al., 2003; Mishra and Simonson, 2005). Nevertheless, knowledge of specific NEFA type actions is limited and more research is needed in this field.

3.3. Mechanistic insights: the actions through which NEFAs can impact.

Several studies, performed in somatic cell lines, assessed mechanisms through which elevated NEFA levels interact at the cellular level. However, the reports are not always univocal and exact mechanisms through which elevated NEFA concentrations impact, are not fully elucidated as multiple signaling pathways seem to be involved. The type of pathways affected seems to be particular to the NEFA profile and quantity, but is also dependent on the species in which the study is performed.

NEFA uptake and intracellular trafficking appear to be highly regulated. Upon entry in the cell, NEFAs are esterified into their active form, acyl-CoA, and then further metabolized. Esterification of the fatty acids by long chain fatty acyl-CoA synthase (ACSLs) is a driving force for NEFA uptake. By converting NEFA to acyl-CoA, the ACSLs effectively trap the fatty acid within the cell (Black and Dirusso, 2003). Fatty acid transport in the cells might be facilitated by plasma membrane proteins, but to retain fatty acids in the cell, they only have to be converted to acyl-CoA or to down-stream metabolites that cannot exit (Weimar et al., 2002; Dirusso and Black, 2004; Li et al., 2010). Long chain acylCoA are substrates for most pathways (Figure 2.3) that use fatty acids for I) energy production, II) ceramide generation, III) complex lipogenic pathways, or IV) for other processes (for example omega-oxidation in the endoplasmic reticulum).
Figure 2.3. Survey of potential pathways through which NEFA can be metabolized in the cell.

**PATHWAY I - Oxidative metabolism.** Data for pancreatic β-cells (Carlsson et al., 1999; Koskin et al., 2003), adipocytes (Furukawa et al., 2004), monocytes (Zhang et al., 2006) and skeletal muscle cells (Bonnard et al., 2008) suggest that mitochondrial metabolism plays a decisive role in the etiology of fatty acid induced metabolic disturbances. The role of mitochondrial metabolism can be brought down to the increased production of reactive oxygen species (ROS). Fatty acids are in principle metabolized by mitochondrial β-oxidation and when their supply is plentiful, there is an up-regulation of mitochondrial activity (Iossa et al., 2002), which results in an elevated ROS production (Burton et al., 2003). Moreover, the mitochondrial genome possesses only limited DNA repair mechanisms compared with nuclear DNA and will thereby be more sensitive to increased ROS concentrations (Wallace, 1987; Tarin, 1995).

**PATHWAY II - Ceramide formation.** NEFA-induced cell death can be mediated via the formation of ceramides (Shimabukuro et al., 1998; Maedler et al., 2001; Lu et al., 2003) as ceramides can be generated from *de novo* synthesis from long-chain fatty acids or by the hydrolysis of the
cell membrane phospholipid and sphingomyelin fractions (Bose et al., 1995). Ceramides serve as second messengers for cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis (Bose et al., 1995).

**PATHWAY III - Lipogenesis.** Stored within cells in lipid droplets, TAG itself is probably harmless (Li et al., 2010), but when TAG is lipolyzed, it expands the availability of fatty acids that can enter toxicity related pathways (for example: pathway I and/or II). Some studies attribute the mechanisms of NEFA toxicity to modifications in cell membrane phospholipids (Calder et al., 1994). NEFAs may exert their effects by affecting membrane stability as saturated fatty acids decrease and unsaturated ones increase membrane fluidity. Indeed, enrichment of phospholipids by saturated fatty acids lowers membrane fluidity, which severely hinders membrane function (MacDonald and MacDonald, 1988).

**PATHWAY IV - Other pathways.** Endoplasmic reticulum stress has been proposed as another pathway involved in NEFA-induced toxicity (Li et al., 2010). Apparently, responses to elevated NEFA concentrations depend on the NEFA type to which cells are exposed. A difference in toxicity has been observed in response to saturated or unsaturated NEFA exposure (Cnop et al., 2001). One remarkable theory, in support of this observation, has been provided by Gordon (1978) saying that the difference is based on the relative high melting point of saturated fatty acids (63°C for PA) compared with unsaturated ones (0.5°C for palmitoleic acid; C16:1 n-7). Consequently, TAGs synthesized from saturated fatty chains are insoluble at 37°C. Therefore, immediately after their formation, saturated TAG molecules may precipitate at the site of synthesis (e.g. the sarcoplasmic reticulum). These precipitations are thought to hamper sarcoplasmic reticulum function (Gordon, 1978).
3.4. Implications of elevated NEFA concentrations in the development of insulin resistance.

Insulin resistance is defined by the requirement of a ‘higher-than-normal’ concentration of insulin to effect a normal response (usually in glucose uptake). Studies show that elevated NEFA concentrations contribute to the onset of insulin resistance (Dimopoulos et al., 2006; Bilan et al., 2009). Elevated NEFA concentrations and intracellular lipid content seem to inhibit insulin signalling, leading to a reduction in insulin-stimulated muscle glucose transport that may be caused by a decrease in glucose transporter, more specifically Glut4, translocation (Bilan et al., 2009).

Although the PA dose (200-1000 µM) and time of incubation (6-24 h) has varied among studies, this saturated NEFA provokes insulin resistance of glucose uptake in muscle cells (Perdomo et al., 2004; Sinha et al., 2004; Pimenta et al., 2008; Hommelberg et al., 2009). The groups of Holland et al. (2007) and Watson et al. (2009) have put forward the hypothesis that PA-induced insulin resistant glucose uptake results from changes in the lipid metabolite ceramide (see Figure 2.3: Pathway II). Importantly, a causal effect of ceramide accumulation on impaired insulin-stimulated muscle cells was demonstrated by over-expression of acid ceramidase to convert ceramide into sphingosine (Chavez et al., 2005). Under these conditions, PA could no longer cause insulin resistance, presumably due to the inability of the cells to accumulate ceramide (Chavez et al., 2005). Another important NEFA is the mono-unsaturated OA, which can cause insulin resistance as well, when applied to muscle cells at elevated levels. However, the cellular mechanisms that PA and OA activate to cause insulin resistance appear to be quite different (Bilan et al., 2009). The mechanisms involve activation of the Protein Kinase C (PKC) pathway (Hajduch et al., 2008), as well as initiation of phosphatases (Stratford et al., 2004). Saturated NEFAs can stimulate de novo diacylglycerol (DAG) synthesis and PKC activity in cultured aortic endothelial cells (Kuroki et al., 1998) and smooth muscle cells (Yu et al., 2001) and subsequently induce increased ROS levels via a PKC-dependent activation of NAD(P)H oxidase (Inoguchi et al., 2000). Thus, elevated NEFA-induced PKC dependent activation of NAD(P)H oxidase may cause the increased ROS production in patients with diabetes or an insulin resistant state (Inoguchi et al., 2003).
NEFAs also directly affect insulin secretion, although the nature of this relationship remains a subject for debate. Type II diabetes arises from insulin resistance, which means the inability of muscle, fat and liver cells to respond normally to insulin, coupled to the inability of pancreatic β-cells to secrete sufficient insulin (Leng et al., 2004; Shulman, 2004; Petersen and Shulman, 2006; Taniguchi et al., 2006). Insulin resistance precedes diabetes, sometimes for several years, while β-cells produce sufficiently high insulin levels to counteract the muscle, fat and liver insulin resistance.


Can we assume that follicles, oocytes and resulting embryos in mothers suffering lipolytic-linked metabolic conditions are affected by the cytotoxic characteristics of elevated NEFA concentrations? To understand the interaction between elevated serum NEFA levels and reproductive function, investigators have mostly relied on animal models of NEB, where energy output exceeds energy input (Jorritsma et al., 2004; Leroy et al., 2005; Vanholder et al., 2005). As dairy cows experience a period of elevated metabolic pressure early post-partum, it is now generally assumed that this metabolic stress can impact on the health of the female gamete. Both the oocyte and the pre-implantation embryo are very sensitive to any perturbation of their micro-environment (review: Leroy et al., 2012). Bovine models revealed that metabolism of the dam can indeed influence quality, gene expression patterns, lipid content and composition of early stage in vivo embryos (Wrenzycki et al., 2000; Leroy et al., 2005). The extensive use of in vitro embryo production in cattle enables large numbers of oocytes and embryos to be collected for research. The results from these bovine models together with human data available from epidemiologic surveys are ideal to expand biological knowledge about the impact of elevated NEFA concentrations on follicle health, oocyte maturation, fertilisation and early embryonic development in these species. While laboratory rodents are particularly useful for some aspects of reproductive research, for example implantation and placentation experiments, they offer relatively few advantages for the
study of ovarian dynamics, oocyte and pre-implantation embryo physiology in humans and larger mammals (Adams and Pierson, 1995; Navara et al., 1995; Anderiesz et al., 2000; Ménézo et al., 2000; Campbell et al., 2003; Adjaye et al., 2007).


5.1. The micro-environment of the growing and maturing oocyte.
Follicular fluid is derived from the blood flowing through capillaries present in the theca folliculi and influenced by follicle cell metabolism (Rodgers and Irving-Rodgers, 2010). Exchange of nutrients occurs between the endothelial cells of the capillary, the basal membrane and granulosa cells of the follicle. One of the best-studied examples of metabolic changes in follicular fluid is the NEB condition in the high-yielding dairy cow (Leroy et al., 2008). In dairy cows, NEFA uptake by the ovary has been described, as well as a strong correlation between the serum NEFA level and the concentration of NEFAs in the follicular fluid (Canfield et al., 1990; Grummer, 1995; Rabiee et al., 1997; Comin et al., 2002). In the study of Leroy et al. (2005), NEFA concentrations and profiles were determined in serum and follicular fluid under physiological conditions and during NEB conditions early post-partum in the dairy cow (Table 2.2). NEFA concentrations in follicular fluid during NEB remained +/- 40% lower compared to serum concentrations. Similar correlations between serum and follicular fatty acid composition have recently been reported in humans (Robker et al., 2009; Valckx et al., 2012).

When studying Table 2.2, it is obvious that the NEFA profile present in the serum differs to that of follicular fluid. When in excess, NEFAs can be, at least in part, allocated to low density lipoproteins (Chung et al., 1995), with particular preference for the saturated NEFA, while the unsaturated will rather bind to albumin. The albumin concentration in bovine follicular fluid is only 7% lower when compared to the serum level (Leroy et al., 2005). However, the LDL fraction does not pass the follicle barrier, which makes that LDL is absent in follicular fluid (Wehrman et al., 1991). This can explain the observed differences when comparing serum (more saturated NEFA fraction) versus follicular fluid (less saturated NEFA.
fraction). Leroy et al. (2005) reported that, under NEB conditions, the NEFA profile of the serum (35% OA, 21% PA, and 25% SA) mirrors the profile of follicular fluid (36% OA, 22% PA, 13% SA) more closely compared to the physiological state. The observation that OA is not preferentially shifted towards follicular fluid compared to the saturated NEFA during NEB conditions, together with the knowledge about ‘saturated versus unsaturated’ NEFA toxicity, might be important. As shown in Table 2.2, similar NEFA profiles and concentrations have been found in serum and follicular fluid of women (when compared to cows): PA, SA, OA, but also linoleic acid (LA; C18:2, n6) have been determined as predominant NEFA types in human follicular fluid (Jungheim et al., 2011; Valckx et al., 2012).

Table 2.2. Bovine and human NEFA concentrations in serum and follicular fluid under pathophysiological conditions.

<table>
<thead>
<tr>
<th>PHYSIOLOGICAL NEFA CONCENTRATIONS</th>
<th>Bovine serum</th>
<th>Bovine follicular fluid</th>
<th>Human serum</th>
<th>Human follicular fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Meyer, 1983) 200 μM</td>
<td>100 – 300 μM (Leroy et al., 2005)</td>
<td>200 – 600 μM (Woliver et al., 1995; Valckx et al., 2012)</td>
<td>300 μM (Valckx et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>PATHOLOGICAL NEFA CONCENTRATIONS</td>
<td>400 – 1200 μM (Meyer, 1983)</td>
<td>200 – 500 μM (Leroy et al., 2005)</td>
<td>400 – 2000 μM (Reaven et al., 1988; Valckx et al., 2012)</td>
<td>300 μM (Valckx et al., 2012)</td>
</tr>
<tr>
<td>NEFA PROFILE (under physiological conditions)</td>
<td>21% oleic, 17% palmitic, and 25% stearic acid (Leroy et al., 2005)</td>
<td>30% oleic, 24% palmitic, and 15% stearic acid (Leroy et al., 2005)</td>
<td>38% oleic, 24% palmitic, 21% linoleic, and 10% stearic acid (Jungheim et al., 2005)</td>
<td>31% oleic, 27% palmitic, 25% linoleic, and 12% stearic acid (Jungheim et al., 2005)</td>
</tr>
</tbody>
</table>

While lipolytic activity in adipose tissue mainly determines serum and follicular fluid NEFA concentrations, diet can also influence the serum NEFA levels (as mentioned above). In this respect, studies document on the fact that dietary induced changes of total fatty acid levels (that means allocated to the different lipid fractions) present in the blood are reflected in the fatty acid composition of the follicular environment (Childs et al., 2008; Fouladi-Nashta et al., 2009). For example, the PUFA content in follicular fluid is highly correlated to that of the diet (Adamiak et al., 2005) and it is generally accepted that alterations in dietary fatty acid intake cause a similar shift in the fatty acid profile of the follicular fluid (Wonnacott et al., 2010; Zachut et al., 2010). However, the ovary can, to some extent, buffer major fluctuations in plasma omega-3 and omega-6 fatty acids (Fouladi-Nashta et al., 2009). Whether dietary
induced changes in serum NEFA levels and profiles are ‘correlated to’ or ‘reflected in’ follicular fluid has not been described so far.

5.2. The micro-environment of the early embryo.

Appropriate determination of the NEFA composition in the oviductal and uterine fluid is almost unmanageable due to technical difficulties in sampling the in vivo embryonic micro-environment. Leese et al. (2008) proposed that the epithelial lining of the endosalpinx and endometrium are the final components that link maternal diet at one end and embryo uptake of nutrients at the other. The concentrations of nutrients in tubal fluid are lower than the plasma nutrient concentrations (Leese and Barton, 1984), which suggests that overall transport across the tube occurs principally by diffusion (Leese and Gray, 1985).

Current research in our laboratory focuses on polarized oviductal cell cultures to identify diffusion and/or active transport pathways through the oviductal cell layer. By doing this, the gatekeeper role that the oviductal cells play, in forming the oviductal fluid, can be studied. It is clear from the work of Childs et al. (2008) that PUFA feeding affects the fatty acid composition of the genital tract. Exchange of nutrients will pass through the endothelial cells of the blood vessel, the basal membrane of the oviduct and the oviductal epithelium. As a result, also saturated NEFAs might be transported from the blood into the oviduct. Preliminary research performed in our laboratory revealed that mean physiological bovine oviductal fluid concentrations of NEFAs (290 µM) and mean bovine serum concentrations of NEFAs (320 µM) were not significantly different (unpublished data).


Folliculogenesis and oogenesis are inextricably linked with the oocyte quality. The oocyte grows and develops in an intimate and mutually dependent relationship with the somatic cells of the follicle (Thibault, 1977). Aberrant metabolic conditions affecting follicular fluid composition and follicle cell viability, can thereby influence the developmental potential of the oocyte and further survival of the embryo (Scaramuzzi et
Many studies demonstrate that an oocyte originating from a compromised follicle, or from a follicle with a deviant growth pattern, will be of inferior quality (Bilodeau-Goeseels and Panich, 2002; Lequarre et al., 2005). The length of follicular dominance influences oocyte and subsequent embryo quality and thus conception rates (Cerri et al., 2009; Santos et al., 2010). Therefore, it is important to consider the consequences of altered intra-follicular NEFA concentrations on follicle cell health. When serum NEFA concentrations increase in the mother, these mainly saturated fatty acids are readily taken up by cells within the ovary (Canfield and Butler, 1990; Rabiee et al., 1997). Thereby, NEFAs may affect follicular growth through direct actions on somatic cell types outlining the follicle (granulosa, theca and cumulus cells). So far, it has been demonstrated that increased NEFA concentrations can directly impair bovine and human granulosa cell viability and steroidogenetic capacity (Table 2.3).

**Table 2.3.** Survey of *in vitro* granulosa cell culture studies focusing on the impact of elevated NEFA concentrations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mu et al., 2001</td>
<td>Arachidonic acid (AA; c20:4n-6) antagonizes saturated NEFA-induced apoptosis in human granulosa cells.</td>
</tr>
<tr>
<td>Mi et al., 2001</td>
<td>Elevated SA and PA concentrations reduce human granulosa cell survival in a time-and-dose-dependent manner.</td>
</tr>
<tr>
<td>Jorritsma et al., 2004</td>
<td>Elevated OA concentrations have a negative effect on the number of bovine granulosa cells and cell growth.</td>
</tr>
<tr>
<td>Vanholder et al., 2005</td>
<td>Elevated PA, SA or OA concentrations stimulate oestradiol production in bovine granulosa cells.</td>
</tr>
<tr>
<td>Vanholder et al., 2005</td>
<td>Elevated PA, SA and OA concentrations jeopardize bovine granulosa cell viability.</td>
</tr>
</tbody>
</table>

Besides reports on granulosa cells, additional studies showed that elevated NEFA exposure also compromises the viability and function of other follicular cell types. For instance, Vanholder et al. (2006) showed that theca cells exposed to a mixture of OA, PA and SA display an inhibited cell proliferation and impaired progesterone production. Furthermore, apoptosis and even cumulus cell necrosis in response to
elevated NEFA concentrations have been described by Leroy et al. (2005). A healthy cumulus investment is indispensable for correct oocyte maturation (Tanghe et al., 2002). Thereby, the detrimental effects of elevated NEFA concentrations on granulosa, theca and cumulus cells might be responsible, at least partly, for ovulatory problems and cystic follicles, reproductive abnormalities frequently observed in cows suffering metabolic disorders (Opsomer et al., 1998). Furthermore, the presence of high NEFA levels in follicular fluid may result in subnormal corpus luteum function after ovulation, causing lower blood progesterone concentrations and a decreased weight of the corpus luteum (Yung et al., 1996). Related to this, NEFAs can destabilize the LH receptor and inhibit LH responsiveness in porcine ovarian granulosa cells (Scsukova et al., 2000).


7.1. The follicular environment is a reflection of the physiological status of the female.

Metabolic changes in the follicular micro-environment are likely to impact on the metabolic activity of the oocyte (for review: Fair, 2010). Studies demonstrate that oocytes originating from a compromised follicle are of inferior quality (Bilodeau-Goeseels and Panich, 2002; Lequarre et al., 2005). During the course of folliculogenesis, oocytes gradually obtain developmental competence, which is a biochemical and molecular state that allows a mature oocyte to undergo normal fertilisation, to support normal pre-implantation embryo development and the subsequent growth of the implanted embryo to term (Hyttel et al., 1997). The oocyte growth phase includes a series of modulations of organelles and inclusions, as well as accumulation of adequate stocks of maternal mRNA, which are necessary for the oocyte to achieve meiotic and developmental competence. Further ultrastructural modifications occur in the oocyte of the dominant follicle and the oocyte attains full developmental competence through a process that may be termed “capacitation” (Hyttel et al., 1997). Final oocyte maturation governs several sequential and critical steps in meiosis, fertilisation and early cleavage (Sirard et al.,
1989; Levesque and Sirard, 1995; Krisher, 2004). Thereby, adverse NEFA conditions within the preovulatory follicle may not only affect the follicle cells, but also the follicle-enclosed oocyte. Alos at oviductal level, there is ample evidence that embryos respond to changes in their environment with altered metabolic and transcriptional activities (Edirisigne et al., 1984; Johnson and Nasr-Esfahani, 1994; Barnett and Bavister, 1996).

7.2. NEFA uptake in oocytes and embryos.

The fatty acid composition of oocytes and embryos in a number of mammalian species has been documented. However, little is known about the uptake of specific fatty acids during these early stages in life, how this may be altered by lipolytic conditions and which consequences this might have for post-fertilisation development and resultant embryo physiology. The lipid profile of the oocyte is dynamic and largely dictated by the micro-environment in which it develops (Ferguson and Leese, 1999; Sata et al., 1999). Spector (1972) showed that cells readily take up fatty acids, phospholipids and TAGs from culture medium containing serum. Kim et al. (2001) demonstrated that fatty acids may be incorporated into the oocyte’s cytoplasm during in vitro maturation. Interestingly, the fatty acid composition of esterified lipids in porcine, cow, and sheep oocytes is dominated by PA, OA, and SA and thus mirrors the composition of NEFAs present in blood and follicular fluid (Homa et al., 1986; McEvoy et al., 2000; Leroy et al., 2005). Furthermore, these fatty acid profiles are similar to those of the adipose tissue from which they are released, suggesting a dynamic fatty acid exchange (Rukkwamsuk et al., 2000). Santos et al. (2008) described the existence of a selective uptake process to ensure that the PUFA content of oocytes is kept to a minimum to minimize risks for degradation. Also Fouladi-Nashta et al. (2009) proposed that the ovary buffers major fluctuations in plasma PUFA. Furthermore, changing the composition of the culture medium changes the relative amounts of fatty acids in neutral and polar lipids of bovine blastocysts (Reis et al., 2003). In this context, embryos are capable of taking up fatty acids from the environment (Hillman and Flynn, 1980; Thompson et al., 1998; Reis et al., 2003).
7.3. NEFAs have a functional role at physiological levels.

Data from animal studies show that, during final maturation, oocytes rely on the oxidative fatty acid metabolism as preferential energy source (Downs et al., 2009; Sturme et al., 2009; Paczkowski et al., 2013). On the contrary, early embryos will rather consume pyruvate and, later on, glucose. Lipids may act as a reservoir for latent energy (Betteridge and Fléchon, 1988), although metabolic studies indicate that ATP requirements of embryos are met by utilization of carbohydrates (Thompson et al., 1996). Interestingly, Khandoker et al. (1998) determined how exogenous fatty acids added to the culture medium are metabolized in the pre-implantation rabbit embryo. Rabbit embryos can oxidize PA to carbon dioxide even at the 1-cell stage, although the rate of fatty acid oxidation was found to increase from the 4-cell stage onwards (Khandoker et al., 1998).

7.4. NEFAs present in excess become toxic.

Using a bovine in vitro maturation model, Leroy et al. (2005) showed that elevated SA and PA concentrations reduce the oocyte’s developmental competence. In this context, Jorritsma et al. (2004) reported that oocyte exposure to elevated OA concentrations delays the progression of meiosis and results in lower successive fertilisation, cleavage, and blastocyst rates. However, the question arises whether elevated NEFA levels during oocyte maturation can induce carry over effects towards the resultant blastocyst physiology. The insight that metabolic changes, even when exclusively applied during the final phase of oocyte maturation, can persuade offspring physiology (Watkins et al., 2008), emphasizes the need to study the physiology of embryos originating from NEFA-exposed oocytes.

At the embryonic level, Quinn and Whittingham (1982) reported that exogenous OA and PA inhibit fertilisation of mouse embryos, but promote blastocyst formation and hatching when added to 8-cell embryos. Nonogaki et al. (1994) showed that elevated PA and PUFA concentrations, including LA, distort embryo development. This could be overcome by addition of antioxidants, which resulted in the speculation that increased oxidative stress might be responsible for the reduced development. We have previously reported that exposure of pre-implantation embryos to
dietary induced hyperlipidemic serum can result in reduced embryo development and quality, hence poorer fertility (Leroy et al., 2010). The mechanistic insights for these findings are lacking. The supplemented sera contained several lipid fractions that were significantly altered in response to the dietary lipid supplements, including doubled cholesterol concentrations, more than doubled total fatty acid concentrations and increased levels of both long chain saturated and unsaturated fatty acids (Leroy et al., 2010). Here, in vitro studies come into play to define and distinguish the potential factors involved. Strangely, in vitro studies evaluating the effect of NEFA exposure during early embryo development are missing. Nevertheless such knowledge becomes extremely important when considering the dramatically increased incidence in embryonic loss in high yielding dairy cows, but also in women suffering lipolytic disorders, such as obesity and type II diabetes.

8. Conclusions.
Fertility in females suffering lipolytic related disorders, such as obesity, type II diabetes and negative energy balance, declines. There is increasing evidence to assume that metabolic changes versus oocyte and embryo quality are major factors in the complex pathogenesis of this reproductive problem.

In this review, we focused on the potential role that elevated NEFA concentrations might play in the onset of subfertility.

✓ Changes in follicular cell function, induced by high NEFA levels, can indirectly affect oocyte development.
✓ The elevated NEFA concentrations are reflected in the microenvironment of the growing and maturing female gamete, and likely result in the ovulation of a developmentally incompetent oocyte.

But there is more. Even after an oocyte is successfully ovulated and fertilized, a full-term pregnancy is still not guaranteed. Bovine oocyte developmental competence is jeopardized in response to elevated NEFA exposure in vitro, but what is happening with the oocytes that do succeed to develop into blastocysts? Is the phenotype of these embryos different compared to non-exposed counterparts? What about
early embryos fulfilling their first steps of development in the oviduct? Do they suffer from elevated NEFA concentrations present in this reproductive tract? These questions remain unanswered, though are relevant. NEFAs might be involved in the low conception rates and the high incidence of early embryonic mortality in females suffering metabolic, lipolytic linked disorders, such as obesity, type II diabetes and negative energy balance. Step by step, one cannot deny the warnings/signals for a potential link between elevated NEFA concentrations and fertility problems in females suffering metabolic disorders. However, our understanding of the biological significance of these elevated serum NEFA concentrations at periconceptional level remains poor/fragmentary.
List of References


Edirisinghe WR, Wales RG, Pike IL (1984) Degradation of biochemical pools labeled with (14C)glucose during culture of 8-cell and morula/early


CHAPTER 3

Aims of the Study.

Suboptimal metabolic health in the mother has been associated with a disappointing reproductive performance both in animals and human settings and there is growing evidence to assume that decreased oocyte and embryo quality are major factors causing this phenomenon. Expertise is available on how metabolic changes can disrupt the hypothalamus-pituitary-ovary axis and thereby interact with reproductive pathways. Only recently, direct consequences of metabolic changes in the blood of the mother on oocytes and early embryos have gained scientific attention.

Several metabolic disorders are characterized by elevated non-esterified fatty acid (NEFA) concentrations. As it is well established that elevated NEFA concentrations are cytotoxic for many cell types, they have also been put forward as causative key between a distorted maternal metabolic health and problems at the oocyte and embryonic level. However, data corroborating this link are scarce. So far, it has been reported that elevated serum NEFA concentrations are reflected in the reproductive tract fluids, the micro-environment in which oocyte maturation, fertilisation and embryo development occur. Furthermore, it has been recognized that such elevated NEFA concentrations can jeopardize oocyte development.

Our main hypothesis is that elevated NEFA concentrations hamper female fertility not only through reduced oocyte development, but also by affecting (resultant) embryo physiology.
More specifically, to investigate the consequences of oocyte maturation or embryo culture under elevated NEFA conditions, the aims of this study were:

- **To expose bovine oocytes to elevated NEFA concentrations during 24 h of *in vitro* maturation in order to:**
  I. evaluate consequences on oocyte development and resultant embryo morphology, physiology and gene transcription.
     ~ **Chapter 4**
  II. describe the mechanisms through which NEFAs exert their effects.
     ~ **Chapter 5**
  III. define the pathways through which the specific NEFA types influence resultant blastocysts by mapping effects on gene expression profiles.
     ~ **Chapter 6**

- **To expose bovine pre-implantation embryos to elevated NEFA concentrations during 6.5 days of *in vitro* culture in order to:**
  IV. evaluate consequences on embryo development and define the pathways through which the specific NEFA types influence resultant blastocysts by mapping effects on gene expression profiles.
     ~ **Chapter 7**
CHAPTER 4

Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology.


Abstract

Elevated concentrations of serum non-esterified fatty acids (NEFAs), associated with maternal disorders such as obesity and type II diabetes, alter the ovarian follicular micro-environment and have been associated with subfertility arising from reduced oocyte developmental competence. We have asked whether elevated NEFA concentrations during oocyte maturation affect the development and physiology of zygotes formed from such oocytes, using the cow as a model. The zygotes were grown to blastocysts, which were evaluated for their quality in terms of cell number, apoptosis, expression of key genes, amino acid turnover and oxidative metabolism. Oocyte maturation under elevated NEFA concentrations resulted in blastocysts with significantly lower cell number, increased apoptotic cell ratio and altered mRNA abundance of DNMT3A, IGF2R and SLC2A1. In addition, the blastocysts displayed reduced oxygen, pyruvate and glucose consumption, up-regulated lactate consumption and higher amino acid metabolism. These data indicate that exposure of maturing oocytes to elevated NEFA concentrations has a negative impact on fertility not only through a reduction in oocyte developmental capacity but through compromised early embryo quality, viability and metabolism.
Introduction
Up-regulated lipolysis, a feature of metabolic disorders such as obesity and type II diabetes, results in increased plasma non-esterified fatty acid (NEFA) concentrations (Jorritsma et al., 2004; Leroy et al., 2005; Van Gaal et al., 2006; Mooradian et al., 2008). It is well established that elevated NEFA concentrations are cytotoxic for several cell types such as Leydig cells (Lu et al., 2003), nerve growth factor differentiated cells (Ulloth et al., 2003) and hepatocytes (Wu and Cederbaum, 2000). Furthermore, exposure of pancreatic β-cells to elevated NEFA concentrations impairs insulin secretion and is considered as an important factor in the pathogenesis of diabetes (McGarry, 2002). Using a bovine model, we have demonstrated that elevated serum NEFA concentrations are reflected in the follicular fluid of the preovulatory ovarian follicle (Leroy et al., 2005) and can directly affect the granulosa cell viability and steroidogenesis (Jorritsma et al., 2004; Vanholder et al., 2005) as well as the developmental capacity of the oocyte (Jorritsma et al., 2004; Leroy et al., 2005; Aardema et al., 2011). In the human, saturated NEFAs, such as palmitic acid (PA) and stearic acid (SA), suppress granulosa cell survival in a time-and-dose-dependent manner (Mu et al., 2001), which might impair fertility. Epidemiological studies have shown that lipolysis-linked maternal metabolic disorders, such as obesity and type II diabetes (Van Gaal et al., 2006; Mooradian et al., 2008), are potential risk factors for reproductive disorders (Van der Steeg et al., 2008; Lash and Armstrong, 2009), which could, at least in part, be related to early pregnancy loss (Fedorcsak et al., 2000; Pasquali et al., 2003). As such, obesity in women not only increases the risk of miscarriage but impairs the outcome of assisted reproductive technologies (Clark et al., 1998; Wittemer et al., 2000; Pasquali et al., 2007). Appropriate oocyte development governs several sequential and critical steps in meiosis, fertilisation and early cleavage (Sirard et al., 1989; Levesque et al., 1995; Krisher, 2004). Furthermore, molecular events during oogenesis impact on the subsequent activation of the embryonic genome, manifested later in embryogenesis (Latham and Sapienza, 1998; Watson et al., 1999; Wrenzycki et al., 2007). However, the extent of the
relationship between these maternal metabolic disorders and subfertility is unclear.
In the present study, we hypothesized that elevated NEFA concentrations are a key metabolic factor in the relationship between maternal metabolic disorders and subfertility, through a negative effect on the oocyte. To address this, we have used a bovine oocyte in vitro culture model to investigate whether elevated NEFA concentrations during bovine oocyte maturation influence subsequent embryo phenotype.
We report that elevated NEFA concentrations, specifically oleic acid (OA), palmitic acid (PA) and stearic acid (SA), during oocyte maturation have negative consequences for the resulting pre-implantation embryo, measured 7 days later at the blastocyst stage. These include altered blastocyst gene expression and reduced embryo quality, determined in terms of energy and amino acid metabolism; known markers of embryo viability. Our data provide evidence of a mechanism for metabolic deregulation appearing in the pre-implantation embryo as a consequence of elevated NEFA concentrations during oocyte maturation and help to explain the higher rate of early pregnancy loss and miscarriage (Pasquali et al., 2003; Metwally et al., 2007) observed in women suffering metabolic disorders.

**Methods**

**Preparation of NEFA treatments**
All chemicals were purchased from Sigma® (Bornem, Belgium), unless otherwise stated. SA, PA and OA were dissolved in a stock solution of pure ethanol at concentrations of 25, 150 and 200 mM, respectively. These ethanol stock solutions were vortex-mixed for 4 min and diluted in working solutions to obtain the desired final concentration in maturation medium. The serum-free maturation medium contained TCM199 supplemented with 0.75% BSA free of fatty acids, 0.4 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/mL Gentamycin and murine epidermal growth factor (mEGF, 20 ng/ml). All treatments were vigorously shaken for 45 min and filter-sterilised under aseptic conditions.
In vitro embryo production

Bovine ovaries were collected from dairy heifers at local slaughterhouses as soon as possible after slaughter, and transported immediately to the laboratory. They were then washed 3 times in warm saline solution (38°C) supplemented with 0.5% kanamycin. Subsequently, follicles with a diameter of 2-6 mm were aspirated. Only unexpanded cumulus oocyte complexes (COCs) surrounded by five or more cumulus cell layers (quality grade I) were matured in vitro as described before (Leroy et al., 2010). Briefly, the COCs were washed three times in Hepes-TALP, a salt solution with 114 mM NaCl, 3.1 mM KCl, 0.3 mM Na₂HPO₄, 2.1 mM CaCl₂.2H₂O, 0.4 mM MgCl₂.6H₂O, 2 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, 1 M Hepes, 2 µL/mL phenol red, 0.4 mg/mL BSA and 50 µg/mL Gentamycin. The COCs were first washed in 500 µL maturation medium and then matured in groups of 50-60 COCs in 500 µL maturation medium in 4-well plates (Nunc®, Langenselbold, Germany) for 22-24 h in humidified air with 5% CO₂ at 38.5°C. After in vitro maturation (IVM), all COCs were coincubated per 100-120 with spermatozoa at a final concentration of 10⁶ sperm cells/ml for 20 h at 38.5 °C in fertilisation medium in a humidified 5% CO₂ incubator. For all experiments, frozen bull semen of proven fertility (Leroy et al., 2010) was thawed and live spermatozoa were selected by centrifugation on a discontinuous Percoll® gradient (90 and 45%, Amersham Biosciences, Roosendaal, The Netherlands). The final sperm-egg ratio was adjusted to 5000:1. Fertilisation medium contained 114 mM NaCl, 3.1 mM KCl, 0.3 mM Na₂HPO₄, 2.1 mM CaCl₂.2H₂O, 0.4 mM MgCl₂.6H₂O, 25 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, 2 µL/mL phenol red, 6 mg/mL BSA, 50 µg/mL Gentamycin and 10 µL/mL heparin. After co-incubation with spermatozoa, the presumptive zygotes were vortexed for 4 min to remove excess sperm and cumulus cells. After 3 wash steps with HEPES-TALP and modified SOF medium, presumptive zygotes were cultured per 25 ± 4 in 50 µl modified SOF medium with mineral oil overlay (modular incubator: 38.5 °C, 5% CO₂, 5% O₂ and 90% N₂) until day 7 post insemination (p.i.). The SOF medium contained 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH₂PO₄, 0.8 mM MgSO₄·7H₂O, 0.6% v/v sodium lactate, 25 mM NaHCO₃, 0.0266 mM phenol red, 0.73 mM sodium pyruvate, 1.78 mM CaCl₂·2H₂O, 0.34 mM trisodium citrate, 2.755 mM myoinositol, 3%
v/v BME 50x, 1% v/v MEM 100x, 0.4 mM glutamine, 5% serum and 50 μg/mL Gentamycin. Embryos originating from oocytes matured in control, HIGH SA and HIGH COMBI maturation medium were labelled as control, HIGH SA and HIGH COMBI embryos, respectively.

**Composition of the oocyte maturation treatments**

The types and concentrations of free fatty acids used in the present study are based on bovine *in vivo* studies (Leroy *et al*., 2005) and are pathophysiologically appropriate, since circulating free fatty acid concentrations in women suffering lipolysis-linked metabolic disorders, including obesity (Stolba *et al*., 1993; Reaven *et al*., 1998), are very similar to NEFA concentrations detected in bovine during an episode of up-regulated lipolysis (Leroy *et al*., 2005). Standard serum-free maturation systems are devoid of fatty acids, although the physiological environment, in which the oocyte matures *in vivo*, contains physiological, basal concentrations of NEFAs (Leroy *et al*., 2005). In order to improve the relevance of our *in vitro* model, we therefore used a maturation medium supplemented with basal NEFA concentrations as control medium.

In preliminary experiments, we identified stearic acid (SA) as the most toxic NEFA for oocyte developmental competence. The effect of elevated palmitic acid (PA) concentrations on developmental competence revealed similar findings as for elevated stearic acid (SA) treatment, though less pronounced, supporting the findings of our previous study (Leroy *et al*., 2005). Elevated oleic acid (OA) treatment had no influence on developmental competence nor on total blastocyst cell number. Only the blastocyst apoptotic cell index was significantly elevated after OA treatment during oocyte maturation.

For additional experiments we therefore focussed on the HIGH SA and the HIGH COMBI treatments. The following NEFA treatments were used in the further study:

1. control = physiological NEFA concentrations (150 μM total NEFA containing 25 μM SA, 50 μM PA and 75 μM OA).
2. HIGH SA = elevated stearic acid concentrations (75 μM SA).
3. HIGH COMBI = combination of elevated NEFA concentrations (425 μM total NEFA, containing 75 μM SA, 150 μM PA and 200 μM OA).
Oocyte developmental competence assessment
Cleavage (2 days p.i.), and blastocyst rates (7 days p.i.) were defined as the number of cleaved zygotes or formed blastocysts per oocyte matured, respectively. The number of blastocysts from cleaved zygotes was also recorded.

Measurement of blastocyst cell number and apoptotic cell index
Cell number and apoptotic cell index (number of apoptotic cells over total cell count) were assessed by staining normal and expanded day 7 blastocysts with propidium iodide (PI) and with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), respectively, as described (Vandaele et al., 2006).

Blastocyst RNA extraction, reverse transcription and quantification of mRNA transcript abundance
Molecular biology procedures were carried out as previously described (Bermejo-Alvarez et al., 2010). Poly(A) RNA was extracted from eight groups of 8 blastocysts per experimental group following the manufacturer’s instructions, using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech) with minor modifications. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer’s instructions (Bioline), using poly(T) primer, random primers and the MMLV reverse transcriptase enzyme, in a total volume of 40 µl, in order to prime the RT reaction and to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. They were then incubated at 42°C for 60 min to allow the reverse transcription of RNA, followed by 70°C for 10 min to denature the RT enzyme. The quantification of all mRNA transcripts was carried out by real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For qRT-PCR, 4 groups of cDNA per experimental group, were used with two repetitions for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone H2AFZ in every sample. PCR was performed by adding a 2 µl aliquot of each sample to the PCR mix, containing the specific primers, to amplify histone H2AFZ (H2AFZ), tumor protein p53
(TP53), Bcl-2-associated X protein (BAX), (Src homology 2 domain containing) transforming protein 1 (SHC1 SHC, also known as P66), placenta-specific 8 (PLAC8), prostaglandin G/H synthase-2 (PTGS2, also known as COX2), DNA (cytosine-5-) methyltransferase 3 alpha (DNMT3A), insulin-like growth factor receptor 2 (IGFR2), solute carrier family 2 (facilitated glucose transporter) member 1 (SLC2A1, also known as GLUT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PD). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 4.1. For quantification, real time PCR was performed as previously described (Bermejo-Alvarez et al., 2010). PCR conditions were optimized to achieve efficiencies close to 1 and then the comparative cycle threshold (CT) method was used to quantify expression levels. Quantification was normalized to the endogenous control (H2AFZ). Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the H2AFZ CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Changes in the relative gene expression of the target were determined using the formula 2-ΔΔCT.
Table 4.1. Details of primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Fragment Size, bp</th>
<th>Gene Bank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2AFZ</td>
<td>AGGAGCAGTACGAGCAGCGGCGTGTG</td>
<td>209</td>
<td>NM_174809.2</td>
</tr>
<tr>
<td>TP53</td>
<td>CCAACACCAAGAATTGACGCTTG</td>
<td>364</td>
<td>NM_174201.2</td>
</tr>
<tr>
<td>BAX</td>
<td>CTCAGTCTCTTGCAATACTTA</td>
<td>158</td>
<td>NM_173894.1</td>
</tr>
<tr>
<td>SHC1</td>
<td>GGAACAGGATAAGGAGGC</td>
<td>335</td>
<td>NM_001075305.1</td>
</tr>
<tr>
<td>PLCB8</td>
<td>CTACCTTGGCCAGCAACACTTG</td>
<td>166</td>
<td>NM_001025325.1</td>
</tr>
<tr>
<td>PTGS2</td>
<td>TTCCAAAGTAGAGAGAGGA</td>
<td>187</td>
<td>NM_174445.2</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>GGTCCGGGACAGGAAGAGATAC</td>
<td>317</td>
<td>XM_867643.3</td>
</tr>
<tr>
<td>IGFB3</td>
<td>GTGAGGGTCTGGGGAGAGGC</td>
<td>201</td>
<td>NM_174352.2</td>
</tr>
<tr>
<td>SCL2A1</td>
<td>CGCTGTCTCCAAGGTTCCTCC</td>
<td>68</td>
<td>NM_174602.2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAGATGCCAGTTCGCGAGTC</td>
<td>247</td>
<td>NM_001034034.1</td>
</tr>
<tr>
<td>G6PD</td>
<td>ATCTACCCGCGCTCATGTTCCT</td>
<td>347</td>
<td>XM_583628.5</td>
</tr>
<tr>
<td>H2AFZ</td>
<td>GGATTAGCCGTCGCTTTGGCA</td>
<td>209</td>
<td>NM_174809.2</td>
</tr>
<tr>
<td>TP53</td>
<td>CTGTTGCTGAAGGACTTGGGGC</td>
<td>364</td>
<td>NM_174201.2</td>
</tr>
<tr>
<td>BAX</td>
<td>CAGAAGAAGGGCGGGGTTCATC</td>
<td>158</td>
<td>NM_173894.1</td>
</tr>
</tbody>
</table>

Amino acid profiling of blastocysts

The amino acid content of spent culture media was determined by reverse-phase HPLC as previously described (Sturmey et al., 2010). Day 7 blastocysts were placed in 4 µl fresh, pre-equilibrated modified SOF medium, overlaid with mineral oil and cultured for 24 h, alongside empty medium control droplet. Both the time of incubation and the stage of the embryo development were recorded. The embryos were then removed and the medium of the spent culture drops was frozen at −80°C until analysis for amino acid content within 21 days. A net fall in amino acid concentration in the spent culture droplet is interpreted as ‘consumption’. A net increase in concentration of an amino acid was interpreted as ‘production’. The sum of consumption and production is used as an overall indicator of amino acid metabolic activity and is defined as ‘turnover’.

Oxygen consumption analysis of blastocysts

Individual blastocysts day 7 p.i. were loaded into a PCR Glass micropipette (Drummond) and allowed to respire for 30 min to form an oxygen gradient. This oxygen gradient was measured in real time using a nanorespirometer (Unisense) and converted to oxygen consumption rate using SensorTrace Pro (Unisense) according to a previous study (Lopes et al., 2005).
**Pyruvate, lactate and glucose consumption**

Day 7 blastocysts were cultured individually for 3 h in 4 µl droplets of modified SOF medium alongside empty control droplets. After the incubation period, the embryos were removed and spent culture droplets frozen at -80°C until analysis. Glucose, lactate and pyruvate utilization was determined by ultrafluorometric assays of spent medium as previously described (Gardner and Leese, 1990).

**Statistical analyses**

All statistical procedures were carried out with SPSS 15.0 (for Windows, Chicago, IL, USA), unless otherwise stated. Cleavage and blastocyst rates were compared between the three treatments using a binary logistic regression model. For the other parameters a mixed model ANOVA, taking treatment as fixed factor and replicate as random factor, was used to compare differences between the three groups. No data transformations were necessary for inequality of variance between groups or for achieving normality for any data with the exception of amino acid metabolism. Chi square tests were used to analyze the effect of blastocyst developmental stage on oxygen, glucose, pyruvate and amino acid profiles. Relative transcript abundance was analyzed by the SigmaStat (Jandel Scientific) software package using one-way ANOVA with multiple pair-wise comparisons using Student-Newman-Keuls method post-hoc.

**Results**

**Validation of physiologically relevant control medium**

We showed that inclusion of physiological concentrations of the key NEFAs in maturation media formulations (3 replicates; 595 oocytes) does not affect developmental competence compared to standard serum-free maturation media (\(P > 0.05\), details in Table 4.2).
Table 4.2. Validation of a physiological relevant control medium.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>standard serum-free maturation medium</th>
<th>physiologically relevant control medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes</td>
<td>291</td>
<td>304</td>
</tr>
<tr>
<td>Cleaved</td>
<td>222 (76.2)*</td>
<td>179 (64.8)*</td>
</tr>
<tr>
<td>Blastocysts from Oocytes matured</td>
<td>88 (30.2)*</td>
<td>89 (29.3)*</td>
</tr>
<tr>
<td>Blastocysts from Cleaved zygotes</td>
<td>88 (39.6)*</td>
<td>89 (49.7)*</td>
</tr>
</tbody>
</table>

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Oocytes (n = 595; 3 replicates) were matured in standard serum-free maturation medium devoid of all fatty acids (Leroy et al., 2005) and in physiologically relevant, NEFA containing maturation medium with physiological, non toxic NEFA concentrations (= control medium used in this study: 150 µM of total NEFA, i.e. OA, SA and PA). Data marked with different superscripts per row are significantly different between treatments (P < 0.05).

**Developmental competence of oocytes matured in the presence of elevated NEFA**

Supplementing maturation medium with different combinations of NEFAs had no significant effect on cleavage rates but based on the odds ratios, maturing oocytes in medium with elevated stearic acid (HIGH SA) or in medium supplemented with a combination of elevated stearic acid (SA), palmitic acid (PA) and oleic aid (OA) concentrations (HIGH COMBI) resulted in a significant reduction in the number of oocytes reaching the blastocyst stage at day 7 post insemination (p.i.) (P < 0.05). The HIGH COMBI treatment also reduced the number of cleaved zygotes reaching the blastocyst stage compared with the control group (P < 0.01). The capacity of cleaved zygotes to become blastocysts tended to be lower in the HIGH SA group (P = 0.06) compared with the control group. The data of the effects of HIGH SA and HIGH COMBI exposure during maturation on developmental competence are presented in Table 4.3.

**Cell number and apoptotic cell index in bovine blastocysts arising from oocytes matured in the presence of elevated NEFA**

Total blastocyst cell number was significantly lower in the HIGH COMBI (104.7 ± 26.1) and the HIGH SA (105.4 ± 24.7) group compared with their
Furthermore, the apoptotic cell index was significantly higher in the HIGH SA embryos (0.18 ± 0.08) compared with the control (0.09 ± 0.05) and the COMBI embryos (0.14 ± 0.12) (P < 0.05). Data are listed in Table 4.3.

Table 4.3. NEFA exposure of bovine oocytes significantly reduced the oocyte developmental competence and the quality of the resultant blastocysts.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>control</th>
<th>HIGH SA</th>
<th>HIGH PA</th>
<th>HIGH OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes</td>
<td>240</td>
<td>255</td>
<td>243</td>
<td>286</td>
</tr>
<tr>
<td>Cleaved</td>
<td>133 (55.4)</td>
<td>126 (52.1)</td>
<td>155 (63.8)</td>
<td>194 (67.8)</td>
</tr>
<tr>
<td>Blastocysts from Oocytes matured</td>
<td>60 [25.0] a</td>
<td>45 (18.6) b</td>
<td>42 (17.3) b</td>
<td>64 (22.4) a</td>
</tr>
<tr>
<td>Blastocysts from cleaved zygotes</td>
<td>60 (45.1) a</td>
<td>45 (35.7) a</td>
<td>42 (27.1) b</td>
<td>64 (33.0) a</td>
</tr>
<tr>
<td>Blastocyst’s cell number ± SD</td>
<td>125.8 ± 29.4 a</td>
<td>105.4 ± 24.7 b</td>
<td>118.5 ± 34.5 a</td>
<td>122.7 ± 23.9 a</td>
</tr>
<tr>
<td>Blastocyst’s apoptotic cell ratio ± SD</td>
<td>0.09 ± 0.05 a</td>
<td>0.18 ± 0.08 b</td>
<td>0.20 ± 0.12 b</td>
<td>0.16 ± 0.08 b</td>
</tr>
</tbody>
</table>

Oocytes (n = 1024; 3 replicates) were matured in maturation medium supplemented with 1) physiological NEFA = control (150 μM of total NEFA, e.g. OA, SA and PA); 2) elevated stearic acid = HIGH SA (75 μM SA); 3) elevated palmitic acid = HIGH PA (150 μM PA) and 4) elevated oleic acid = HIGH OA (200 μM OA). Data marked with different superscripts per row are significantly different between treatments (P < 0.05).
Expression patterns of key genes in bovine blastocysts arising from oocytes matured in the presence of elevated NEFA

We next investigated the expression of key genes representative of cellular processes linked to apoptosis, development, quality and metabolism in the blastocyst. Surprisingly, the expression patterns of many of these genes did not differ between embryos arising from oocytes matured with HIGH SA or the controls (Figure 4.1). However, these data did indicate differences in regulation of DNA methylation and glucose transport, i.e. both DNMT3A and SLC2A1 were up-regulated in blastocysts originating from oocytes matured under HIGH COMBI conditions compared with the control group (P < 0.05). The expression of the IGF2R gene was up-regulated in both groups matured under either HIGH SA or HIGH COMBI conditions, compared to the control group (P < 0.05, Figure 4.1).

**Figure 4.1.** Expression patterns of key genes are altered in bovine blastocysts arising from oocytes matured in the presence of elevated NEFAs. Blastocysts were derived from oocytes matured under control, HIGH SA and HIGH COMBI conditions (n = 192; 5 replicates). Quantification was normalized to the endogenous control H2AFZ. Bars with a and b superscripts are significantly different between treatments with P < 0.05.
Amino acid metabolism of blastocysts originating from NEFA-exposed oocytes

As shown in Figure 4.2A, blastocysts arising from oocytes matured in the presence of all three NEFAs had a significantly elevated amino acid consumption ($P < 0.01$), production ($P < 0.05$) and overall turnover ($P < 0.01$) compared with control embryos. HIGH SA embryos displayed significant higher amino acid consumption ($P < 0.01$) and turnover ($P < 0.05$) compared with control embryos (Figure 4.2A). The profiles of individual amino acids showed that significantly higher levels of serine ($P < 0.01$), histidine ($P < 0.01$) and threonine ($P < 0.05$) were depleted from the culture medium containing blastocysts derived from HIGH SA oocytes compared with control and HIGH COMBI embryos, whereas more valine ($P < 0.05$) was depleted by HIGH COMBI blastocysts compared with control embryos (Figure 4.2B). Glycine concentrations decreased in the medium containing blastocysts from HIGH SA-exposed oocytes, suggesting consumption of glycine, whereas control and HIGH COMBI embryos showed net glycine production ($P < 0.05$). Chi square tests revealed that there were no significant differences in developmental stage among treatment groups. Therefore, developmental stage can be excluded as possible confounder.
**Figure 4.2.** Amino acid metabolism of blastocysts originating from NEFA-exposed oocytes is compromised.

**A.** Amino acid ‘turnover’ of day 7 blastocysts as calculated by summing all amino acids produced and consumed on a per-embryo basis. Comparison was done between blastocysts (n = 135; 3 replicates) derived from oocytes matured under control, HIGH SA and HIGH COMBI conditions.

**B.** Overall mean profiles (± SEM) of individual amino acids by blastocyst in the three treatments. Bars with a and b superscripts are significantly different between treatments with P < 0.05.
Key processes of ATP production in blastocysts arising from oocytes exposed to NEFA

Given the observations that exposing oocytes to elevated NEFA impacted on blastocyst quality, gene expression patterns and amino acid metabolism, we next investigated the oxygen consumption by these embryos. Using a non-invasive real time assay of oxygen consumption, we found that blastocysts arising from oocytes matured under HIGH COMBI conditions consumed significantly less oxygen than control embryos ($P < 0.05$, Figure 4.3A). There was no significant difference in oxygen consumption between the HIGH SA and the control group or between HIGH SA and HIGH COMBI embryos. We also determined the consumption of pyruvate, glucose and lactate by blastocysts originating from oocytes matured in the presence of NEFAs. HIGH COMBI embryos consumed significantly less pyruvate and glucose when compared with control embryos ($P < 0.05$, Figure 4.3B). By contrast, the HIGH COMBI embryos consumed significantly more lactate compared with the control group ($P < 0.05$, Figure 4.3B). There was no significant difference in the level of pyruvate, glucose or lactate consumption between the HIGH SA and the control embryos. Chi square tests revealed that there were no significant differences in developmental stage among treatment groups. Developmental stage can therefore be excluded as a possible confounder.
Discussion
Elevated serum NEFA concentrations, arising from up-regulated lipolysis, have been implicated as a key factor in the association between metabolic imbalances, cellular dysfunction and related pathologies (Wu and Cederbaum, 2000; McGarry, 2002; Lu et al., 2003; Ulloth et al., 2003). In this context, we demonstrate, using a bovine model, that elevated NEFA concentrations during oocyte maturation have a profound negative impact on embryo quality: effects which persist some seven days after removal of NEFA exposure, in terms of blastocyst formation, viability, metabolism and gene transcription.
Support for the bovine as model of early mammalian reproduction, especially of the human, has grown steadily over the last decade. The
human and bovine are single ovulators and there are close similarities between human and bovine ovarian function and oocyte characteristics, in contrast to major differences in ovarian physiology and reproductive function between rodents and humans (Campbell et al., 2003). Bovine and human embryos are remarkably similar with respect to microtubule timing of genome activation, metabolic requirements, interactions with the culture medium and duration of pre-implantation development (Navara et al., 1995; Anderiesz et al., 2000; Ménézo et al., 2000), which makes the cow an excellent model for human reproductive research.

Our data show that elevated NEFA exposure during oocyte maturation does not impair the ability of zygotes to reach the 2-cell stage. However, there is a reduction in the number of the high NEFA-exposed oocytes capable of forming blastocysts. In bovine and human embryos, the developmental processes of the first 3-to-4 cleavage divisions occur under the control of maternally-derived mRNAs and proteins stored in the oocyte, until major embryonic genome activation (EGA) occurs at the 8-16 cell stage. The reduction in blastocysts formed indicates that NEFA-exposure during oocyte development has a significant negative impact on post-genome activated development as well as on the pattern of gene transcription after EGA. Data from recent studies on an obese female mouse model (Minge et al., 2008; Igosheva et al., 2010) lend further support to the hypothesis that elevated NEFA concentrations contribute to the reduced fertility seen in females with compromised metabolic status.

The total cell number in blastocysts from high NEFA-exposed oocytes was significantly reduced and the corresponding apoptotic cell ratio increased, compared with control counterparts. It is generally recognized that saturated NEFAs, such as PA (C16:0) and SA (C18:0), can directly exert negative effects on cell viability (Mu et al., 2001; Lu et al., 2003; Leroy et al., 2005). In the present work, apoptotic cell ratio was significantly higher in blastocysts from oocytes matured in HIGH SA medium compared with those originating from oocytes matured in HIGH COMBI or control medium. One interpretation of these findings is that the unsaturated OA (C18:1), present in the HIGH COMBI mixture, may compensate for the negative effects induced by the saturated NEFAs (PA and SA) in the COMBI mixture. This hypothesis is in line with the recent findings of
Aardema et al. (2011), who recognized that OA prevents detrimental effects of saturated fatty acids. NEFA exposure during oocyte maturation also affected the gene expression pattern of the resulting blastocysts. A significant increase in relative mRNA abundance of the HIGH COMBI embryos was found for the SLC2A1 gene, i.e. a classic indication of cellular stress (Sviderskaya et al., 1996). Furthermore, the DNMT3A gene was significantly up-regulated in HIGH COMBI blastocysts. This gene encodes the de novo DNA methyltransferase whose regulation is essential during pre-implantation development for the proper establishment of epigenetic marks. Among the epigenetic events occurring during pre-implantation development, the establishment of genomic imprinting is crucial for subsequent placentation and embryo development. IGF2R is a well-known maternal imprinted gene whose dysregulation causes perturbed placentation and fetal growth (Killian et al., 2001; Wutz et al., 2001; Young et al., 2001). In this regard, we observed that this gene was significantly up-regulated in HIGH COMBI embryos compared with the other treatments in the current study. No differences were found in the relative mRNA abundance of apoptosis related genes, such as BAX, SHC1 and TP53, which may indicate that the increased apoptotic cell index observed in blastocysts may have originated earlier in development. Moreover, it has been proposed that translational and post-translational regulation of apoptosis related genes in embryos appears to be more important than transcriptional regulation (Vandaele et al., 2008).

In 2002, the ‘quiet embryo hypothesis’ was presented (Leese, 2002), which states that the most viable early embryos exhibit a ‘quieter’ metabolism, well-illustrated with regard to amino acids. This general pattern has been reported for bovine (Sturmey et al., 2010), porcine (Humpherson et al., 2005) and human embryos (Houghton et al., 2002) and has been suggested as a general marker of embryo viability. Furthermore, data on the amino acid metabolism in cleavage stage embryos are predictive of the ability to form a blastocyst (Sturmey et al., 2010), with elevated metabolism indicating reduced embryo competence. We therefore determined the amino acid profiles of blastocysts arising from oocytes matured under the NEFA conditions. Our findings indicated that blastocysts from HIGH SA and HIGH COMBI had increased amino acid
metabolism and were most likely to be less viable than the controls. The observation that in HIGH SA embryos the increased amino acid metabolism was accompanied by an increase in nuclear fragmentation, reflected in the higher apoptotic cell ratio, corroborates previous findings in bovine blastocysts that an elevated metabolism is associated with DNA damage (Sturmey et al., 2009).

It is increasingly recognized that oocyte and embryo metabolism are closely linked with subsequent developmental capacity (Dunning et al., 2010). The consumption of oxygen provides an indicator of global metabolic activity (Leese et al., 2008) and has previously been identified as an important viability indicator for bovine embryos (Sviderskaya et al., 1996). In contrast with the up-regulated amino acid metabolism, we observed a reduced oxidative metabolism in the HIGH SA and HIGH COMBI embryos suggesting that oxygen consumption must be at a certain threshold to sustain appropriate levels of ATP, since chronically low ATP content can be responsible for implantation failure in seemingly normal human embryos (Van Blerkom et al., 1995; Steuerwald et al., 2000). Our data in the bovine are generally in line with the findings of Lopes et al. (2007) that bovine blastocysts capable to giving rise to a pregnancy post-transfer have values for oxygen consumption in the mid-range of the distribution and that a decrease in oxygen consumption is not conducive to embryo viability. Both the reduced oxygen consumption in HIGH SA and HIGH COMBI embryos and the concomitant reduction in pyruvate and glucose consumption in the same embryos (Figure 4.3) strongly suggest a drop in the rate of oxidative phosphorylation. Unusually, all of the blastocysts in the current work removed significant amounts of lactate from the medium, most probably due to the low amounts of glucose in our culture medium only present in the fetal calf serum. Lactate can serve as an alternative energy source and may sustain normal development to the blastocyst stage when glucose availability is low (Pantaleon et al., 1997; Harding et al., 1999).

Surprisingly, HIGH COMBI embryos consumed significantly more lactate compared with control embryos. One explanation for this might be that NEFA exposure results in an imbalance of the intracellular oxidation-reduction (REDOX) potential, as previously reported (Kakinuma and Minikami, 1978; Listenberger et al., 2001). Lactate has been recognized as
a strong cytosolic reductant in mouse oocytes (Lane and Gardner, 2000) and may thus be consumed by the HIGH COMBI embryos in our study to act as REDOX-regulator in the face of metabolic stress. Lactate can be converted by Lactate Dehydrogenase (LDHA) into pyruvate, which may then enter the tricarboxylic acid cycle after conversion to oxaloacetate (Dumollard et al., 2007; Wilding et al., 2009), with the concomitant regeneration of NADH (Pantaleon et al., 1997). Alternatively, we observed elevated alanine production in HIGH COMBI embryos. It is plausible that the excess in lactate-derived pyruvate may be converted preferentially in the cytosol to alanine by alanine aminotransferase (Harvey et al., 2002). Such an alteration in REDOX status may affect the activity of REDOX sensitive transcription factors. For example, the amount of SLC2A1 mRNA and the REDOX state are directly associated (Harding et al., 1999; Wenger and Gassman, 1999). Elevated reactive oxygen species (ROS) concentrations are known to up-regulate SLC2A1 transcription (Sviderskaya et al., 1996), a pattern we have observed in the present study. Despite the increased expression of SLC2A1, also known as the facilitated glucose transporter 1, there was no difference in the glucose consumption by HIGH COMBI embryos. Indeed there was no detectable glucose consumption by embryos in this group. SLC2A1 up-regulation in the absence of glucose uptake could be caused by a failure in the SLC2A1 protein production or impairment in the glucose transport mechanisms. Alternatively, the decreased glucose uptake in HIGH COMBI embryos may be considered in terms of the glucose:fatty acid cycle (Randle, 1998), that, in cardiac cells provision of NEFAs, promotes fatty acid oxidation and inhibits glucose oxidation.

In conclusion, our data show that maternal metabolic conditions, associated with elevated NEFAs during oocyte maturation, may compromise fertility through a reduction in oocyte developmental competence and the viability of the subsequent embryo. In highlighting the metabolic problems associated with obesity and their potential relationship with subfertility, our findings are consistent with public health recommendations which emphasize the importance of women being at healthy weight before starting a pregnancy.
List of References


CHAPTER 5

Oocyte developmental failure in response to elevated non-esterified fatty acid concentrations: mechanistic insights.


Abstract

Elevated plasma non-esterified fatty acid (NEFA) concentrations are associated with negative energy balance (NEB) and metabolic disorders such as obesity and type II diabetes. Such increased plasma NEFA concentrations induce changes in the micro-environment of the ovarian follicle, which can compromise oocyte developmental competence. Exposing oocytes to elevated NEFA concentrations during maturation affects the gene expression and phenotype of the subsequent embryo, notably prompting a disrupted oxidative metabolism. We hypothesized that these changes in the embryo are a consequence of modified energy metabolism in the oocyte. To investigate this, bovine cumulus oocyte complexes were matured under elevated NEFA conditions, and energy metabolism-related gene expression, mitochondrial function and ultrastructure evaluated. It was found that expression of genes related to oxidation-reduction (REDOX) maintenance was modified in high NEFA-exposed oocytes, cumulus cells and resultant blastocysts. Moreover, the expression of genes related to fatty acid synthesis in embryos that developed from NEFA-exposed oocytes, was up-regulated. From a functional perspective, inhibition of fatty acid β-oxidation in maturing oocytes exposed to elevated NEFA concentrations restored developmental competence. There were no clear differences in mitochondrial morphology or oxygen consumption between treatments.
although there was a trend for a higher mitochondrial membrane potential in zygotes derived from NEFA-exposed oocytes. These data show that the degree of mitochondrial fatty acid β-oxidation has a decisive impact on the development of NEFA-exposed oocytes. Furthermore, the gene expression data suggest that resulting embryos adapt through altered metabolic strategies, which might explain the aberrant energy metabolism previously observed in these embryos originating from NEFA exposed maturing oocytes.

Introduction
Elevated plasma NEFA concentrations, arising from up-regulated lipolysis, have been implicated as a key factor in the association between metabolic imbalances, cellular dysfunction and related pathologies such as insulin resistance in mammals (Shimabukuro et al., 1998; Carlsson et al., 1999; Kruszynska et al., 2002; McGarry et al., 2002). Elevated serum NEFA concentrations are also reflected in the bovine and human ovarian follicular micro-environments (Leroy et al., 2004; 2005; Robker et al., 2009; Jungheim et al., 2011) and, as a result, have been associated with reduced oocyte developmental competence (Jorritsma et al., 2004; Leroy et al., 2005; Aardema et al., 2011) and compromised human and bovine granulosa cell viability (Mu et al., 2001; Vanholder et al., 2005). In our recent study (Van Hoeck et al., 2011), it was demonstrated that embryos arising from fertilized, NEFA-exposed oocytes have a significantly lower cell number, increased apoptotic cell index, aberrant transcriptional activities, altered amino acid turnover and compromised oxidative metabolism; all indicators for a lower embryo quality and viability. Although such embryos displayed up-regulated expression of the SLC2A1 glucose transporter, they did not consume more glucose compared to control embryos, an intriguing finding, since this situation is similar to that in insulin resistant somatic cells.

A strong metabolic role for lipid oxidation during oocyte maturation is widely acknowledged (Sturmey et al., 2006; Downs et al., 2009; Dunning et al., 2010). Aardema et al. (2011) showed that the number and size of lipid droplets in oocytes change following exposure to NEFA during in vitro maturation, indicating that bovine oocytes are able to incorporate
and metabolize fatty acids from the external environment. However, the mechanisms through which elevated NEFA concentrations affect oocyte development and subsequent embryo physiology are unclear.

In contrast, the mechanisms underlying NEFA cytotoxicity have been extensively investigated in somatic cells where they involve modifications to cell membrane phospholipids (Calder et al., 1994), increased nitric oxide production (Shimabukuro et al., 1997) and elevated ceramide concentrations leading to apoptosis (Shimabukuro et al., 1998; Maedler et al., 2001; Lu et al., 2003). However, Mu et al. (2001) reported that, in granulosa cells, the negative effects of NEFA were unrelated to such mechanisms.

Data for somatic cells, including pancreatic β-cells (Carlsson et al., 1999; Koskin et al., 2003), adipocytes (Furukawa et al., 2004), monocytes (Zhang et al., 2006) and skeletal muscle cells (Bonnard et al., 2005) suggest that the mitochondrial metabolism plays a decisive role in the etiology of fatty acid-induced metabolic disruption. Fatty acids are principally metabolized by mitochondrial β-oxidation and when their supply is plentiful, there is up-regulation of mitochondrial activity (Lossa et al., 2002), which results in elevated reactive oxygen species (ROS) production (Burton et al., 2003). Moreover, the mitochondrial genome possesses limited DNA repair mechanisms, compared with nuclear DNA (Clayton, 1991) and will potentially be more sensitive to increased ROS concentrations (Wallace, 1987; Tarin, 1995). In oocytes, this might be of particular relevance because mitochondria do not replicate during the early stages of pre-implantation development (Cummins, 2002) and each blastomere must rely on oocyte-inherited mitochondria until the blastocyst stage, when mitochondrial replication begins (Cummins, 1998). Perturbations in mitochondrial function are therefore critical for oocyte development and have previously been related to embryo quality (Van Blerkom et al., 2004).

Building on our previous work which showed that elevated NEFA concentrations during oocyte maturation affect oocyte developmental competence (Leroy et al., 2005) and the phenotype of the resulting embryos (Van Hoeck et al., 2011), we have sought to provide basic insights into the etiological mechanisms and have hypothesized that the negative effects of elevated NEFA exposure during oocyte in vitro
maturation are linked to modifications in energy metabolism. To address this hypothesis, we have used our established bovine oocyte in vitro culture model (Van Hoeck et al., 2011) in which cumulus oocyte complexes (COCs) are exposed during maturation to elevated concentrations of stearic acid (SA), palmitic acid (PA) and oleic acid (OA) to examine whether:

1) exposure to elevated NEFA concentrations alters expression profiles of target genes related to fatty acid metabolism in oocytes, cumulus cells and the resulting 7.5 day old embryos.

2) elevated NEFA exposure affects oocyte mitochondrial distribution and ultrastructure.

3) reduced oocyte developmental potential arising from elevated NEFA exposure can be restored by modulating mitochondrial fatty acid oxidation.

4) mitochondrial membrane polarity and oxygen consumption of zygotes are affected by exposing oocytes to elevated NEFA concentrations.

**Materials and methods**

**Preparation of NEFA treatments**

All chemicals were purchased from Sigma® (Bornem, Belgium), unless otherwise stated. SA, PA and OA were dissolved in a stock solution of pure ethanol at concentrations of 25, 150 and 200 mM, respectively. These ethanol stock solutions were vortex-mixed for 4 min and diluted in working solutions to obtain the desired final concentration in maturation medium. The serum-free maturation medium contained TCM199 supplemented with 0.75% BSA free of fatty acids, 0.4 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/mL Gentamycin and murine epidermal growth factor (mEGF, 20 ng/ml). All solutions were shaken for 45 min and filter-sterilized under aseptic conditions.
**In vitro embryo production**

Bovine ovaeries were collected at local abattoirs as soon as possible after slaughter, and transported immediately to the laboratory. They were washed 3 times in warm saline solution (38°C) supplemented with 0.5% kanamycin sulphate. Follicles with a diameter of 2-6 mm were aspirated. Unexpanded COCs surrounded by five or more cumulus cell layers (quality grade I) were matured in vitro as described by Leroy et al., 2010. The COCs were washed in 500 µL maturation medium and matured in groups of 50-60 COCs in 500 µL maturation medium in 4-well plates (Nunc®, Langenselbold, Germany) for 22-24 h in humidified air with 5% CO₂ at 38.5°C. After in vitro maturation, all COCs were co-incubated in groups of 100-120 with spermatozoa at a final concentration of 10^6 sperm cells/ml for 20 h at 38.5 °C in 500 µl fertilisation medium in a humidified 5% CO₂ incubator. For all experiments, frozen semen from a bull of proven in vitro fertility (Van Hoeck et al., 2011) was thawed and live spermatozoa were selected by centrifugation on a discontinuous Percoll® gradient (90% and 45%, Amersham Biosciences, Roosendaal, The Netherlands). The final sperm-egg ratio was adjusted to 5000:1. Fertilisation medium contained 114 mM NaCl, 3.1 mM KCl, 0.3 mM Na₂HPO₄, 2.1 mM CaCl₂·2H₂O, 0.4 mM MgCl₂·6H₂O, 25 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, 2 µL/mL phenol red, 6 mg/mL BSA, 50 µg/mL Gentamycin and 10 µL/mL heparin. After co-incubation with spermatozoa, the presumptive zygotes were vortexed for 4 min to remove excess sperm and cumulus cells. After 3 wash steps in HEPES-TALP and modified SOF medium, presumptive zygotes were cultured in groups of 25 ± 4 in 50 µl modified SOF medium with mineral oil overlay (modular incubator: 38.5 °C, 5% CO₂, 5% O₂ and 90% N₂) until day 7 post insemination (p.i.). The SOF medium contained 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH₂PO₄, 0.8 mM MgSO₄·7H₂O, 0.6 mM sodium lactate, 25 mM NaHCO₃, 0.0266 mM phenol red, 0.73 mM sodium pyruvate, 1.78 mM CaCl₂·2H₂O, 0.34 mM trisodium citrate, 2.755 mM myoinositol, 3% v/v BME 50x, 1% v/v MEM 100x, 0.4 mM glutamine, 5% fetal bovine serum and 50 µg/mL Gentamycin.
Composition of the oocyte maturation treatments

The types and concentrations of free fatty acids used in the present study are based on bovine in vivo studies in serum and in follicular fluid (Leroy et al., 2005) and are pathophysiologically appropriate, since circulating NEFA concentrations in women suffering lipolysis-linked metabolic disorders, including obesity (Reaven et al., 1988; Stolba et al., 1993), are very similar to NEFA concentrations detected in bovine during an episode of up-regulated lipolysis (Leroy et al., 2005). Standard serum-free maturation systems are devoid of fatty acids, although the physiological environment, in which the oocyte matures in vivo, contains physiological, basal concentrations of NEFA (Leroy et al., 2005). In order to improve the relevance of our in vitro model, we used a maturation medium supplemented with basal NEFA concentrations as control medium. We showed that inclusion of physiological concentrations of the key NEFAs in maturation media formulations (3 replicates; 595 oocytes) does not affect developmental competence compared to standard serum-free maturation media ($P > 0.1$, details in Van Hoeck et al., 2011). Moreover, in previous studies, we identified stearic acid (SA) as the most toxic NEFA for oocyte developmental competence (Leroy et al., 2005; Van Hoeck et al., 2011). We therefore focussed on the following NEFA treatments in the present study:

1) control = physiological NEFA concentrations (150 μM total NEFA containing 25 μM SA, 50 μM PA and 75 μM OA).
2) HIGH SA = elevated stearic acid concentrations (75 μM SA).
3) HIGH COMBI = combination of elevated NEFA concentrations (425 μM total NEFA, containing 75 μM SA, 150 μM PA and 200 μM OA).

RNA extraction, reverse transcription and quantification of mRNA transcript abundance

For gene expression analysis, cumulus cells were separated from the mature oocytes by repeated aspiration with a small-bore glass pipette. Poly(A) RNA of cumulus cells, oocytes and day 7 blastocysts was extracted using a Dynabead mRNA Direct Extraction KIT (Dynal Biotech, Madrid, Spain) according to the manufacturer’s instructions, with minor modifications. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer’s instructions.
(Bioline), using poly(T) primer, random primers and the MMLV reverse transcriptase enzyme, in a total volume of 40 µl, in order to prime the RT reaction and to synthesize cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and the RT reaction was completed with the addition of 100 units of reverse transcriptase. The mixture was incubated at 42°C for 60 min to allow the reverse transcription of RNA, followed by 70°C for 10 min to denature the RT enzyme. The quantification of all mRNA transcripts was carried out by real time quantitative reverse transcription-polymerase chain reaction (qPCR).

For qPCR, 4 groups of cDNA per experimental group were used with 2 replicates for all genes of interest. Experiments were conducted to compare relative levels of each transcript and histone H2AFZ in every sample. The PCR was performed by adding a 2 µl aliquot of each sample to the PCR mix, containing the specific primers, to amplify the genes of interest. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 5.1. For quantification, qPCR was performed as previously described (Bermejo-Alvarez et al., 2010). PCR conditions were optimized to achieve efficiencies close to 1 and the comparative cycle threshold (CT) method used to quantify expression levels as described by Schmittgen and Livak (2008). Quantification was normalized to the endogenous control H2AFZ. The genes chosen have been associated with aspects of embryo energy metabolism: Lactate Dehydrogenase (LDHA), Glyceraldehyde 3-phosphate Dehydrogenase (GADH), Facilitated Glucose Transporter 1 (SL2A1), and Glucose-6-phosphate Dehydrogenase (G6PD); oxidative stress: Manganese Superoxide Dismutase (MNSOD), Glutathione Peroxidase 1 (GPX1); de novo methylation: DNA cytosine-5-methyltransferase 3A (DNMT3A): mitochondrial biogenesis: Transcription Factor A Mitochondria (TFAM); and fatty acid metabolism: Carnitine Palmitoyl Transferase (CPT1), Adipophilin 2 (PLIN2), Acetyl CoA Carboxylase (ACCA), Mitochondrial uncoupling protein 2 (UCP2) and Transcription factor A, mitochondrial (ACSL1), all of which have been reported to play a key role in NEFA toxicity pathways in several cell types. A total of 180 COCs was used to analyze gene expression in cumulus cells and oocytes in 3 independent repeats and 192 blastocysts were used for gene expression...
analysis in 5 independent repeats (samples equally distributed along treatments).

**Table 5.1.** Details of primers used for qRT-PCR.

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**Mitochondrial morphological evaluation, ultrastructure and distribution**

Immediately following maturation, COCs were fixed in 4% formaldehyde for 1-3 h and prepared for transmission electron microscopy according to Abe et al. (1999). COCs were washed in PBS and post-fixed for 1 h with 1% osmium tetroxide in PBS at 0-4°C. Subsequently, the COCs were individually embedded in 1% agar prior to the epoxy resin embedding process. All samples were dehydrated in ascending concentrations of ethanol solutions (50%-100%), substituted in propylene oxide, and embedded in epoxy resin. Ultra-thin sections were prepared using an ultramicrotome. These sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope operated at 60 kV. In total 15 COCs, equally distributed along treatments, were visualized.

**Evaluation of the effect of β-oxidation inhibition**

β-oxidation was inhibited by incubation with β-mercaptoacetate (β-MA), a competitive inhibitor of 3-hydroxyl CoA dehydrogenase, a component of the β-oxidation pathway (Sturmey et al., 2006). During a serum-free maturation period of 24h, bovine COCs were exposed to the following treatments: 1) control, 2) HIGH COMBI, 3) control + 0.1mM β-MA and 4) HIGH COMBI + 0.1mM β-MA. Following IVF, zygotes were cultured in SOF (+ 5% of FCS) medium as described above. Cleavage (2 days p.i.), and
blastocyst rates (7 days p.i.) were defined as the number of cleaved zygotes or blastocysts formed per oocyte matured, respectively. The number of blastocysts from cleaved zygotes was also recorded. A total of 820 COCs, equally allocated over treatments, were used in 5 independent replicates.

**Oxygen consumption analysis**

As the maturing oocyte is surrounded by cumulus cells, it is not relevant to measure respiration of the oocyte alone (Wilding *et al.*, 2009). Oxygen consumption and mitochondrial membrane potential were therefore measured in zygotes after they had been denuded of cumulus oophorus. Individual zygotes day 1 p.i. were loaded into a PCR Glass micropipette (Drummond) and allowed to respire for 30 min to form an oxygen gradient, which was measured in real time using a nanorespirometer (Unisense) and converted to oxygen consumption rate using SensorTrace Pro (Unisense) according to previous studies (Lopes *et al.*, 2005; Van Hoeck *et al.*, 2011). In total, 54 zygotes, equally distributed along treatments, were analyzed on 3 independent occasions.

**Analysis of the ratio of high versus low polarized mitochondrial membranes**

Bovine zygotes were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) following the method of Van Blerkom *et al.* (2002). Briefly, zygotes at day 1 p.i. were washed three times in PBS and incubated in SOF culture medium containing 2 µg/ml JC-1 in a modular incubator (38.5°C, 5% CO₂, 5% O₂ and 90% N₂) for 25 minutes. Subsequently, the zygotes were washed three times and transferred into imaging dishes, protected from light, and immediately evaluated on a PerkinElmer UltraVIEW VoX confocal microscope in the fluorescein isothiocyanate (FITC; bandpass 500-550 nm), rhodamine isothiocyanate (RITC; bandpass 580-650 nm) channels with narrow band filter sets. Images were acquired using Volocity 6.0.1 software and data on pixel intensity in each channel determined with ImageJ, a Java image-processing program, with sample origin blinded to the scorer. In each zygote, the ratio of high versus low polarized mitochondrial membranes was estimated as the percentage of the red pixel intensity (highly polarized membrane) out of the total red and green pixel intensity (total...
Statistical analyses
Data are expressed as mean ± SEM except for the β-MA experiment. Relative transcript abundance was analyzed by the SigmaStat (Jandel Scientific) software package using one-way ANOVA with multiple pairwise comparisons using the Student-Newman-Keuls method post-hoc. Other statistical procedures were carried out with PASW Data Collection 5.6 (for Windows, Chicago, IL, USA). For the β-Mercaptoacetate experiment, cleavage and blastocyst rates were compared between the three treatments using a binary logistic regression model taking replicate, treatment and the interaction term into account. Where no significant interaction was present, the term was omitted from the final model. For oxygen consumption and ratio of high versus low polarized mitochondrial membranes, a mixed model ANOVA, taking treatment as fixed factor and replicate as random factor, was used to compare differences between the three groups. Replicate, treatment and the interaction term were taken into account. If no significant interaction was present, the term was omitted from the final model. Data transformations were not required for inequality of variance between groups or for achieving normality for any data.

Results
Gene expression in oocytes matured in the presence of elevated NEFA concentrations
As shown in Figure 5.1, oocytes exposed to HIGH COMBI medium displayed a significant increase in mRNA expression of Lactate Dehydrogenase (LDHA) and Glyceraldehyde 3-phosphate Dehydrogenase (GADH), key genes involved in energy metabolism, when compared to control oocytes (P < 0.05). Elevated GADH expression was also observed in the HIGH SA oocytes (P < 0.01). Furthermore, the expression of
Glutathione Peroxidase 1 (GPX1), a gene related to oxidative stress, was significantly up-regulated in HIGH SA and HIGH COMBI oocytes \((P < 0.01)\).

![Figure 5.1](image.png)

**Figure 5.1.** Comparison of relative transcript abundance in oocytes. Oocytes were matured under control (150 µM total, physiological, NEFA); HIGH SA (75µM SA); and HIGH COMBI (425µM total, elevated, NEFA) conditions \((n = 180; 3\) replicates). Quantification was normalized to the endogenous control \(H2AFZ\). Bars with a and b superscripts are significantly different between treatments with \(P < 0.05\).

**Gene expression in cumulus cells from COCs matured in the presence of elevated NEFA concentrations**

Figure 5.2 shows that expression of the Facilitated Glucose Transporter 1 \(\textit{SL2A1}\), \(LDHA\) and Glucose-6-phosphate Dehydrogenase \((G6PD)\); genes related to glucose metabolism, were significantly lower in HIGH SA cumulus cells compared to cumulus cells harvested from control COCs \((P < 0.05)\). By contrast, the abundance of \(GADH\) mRNA, was significantly higher in HIGH SA cumulus cells. Cumulus cells collected from COCs matured under HIGH COMBI conditions displayed a significantly lower expression of \(LDHA\), \(GADH\) and \(G6PD\) \((P < 0.05)\). Expression of \(GPX1\) and DNA cytosine-5-methyltransferase 3A \((DNMT3A)\) was lower in cumulus cells from COCs matured in HIGH SA and HIGH COMBI conditions \((P < 0.05)\) compared to control COCs. Additionally, Carnitine Palmitoyl Transferase \((CPT1)\) and Adipophilin 2 \((PLIN2)\) expression were reduced in cumulus cells collected from HIGH SA COCs \((P < 0.05)\).
Figure 5.2. Comparison of relative transcript abundance in cumulus cells. Cumulus cells from cumulus oocyte complexes (n = 180; 3 replicates) matured under control (150 µM total, physiological, NEFA); HIGH SA (75µM SA); and HIGH COMBI (425µM total, elevated, NEFA) conditions. Quantification was normalized to the endogenous control H2AFZ. Bars with a and b superscripts are significantly different between treatments with $P < 0.05$.

Gene expression in blastocysts originating from oocytes matured in the presence of elevated NEFA concentrations

Four of the seven genes analyzed were differentially expressed amongst treatment groups (Figure 5.3). Firstly, the expression of a Fatty Acid Synthetase (ACSL1) was dramatically up-regulated in embryos originating from oocytes matured under HIGH COMBI concentrations compared to embryos arising from control and HIGH SA oocytes ($P < 0.05$). Secondly, Acetyl CoA Carboxylase (ACCA) expression was significantly higher in the group matured under HIGH COMBI conditions compared to the control embryos ($P < 0.05$), although the group matured with HIGH SA concentrations did not differ from the controls or HIGH COMBI embryos. Finally, the expression of genes related to mitochondrial biogenesis and oxidative stress (Transcription Factor A Mitochondria; TFAM and Manganese Superoxide Dismutase; MNSOD, respectively), tended to be increased in HIGH COMBI embryos ($P < 0.1$).
Expression patterns of key genes in bovine day 7 blastocysts arising from oocytes matured under control (150 µM total, physiological, NEFA); HIGH SA (75 µM SA); and HIGH COMBI (425 µM total, elevated, NEFA) conditions (n = 192; 5 replicates). Bars with a and b superscripts are significantly different between treatments with \( P < 0.05 \). Quantification was normalized to the endogenous control \( H2AFZ \). Bars with * and $ superscripts tend to be significantly different between treatments with \( P < 0.1 \).

Mitochondrial ultrastructure and distribution in oocytes matured in the presence of elevated NEFA concentrations

Transmission electron microscopic analysis of matured oocytes did not reveal any obvious differences in spatial distribution of mitochondria (Figure 5.4, 1A and 2A). As expected (Clararo et al., 1976; Bracket et al., 1980; Mohr and Trounson, 1981), the mitochondria of the oocyte appeared small and electron dense with few cristae, and there was little obvious difference between oocytes from the different treatments. In all of the treatments, the cytoplasm was divided into organelle-rich and organelle-free zones. The organelle-rich zones displayed a slightly more peripheral localization in the ooplasm and were characterized by an apparently random distribution of mitochondria. Moreover, mitochondrial ultrastructure did not appear to change in response to high NEFA exposure (Figure 5.4, 1B and 2B).
Figure 5.4. Electron microscopic images of mitochondria in oocytes matured under control (150 µM total, physiological, NEFA) conditions (1A and 1B) and HIGH COMBI (425µM total, elevated, NEFA) conditions (2A and 2B) at magnification 6000x (A) and magnification 25000x (B). Mitochondria (M), vesicles (V) and lipid droplets (LD) were distributed randomly in the cytoplasm.

Development of oocytes matured in the presence of elevated NEFA concentrations after inhibiting β-oxidation

As shown in Table 5.2, the presence of β-mercaptoacetate (β-MA) had no significant effect on cleavage and blastocyst rates obtained from the fertilisation of oocytes matured in control maturation medium. However, blastocyst production from oocytes matured in the presence of β-MA and HIGH COMBI NEFA was higher than when β-MA was absent (26.5 vs. 18.8%; P = 0.05).
Table 5.2. Developmental competence of NEFA-exposed oocytes in the presence and absence of β-Mercaptoacetate (β-MA), an inhibitor of the mitochondrial β-oxidation.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>CONTROL + β-MA</th>
<th>HIGH COMBI</th>
<th>HIGH COMBI + β-MA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oocytes</strong></td>
<td>208</td>
<td>204</td>
<td>208</td>
<td>200</td>
</tr>
<tr>
<td><strong>Cleaved</strong></td>
<td>172 (82.7)</td>
<td>160 (78.4)</td>
<td>162 (77.9)</td>
<td>154 (77.0)</td>
</tr>
<tr>
<td><strong>Blastocysts from oocytes matured</strong></td>
<td>54 (26.0)</td>
<td>49 (24.0)</td>
<td>39 (18.8)</td>
<td>53 (26.5) a</td>
</tr>
<tr>
<td><strong>Blastocysts from cleaved zygotes</strong></td>
<td>54 (31.4)</td>
<td>49 (30.6)</td>
<td>39 (24.1)</td>
<td>53 (34.4) b</td>
</tr>
</tbody>
</table>

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Oocytes (n = 820; 5 replicates) were matured under the following conditions: 1) control (150 µM total, physiological, NEFA), 2) HIGH COMBI (425µM total, elevated, NEFA), 3) control (150 µM total, physiological, NEFA) + 0.1mM β-MA and 4) HIGH COMBI (425µM total, elevated, NEFA) + 0.1mM β-MA. Data with a and b superscripts are significantly different between treatments with $P < 0.05$.

**Oxygen consumption in zygotes originating from oocytes matured in the presence of elevated NEFA concentrations**

No significant difference was observed in the average oxygen consumption of zygotes originating from oocytes matured under control (1.25 ± 0.07 nl/embryo/h) and HIGH COMBI (1.11 ± 0.11 nl/embryo/h) conditions.

**Ratio of high versus low polarized mitochondrial membranes in zygotes originating from oocytes matured in the presence of elevated NEFA concentrations**

Confocal images (Figure 5.5) showed that the proportion of red pixel intensity (highly polarized membrane) out of the total red and green pixel intensity (total mitochondrial mass) was not significantly different between treatments. However, the latter percentage tended to be higher in HIGH COMBI zygotes compared to the controls for the top plane (55.0% ± 1.2 vs. 51.2% ± 2.6, respectively; $P = 0.09$) and for the average of both the top and midequatorial planes (53.8% ± 1.2 vs. 50.9% ± 2.4,
respectively; \( P = 0.09 \). No difference in mitochondrial membrane polarity ratios could be detected between treatments for the midequatorial plane (52.9\% \pm 1.2 \text{ vs. } 50.7\% \pm 2.5, \text{ respectively; } P = 0.17).

**Figure 5.5.** Confocal microscopic images (400x magnification) of JC-1 stained zygotes (1d p.i.) originating from oocytes matured under 1) control (150 \( \mu \text{M} \) total, physiological, NEFA) and 2) HIGH COMBI (425\( \mu \text{M} \) total, elevated, NEFA) conditions with A) view at mid equatorial plane and B) view at top plane. Blue fluorescence represents chromatin material. Green fluorescence indicates low polarized mitochondria. Red fluorescence indicates high polarized mitochondria.
Discussion

Elevated NEFA concentrations represent a common feature of a distorted maternal metabolism, typically present in obese and type II diabetes patients (Reaven et al., 1988; Mooradian et al., 2008). Epidemiological studies suggest that such metabolic disorders are associated with decreased fertility (Pasquali et al., 2003; Metwally et al., 2007) and there is growing evidence that elevated NEFA concentrations play a key role in this process as they may alter the biochemical composition of the follicular micro-environment (Leroy et al., 2005; Robker et al., 2009; Jungheim et al., 2011). Such adverse follicular conditions during oocyte maturation can interfere with the oocyte’s developmental capacity (Jorritsma et al., 2004; Leroy et al., 2005; Aardema et al., 2011; Leroy et al., 2012). Furthermore, embryos originating from oocytes exposed to elevated NEFA concentrations exhibit an altered oxidative metabolism, compromised glucose metabolism and an altered gene expression pattern as they develop up to day 7.5 (Van Hoeck et al., 2011).

In the present study, we investigated the etiological mechanisms by which these events occur. We have used cows in negative energy balance as a model system to investigate the lipotoxic effects of elevated NEFA on the oocyte. Although there are obvious differences between ruminant NEB and human obese of type II diabetes conditions, both ultimately lead to up-regulated lipolysis and to elevated serum NEFA concentrations. The types and the concentrations of the NEFA used in the present model are based on studies on follicular fluid in situ under conditions of up-regulated lipolysis and thus are physiologically relevant (Leroy et al., 2005). We know from human follicular fluid analyses that our bovine observations accurately replicate the situation in the human. Furthermore, human serum NEFA concentrations under obese conditions (Hall et al., 1979; Reaven et al., 1988; Stolba et al., 1993) are very similar to the bovine serum NEFA concentrations during NEB (Leroy et al., 2005). We therefore hypothesized that the compromised oocyte developmental competence, which results from NEFA-exposure during the final stages of maturation, is caused by modifications in oocyte energy metabolism; a concept based on fundamental research in myocardial cells exposed to elevated NEFA concentrations, in which high fatty acid β-oxidation rates contribute to the development of myocardiopathies (for an overview:
Lopaschuk et al., 2010). Interestingly, Igosheva et al. (2010) also reported that excessive nutrient exposure prior and during conception in obese mice might be associated with a compromised oocyte and embryo mitochondrial metabolism.

At the molecular level, oocytes matured in medium with elevated concentrations of palmitic, stearic and oleic acid showed a significant increase in mRNA encoding GADH and LDHA. GADH catalyzes the sixth step of glycolysis, converting NAD+ to NADH and thus generating reducing power. LDHA is also recognized as a strong cytosolic reductant (Lane and Gardner, 2000) as it preferentially converts lactate into pyruvate (Dumollard et al., 2001; Wilding et al., 2009), with the conversion of NAD+ to NADH. It is also noteworthy that the products of these two genes regulate transcription by virtue of their ability to interact with the transcriptional cofactor Oct-1 in a REDOX-dependent manner (Zheng et al., 2003). Furthermore, the HIGH COMBI oocytes showed significant up-regulation of GPX1 expression, a ROS-related gene, which supports our assumption that the REDOX status of the oocyte is affected by exposure to high NEFA. GPX1 is of fundamental importance since it acts in the detoxification of hydrogen peroxide. Interestingly, increased expression of GADH will spare NAD(P)H, essential for the recycling of glutathione. In other words, the differential expression of these specific genes in response to NEFA-exposure may provide a mechanism for the imbalance in intracellular REDOX potential, as reported in previous studies in leucocytes (Kakinuma and Minikami, 1978; Listenberger et al., 2001); a state characterized by an imbalance between pro-oxidant molecules including reactive oxygen species and antioxidant defenses. A moderate increase in ROS levels can stimulate cell growth and proliferation and is physiologically important. Conversely, excessive ROS will cause cellular injury (e.g. damage to DNA, lipid membranes, and proteins). Mitochondria are major sites for ROS production and excessive ROS can affect their function in oocytes and embryos (for review: Agarwal et al., 2012).

In contrast to the observations in the oocyte, cumulus cells from HIGH NEFA-exposed COCs, exhibited down-regulated expression of genes encoding enzymes involved in REDOX regulation: GADH, GPX1, G6PD and LDHA. Furthermore, expression of the DNMT3A gene was significantly
down-regulated in HIGH COMBI and HIGH SA cumulus cells, pointing to the possibility of altered methylation status. Cumulus cells are vital for the oocyte to complete successful maturation and sustain further development (Tanghe et al., 2002). Moreover, cumulus cells likely play a role in regulating REDOX-homeostasis by providing glutathione to the oocyte (Geshi et al., 2000). If the cumulus cells do not succeed in safeguarding this system and fail to protect the oocytes, there could be a compensating mechanism in oocytes to up-regulate their own defense against ROS. Thus, impaired viability and anti-ROS defense of the cells surrounding the oocyte most likely reflects jeopardized oocyte capacity to defend against ROS, consistent with the significant up-regulated GPX1 and LDHA expression in the oocyte. 

As a result of our own work (Van Hoeck et al., 2011) and that of others (Rizos et al., 2002; Lonergan et al., 2003; Sirard et al., 2006), it can be concluded that the nutritional environment during maturation can affect the resulting embryo. We therefore examined whether blastocysts originating from NEFA-exposed oocytes displayed differences in transcript abundance of key genes. The HIGH COMBI blastocysts tended to display up-regulated expression of MnSOD, an enzyme that plays a protectant role against oxidative stress (Harvey et al., 1995), which might be the result of a compensatory mechanism in response to a shifted REDOX potential at earlier stages of development and in turn, affect the activity of REDOX sensitive transcription factors. A hypothesis based on our previous observation of SLC2A1 mRNA up-regulation in HIGH COMBI embryos (Van Hoeck et al., 2011). Moreover, the HIGH COMBI embryos showed higher expression of TFAM, essential in stabilizing mitochondrial DNA, and for which over-expression has been reported to be related to oxidative stress (Suarez et al., 2008). Interestingly, a significant increase was also observed in the mRNA encoding enzymes involved in fatty acid synthesis pathways, i.e. ACSL1 and ACCA in HIGH COMBI blastocysts. ACSL1 is involved in the activation of long chain fatty acid for β-oxidation, however, when malonyl Co A, the product of ACCA, is also abundant, there is a shift toward synthesis of triglycerides. The parallel increase in the mRNA abundance of ACSL1 and ACCA suggests increased lipogenesis in such embryos since ACSL1 has been closely linked to triglyceride synthesis, and over-expression suggests a mechanism for sequestering
fatty acids as triacylglycerols in lipid droplets (Li et al., 2010). The ability to store NEFA in lipid droplets might have a protective function in channeling fatty acids away from other lipotoxic pathways that may have been triggered at the oocyte level (Cnop et al., 2001; Li et al., 2010). However, previous studies in somatic cell lines have demonstrated that excessive accumulation of lipids in peripheral tissues is closely associated with the pathological state of insulin resistance in type II diabetes (Zhang and Zhang, 2012). Therefore, the question arises whether such adaptations towards lipid storage provide protective/compensatory mechanisms or rather activate other toxicity related pathways. Both options may have an important impact during further embryo development, implantation, fetal development; problems which might not become apparent until post-implantation, fetal or even neonatal development.

As the organization, positioning and morphology of mitochondria within the oocyte is indicative of the energy requirements of key events during oocyte maturation (Sun et al., 2001), we evaluated mitochondrial distribution in oocytes matured in the presence and absence of elevated NEFA. Although mitochondrial organization differs between morphologically good and poor oocytes and may be associated with different developmental capacity after in vitro fertilisation (Stojkovic et al., 2001), transmission electron microscopic analyses did not reveal obvious differences in oocyte mitochondrial distribution or morphology between HIGH COMBI oocytes and controls.

We next sought to investigate the functional effects of exposure to high NEFA concentrations on mitochondrial activity, by testing the effect of β-oxidation inhibition during oocyte maturation. A strong metabolic role for lipid oxidation during oocyte maturation has been substantiated previously in mouse (Downs et al., 2009; Dunning et al., 2010) and bovine oocytes (Sturmey et al., 2006). Using β-mercaptoacetate, an enzyme that inhibits the β-oxidation, we found that the developmental potential of HIGH COMBI oocytes could be rescued if β-oxidation was inhibited during oocyte maturation ($P = 0.05$). This suggests that mitochondrial fatty acid β-oxidation is involved in developmental failure in bovine oocytes exposed to elevated NEFA concentrations. By contrast, control oocytes exposed to physiological NEFA concentrations did not display significant
differences in post-fertilisation development after inhibition of β-oxidation, which is in line with findings from Sturmey et al. (2006). We have previously shown that the oxygen consumption of embryos originating from NEFA-exposed oocytes is significantly reduced (Van Hoeck et al., 2011) and therefore wished to discover whether such a reduction in oxygen consumption was already evident directly after maturation. Since it was not possible to measure the respiration of the oocyte alone, due to the presence of surrounding cumulus cells (Wilding et al., 2009), oxygen consumption was determined in zygotes originating from HIGH NEFA-exposed oocytes. We also measured the ratio of high versus low polarized mitochondrial membranes in these zygotes to provide a global picture of mitochondrial activity since this feature has been positively correlated to the level of respiration (Miwa et al., 2003; Van Blerkom et al., 2006). The oxygen consumption of zygotes was not significantly different between treatments. Likewise, confocal analyses did not reveal major differences in the activity of mitochondria in zygotes arising from the different treatments, although there was a tendency for an increased ratio of high versus low polarized mitochondrial membranes in high NEFA zygotes. One possible explanation for this observation might relate to the metabolic plasticity of the early embryo. Measuring oxygen consumption and the ratio of high versus low polarized mitochondrial membranes provides information on overall oxidative process in terms of ATP synthesis from oxidative phosphorylation. However, the identity of the major substrate oxidized to generate the ATP cannot be determined from such experiments. The observation that inhibition of β-oxidation during maturation restores developmental capacity in NEFA-exposed oocytes does, however, strongly suggest that the pathways adopted by the early embryo to generate substrates for oxidation are crucial in determining ongoing viability, and might be influenced by the conditions under which the oocyte matures. If the oocyte is unable to oxidize fatty acids for the generation of ATP, one can hypothesize that it alters its metabolism to oxidize alternative exogenous substrates such as pyruvate, lactate and glucose (Sutton-McDowall et al., 2010).

The follicular micro-environment prior to conception is crucial for maturing oocytes (for an overview: Leroy et al., 2012). As such, using a bovine in vitro production model, we were able to demonstrate that
elevated NEFA-concentrations during the final phase of oocyte maturation compromise further development and even alter the gene expression pattern of the resultant embryo, which substantiates our previous work (Leroy et al., 2005; Van Hoeck et al., 2011). However, Britt (1992) hypothesized that the developmental competence of oocytes, in high yielding dairy cows, is determined by their biochemical environment during a long period (up to 80 days) of follicular growth prior to ovulation. So far, studies determining the effects of a long-term NEFA-exposure during folliculo- and oogenesis, have not been performed, but might be crucial in determining the final fate of oocytes dwelling in NEFA-exposed follicles.

**In conclusion**, our data indicate that the follicular conditions under which the oocyte completes final maturation have a significant effect on the subsequent embryo in terms of gene and molecular/cellular phenotypic expression, particularly with regard to metabolically critical REDOX- and fatty acid synthesis-related gene function, as well as influencing the metabolic strategy of the early embryo. We can hypothesize that such profound alterations apparent at the oocyte stage may persist in fetal development and in the offspring.
List of References


CHAPTER 6

The interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations.


Abstract
Maternal metabolic disorders linked to lipolysis are major risk factors for reproductive failure. A notable feature of such disorders are increased non-esterified fatty acid (NEFA) concentrations in the blood, which are reflected in the ovarian follicular fluid. Elevated NEFA concentrations impact on the maturing oocyte and even alter subsequent embryo physiology. The etiologic mechanisms are not fully elucidated. Therefore, bovine in vitro maturing cumulus oocyte complexes (COCs) were exposed (24 h) to three different maturation treatments containing 1) physiological NEFA concentrations = control (72µM NEFA), 2) elevated stearic acid concentrations = HIGH SA (75µM stearic acid (SA)) and 3) elevated NEFA concentrations = HIGH COMBI (425µM NEFA). Zygotes were fertilized and cultured following standard procedures. Transcriptomic analyses in resultant day 7.5 blastocysts revealed that major pathways affected are related to lipid and carbohydrate metabolism in HIGH COMBI embryos and to lipid metabolism and cell death in HIGH SA embryos. Furthermore, lower glutathione (GSH) content and a reduced number of lipid droplets per cell were observed in HIGH SA-exposed oocytes and resultant morulae (respectively) compared to HIGH COMBI-exposed counterparts.
Vitrified embryos originating from HIGH SA-exposed oocytes tended to display lower survival rates compared to controls. These data provide possible mechanisms through which females suffering lipolytic disorders experience difficulties in conceiving.

**Introduction**

Maternal metabolic disorders, linked to lipolytic conditions, such as negative energy balance, obesity or type II diabetes are risk factors for reproductive efficiency. Reduced oocyte and embryo quality have been suggested as major contributors (Fedorcsak *et al*., 2000; Pasquali *et al*., 2003; Leroy *et al*., 2008). A notable feature of such metabolic disorders is an up-regulation of lipolysis leading to increased non-esterified fatty acid (NEFA) concentrations in the blood. NEFAs alter the biochemical composition of the follicular microenvironment (Leroy *et al*., 2005; Robker *et al*., 2009; Jungheim *et al*., 2011; Valckx *et al*., 2012) and thereby affect the oocyte’s developmental capacity (Jorritsma *et al*., 2004; Leroy *et al*., 2005; Aardema *et al*., 2011; Van Hoeck *et al*., 2011). In this context, elevated serum NEFA concentrations can directly affect granulosa cell viability and steroidogenesis (Jorritsma *et al*., 2004; Vanholder *et al*., 2005). Furthermore, we demonstrated that elevated NEFA concentrations during *in vitro* oocyte maturation impact on gene expression profiles and even the subsequent embryonic phenotype (Van Hoeck *et al*., 2011; 2013), which suggests that follicular conditions under which the oocyte completes final maturation are crucial in determining subsequent embryo physiology.

Fatty acids play diverse cellular roles in metabolism and signalling and are metabolized by mitochondrial β-oxidation for energy provision (for review: McKeegan and Sturmey, 2011). However, NEFAs have come under intense scrutiny because of their dysfunctional role when excessively present. Reports on research with pancreatic β-cells (Carlsson *et al*., 1999; Koskin *et al*., 2003), adipocytes (Furukawa *et al*., 2004), monocytes (Zhang *et al*., 2006) and skeletal muscle cells (Bonnard *et al*., 2005) suggest that mitochondrial metabolism is involved in the etiology of fatty acid induced metabolic disturbances, an effect initiated by increased levels of reactive oxygen species (ROS). Excessive ROS levels
can cause oxidative damage and might, in turn, jeopardize mitochondrial function, which contributes to additional oxygen radical formation, reduced ATP concentrations, and decreased intracellular glutathione (GSH), all of which have been associated with decreased developmental competence of oocytes (Quinn and Wales, 1973; De Matos et al., 1995; 1996; Grupen et al., 1995; Van Blerkom et al., 1995; Tarin, 1996).

The micro-environment around conception profoundly influences subsequent embryo development, and even affects resultant embryo physiology to such a degree that susceptibility to disease may be increased later in life (Chavatte-Palmer et al., 2008). In our recent study (Van Hoeck et al., 2011), we showed that embryos produced from fertilized, NEFA-exposed oocytes have a significantly lower cell number, increased apoptotic cell index, aberrant transcriptional activities, altered amino acid turnover, and compromised oxidative metabolism, all indicators for a lower embryo quality and viability. Although such embryos displayed an up-regulated expression of the SLC2A1 glucose transporter, the glucose consumption by these embryos was drastically reduced compared to control embryos, an intriguing finding, as this situation is similar to that in insulin-resistant somatic cells. Furthermore, we reported a distinctly affected gene expression pattern in response to either a saturated NEFA treatment versus a combined NEFA treatment, containing both saturated and mono-unsaturated NEFAs, which suggests that resulting blastocysts are involved in different metabolic strategies. However, to better understand the consequences of the oocyte’s environment on embryo physiology, a complete transcriptome analysis of the resulting blastocyst should characterize the specific gene expression patterns originating from NEFA exposure.
Therefore, the present study aimed to:

- unravel relevant metabolic pathways by measuring the relative RNA abundance of thousands of candidate genes in blastocysts originating from oocytes exposed to elevated NEFA concentrations during final maturation.
- link these gene expression data to functional information by evaluating the effect of NEFA exposure during oocyte maturation on key biochemical indicators in the different steps of early development:
  1. glutathione content in mature oocytes.
  2. lipid droplet content in subsequent morulae.
  3. cryotolerance of resulting blastocysts.

In the present study, high-throughput technologies for the study of gene expression patterns were used, that allowed us to integrate and interpret data a posteriori and to subsequently focus on the functional relevance of specific observations which resulted in down-stream interpretations. The combination of the new transcriptomic data and the data already available from our previous studies (Van Hoeck et al., 2011; 2013), together with the novel functional data, should improve our current understanding of the possible mechanisms through which specific NEFA exposure during oocyte maturation affects the oocyte’s developmental capacity and the resultant embryo physiology.

**Materials and methods**

**Preparation of NEFA treatments**

All chemicals were purchased from Sigma (Bornem, Belgium), unless otherwise stated. Stearic acid (SA, C18:0), palmitic acid (PA, C16:0), and oleic acid (OA, C18:1) were dissolved in a stock solution of pure ethanol at concentrations of 25, 150 and 200 mM, respectively. These ethanol stock solutions were vortex-mixed for 4 min and diluted in final solutions to obtain the desired final concentration in serum-free maturation medium. The final medium is supplemented with 0.75% BSA free of fatty in order to improve NEFA solubility. All treatments were vigorously sonificated for 4 h and filter-sterilized under aseptic conditions.
**In vitro embryo production**

*In vitro* embryo production procedures were performed as previously described (Van Hoeck *et al.*, 2011; 2013), using immature oocytes retrieved from bovine ovaries collected within 2 h of slaughter. Briefly, unexpanded COCs surrounded by five or more cumulus cell layers (quality grade I) were matured *in vitro*. Therefore, COCs were washed in 500 µl maturation medium and matured in groups of 50-60 in 500 µl serum-free maturation medium containing TCM199 supplemented with 0.75% BSA free of fatty acids, 0.4 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 mg/mL Gentamycin and murine epidermal growth factor (mEGF, 20 ng/ml) in four-well plates (Nunc, Langenselbold, Germany) for 24 h in humidified air with 5% CO₂ at 38.5°C. After IVM, COCs were co-incubated in groups of 100-120 with spermatozoa at a final concentration of 10⁶ sperm cells/ml for 20 h at 38.5°C in 500 µl fertilization medium (containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM Na₂HPO₄, 2.1 mM CaCl₂·2H₂O, 0.4 mM MgCl₂·6H₂O, 25 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, 2 µl/mL phenol red, 6 mg/mL BSA, 50 µg/mL Gentamycin and 10 µl/mL heparin) in a humidified 5% CO₂ incubator. Frozen semen from a bull of proven *in vitro* fertility (Van Hoeck *et al.*, 2011) was thawed and live spermatozoa were isolated by centrifugation on a discontinuous Percoll gradient (90 and 45%, Biosciences, Amersham, UK). The final sperm-egg ratio was adjusted to 5000:1. Following co-incubation with spermatozoa, the presumptive zygotes were vortex-mixed for 3.5 min to remove excess sperm and cumulus cells. After three wash steps with HEPES-TALP and modified SOF medium, presumptive zygotes were cultured per 25 ± 4 in 50 µl modified SOF medium with mineral oil overlay (modular incubator: 38.5 °C, 5% CO₂, 5% O₂ and 90% N₂) until the day of analysis. The SOF medium contained 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH₂PO₄, 0.8 mM MgSO₄·7H₂O, 0.6% v/v sodium lactate, 25 mM NaHCO₃, 0.0266 mM phenol red, 0.73 mM sodium pyruvate, 1.78 mM CaCl₂·2H₂O, 0.34 mM trisodium citrate, 2.755 mM myoinositol, 3% v/v BME 50x, 1% v/v MEM 100x, 0.4 mM glutamine, 5% serum and 50 µg/mL Gentamycin.
Experimental design
The types and concentrations of free fatty acids used in the present study are based on bovine in vivo studies in serum and in follicular fluid (Leroy et al., 2005) and are pathophysiologically appropriate, since circulating free fatty acid concentrations in women suffering lipolysis-linked metabolic disorders, including obesity (Reaven et al., 1988; Stolba et al., 1993), are very similar to NEFA concentrations detected in bovine during an episode of up-regulated lipolysis (Leroy et al., 2005).

Standard serum-free maturation systems are devoid of fatty acids, although the physiological environment, in which the oocyte matures in vivo, contains physiological, basal concentrations of NEFA (Leroy et al., 2005). In order to improve the relevance of our in vitro model, we therefore used a maturation medium supplemented with basal NEFA concentrations as control medium. As in our previous studies (Leroy et al., 2005a; Van Hoeck et al., 2011) SA was the most toxic fatty acid in terms of disrupting final oocyte maturation and as the chemical structure of SA (C18:0) is mostly reliant to the structure of the mono-unsaturated OA (C18:1), we optioned to implement SA as additional saturated NEFA treatment. For all experiments, COCs were exposed for 24 h to the following NEFA treatments:

1. control: physiological NEFA concentrations (72 µM total NEFA containing 28 µM SA, 23 µM PA, and 21 µM OA).
2. HIGH SA: elevated SA concentrations as under high lipolytic conditions (75 µM SA).
3. HIGH COMBI: combination of elevated NEFA concentrations as under high lipolytic conditions (425 µM total NEFA, containing 75 µM SA, 150 µM PA, and 200 µM OA).

Each treatment was tested for its effect on cleavage rate (48 h post insemination (p.i.)) and blastocyst yield (7.5 days p.i.) (1747 oocytes, equally distributed among treatments, were matured in 7 replicates). Resultant blastocysts were snap frozen to perform pathway analyses, large-scale analysis of gene transcription profiles was performed using the microarray technique (120 blastocysts, equally distributed among treatments, 4 replicates) and results were validated with the qRT-PCR.
method (114 blastocysts, equally distributed among treatments, 3 replicates).

Based on the pathways characterized through transcriptome analyses, other oocytes were matured under different conditions and subsequent functional experiments were designed:

- A total of 720 oocytes (equally distributed among treatments) were used to analyse GSH content in oocytes over 4 independent replicates.
- A total of 74 morulae (equally distributed among treatments) was used to visualize and quantify lipid droplet content in resultant morulae (150 h p.i.) over 3 independent replicates.
- A total of 127 day 7.5 blastocysts (equally distributed among treatments) were used to evaluate cryotolerance over 3 different replicates.

**Quantification of gene transcription through microarray technology**

Gene expression analyses using the bovine EmbryoGENE microarray slides were performed as previously described (Cagnone et al., 2012). Total RNA from each replicate was extracted and purified using the PicoPure RNA Isolation Kit (Life Science, Ottawa, Ontario). After DNase digestion (Qiagen, Toronto, Canada), quality and concentration of extracted RNA were analysed by bioanalyzer (Agilent, Diegem, Belgium). All extracted samples showed good quality with an RNA integrity number > 7.5. For microarray purposes, purified RNA was amplified by *in vitro* transcription by T7 RNA amplification using the RiboAmp HSPlus RNA Amplification Kit (Life Science, Otawa, Ontario) and labelled with Cy3 and Cy5 using the ULS Fluorescent Labelling Kit (Kreatech, Durham, North America), and antisense RNA (825 ng per replicate) was hybridized on the Agilent-manufactured EmbryoGENE slides in a two-colour dye-swap design. Following 17 h of hybridization at 65°C, microarray slides were washed for 1 min in gene Expression Wash Buffer 1 (room temperature), 3 min in gene Expression Wash Buffer 2 (42°C), 10 sec in 100% acetonitrile (room temperature) and 30 sec in Stabilization and Drying Solution (Agilent, Diegem, Belgium). Slides were scanned with PowerScanner (Tecan, Männedorf, Switzerland) and features extraction was done with Array-pro6.3 (MediaCybernetics, Warrendale,
Pennsylvania). Data were analysed through the use of Ingenuity Pathways Analysis (IPA; Ingenuity Systems, www.ingenuity.com), which served to identify pathways that were differentially expressed between treatments. A fold change cutoff of 1.5 with a P value 0.05 was set to identify genes whose expression was significantly differentially regulated.

Re-evaluation of data generated with microarray through qRT-PCR

Re-evaluation of microarray results was done by quantitative real-time PCR (qPCR). Poly(A) RNA of day 7.5 blastocysts was extracted using a Dynabead mRNA Direct Extraction KIT (Dynal Biotech, Madrid, Spain) according to the manufacturer’s instructions, with minor modifications. Immediately after extraction, the RT reaction was carried out following the manufacturer’s instructions (Bioline, London, UK), using poly(T) primer, random primers, and the MMLV reverse transcriptase enzyme, in a total volume of 40 µl, in order to prime the RT reaction and to synthesize cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and the RT reaction was completed with the addition of 100 units of reverse transcriptase. The mixture was incubated at 42°C for 60 min to allow the RT of RNA, followed by 70°C for 10 min to denature the RT enzyme. Four groups of cDNA per experimental group were used with three replicates for all genes of interest. Experiments were conducted to compare relative levels of each transcript and histone H2AFZ in every sample. The PCR was performed by adding a 2 µl aliquot of each sample to the PCR mix, containing the specific primers, to amplify the genes of interest. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 6.1.

Quantification was performed as described previously (Bermejo-Alvarez et al., 2010). PCR conditions were optimized to achieve efficiencies close to 1 and the comparative cycle threshold (CT) method used to quantify expression levels as described by Schmittgen and Livak (2008). Quantification was normalized to the endogenous control H2AFZ. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative
CT method, the ΔCT value was determined by subtracting the control gene \((H2AFZ)\) CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula \(2^{-\Delta\Delta CT}\).

Table 6.1. Details for primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Gene Bank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2AFZ</td>
<td>AGGAGCAGACTAGGACCAGCTGGAGCTGGGCCACCACCCAGCAATTGTAGCCTTG</td>
<td>NM_174809.2</td>
</tr>
<tr>
<td>MSMO</td>
<td>GTGCGCCATTTGACGCTGTTTGAAGGGTCTGCTTGATGAATAAAGGCAAGGGAAGGACACATC</td>
<td>NM_001098863</td>
</tr>
<tr>
<td>AGPAT5</td>
<td>GTTTTCGCTGAGTTGCAAAACACTGGATTTAAATATGATGTAATGTTGCTTGAAGG</td>
<td>NM_001075932</td>
</tr>
<tr>
<td>SCP2</td>
<td>GCGCTTAAAGAGCTGGTGTGTGTCGCACTGTAAGGCTGGTTGCTCCTTTCTACCTTTGCTG</td>
<td>NM_001033990</td>
</tr>
<tr>
<td>GPCPD1</td>
<td>GAGCTTAAAGAGCTGGTGTGTGTCGCACTGTAAGGCTGGTTGCTCCTTTCTACCTTTGCTG</td>
<td>NM_002692364</td>
</tr>
<tr>
<td>SMPD3</td>
<td>CTGTTCTACAGGCTCTGCTGAGGGTCTTATTTCTGCTTGCTGACCAGTGTCCTGCAGTG</td>
<td>NM_002694829</td>
</tr>
<tr>
<td>LPLA1</td>
<td>CTGGTATCCCAGCTGACTAGGACAGTGTAGATGGCATCTCATAAATAGGGTGCTAT</td>
<td>NM_001034688</td>
</tr>
<tr>
<td>ATP6VO2</td>
<td>TCCACATCAAGGCACTGAGAGTGGTTGGAGTCTGCTGCTCAGGTATGGATGGCAGTG</td>
<td>NM_001083653</td>
</tr>
<tr>
<td>S100A11</td>
<td>CCATGAAATCCTTTATTAAGTCTGCCTCTCCCAGAGTAAAATGTGAGGAGGCCCTTTGGGC</td>
<td>NM_001098856</td>
</tr>
<tr>
<td>CD47</td>
<td>CTGCTGTGTTGGATCCAGCCTCTGTGTCTGACTCAGTATCCCAGTGCTGTCCTCT</td>
<td>NM_174708</td>
</tr>
</tbody>
</table>

Assessment of glutathione (GSH) content

GSH content was investigated as described by Bijttebier et al. (2008) with minor modifications. A total of 720 oocytes (equally distributed among treatments) were used for GSH analysis. Matured oocytes were denuded by a vortex-mix step. Oocytes were washed three times in 0.1% PVP and transferred in groups of 30 in 5 μl assay buffer (0.2 M sodium phosphate buffer containing 10 mM EDTA) to which 5 μl of 1.25 M H₃PO₄ was added. Samples were stored at -80°C until assayed. GSH levels were analysed by a validated HPLC-electrochemical detection (ECD) method described in erythrocytes (Magielse et al., 2013). After defrosting, samples were diluted (1/10) with phosphate buffer, acidified with 5 μl H₃PO₄ and analysed by HPLC-ECD. Samples (50 μl) were analysed on a Gilson HPLC system with an ESA-5600 CoulArray 8-channel electrochemical detector. As mobile phase, solvent A: 25 mM NaH₂PO₄ and 1.4 mM OSA in 3% methanol, pH 2.7 and solvent B: 25 mM NaH₂PO₄ and 1.4 mM OSA in 50%
methanol, pH 2.7 were used. The gradient profile consisted of a 25 min linear gradient from 0 to 50% B, a 5 min linear gradient from 50 to 100% B and 5 min isocratic at 100% B before returning to initial conditions. A flow rate of 0.7 ml/min was used. Samples were cooled at 10°C. The cell potentials were 500 – 580 – 660 – 740 – 820 – 900 – 980 – 1060 mV versus palladium reference. A C18 RPe (250 x 4 mm; 5 µm) column (Merck, USA) was used. The retention time of GSH was 8 min. Final GSH content per oocyte was calculated by dividing the total content per sample by the number of oocytes present in the sample.

Analysis of lipid droplet content
Lipid droplet analysis was performed at morula level for two reasons: i) cells of the morula stage are not differentiated yet into specific cell lines with possibly different lipid droplet contents and ii) the absence of a fluid-containing blastocoel (causing fluorescence distraction).
Morulae were fixed at 150 h p.i. in 4% paraformaldehyde. Morulae were washed twice in PBS with 0.3% (w/v) polyvinylpyrrolidone (PVP), permeabilized for 30 min in PBS with 0.1% (w/v) saponin (PBS-S; Riedel-de Haën, Seelze, Germany) and 0.1 M glycine (Merck, Darmstadt, Germany) and washed in PBS-S. The nuclei were stained with 10 µg/ml DAPI (Molecular Probes, Eugene, OR) for 5 min and subsequently washed in PBS-PVP. After this, neutral lipids were stained according to a modified protocol of Aardema et al. (2011). Lipid droplets were stained with the specific neutral lipid stain BODIPY 493/503 (Molecular Probes, Ghent, Belgium) in PBS (20 µg/ml, 1 h), morulae were washed three times in PBS with 3 mg/ml PVP and transferred into dishes for imaging. High resolution images were obtained using a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium) equipped with 405 and 488 nm diode lasers for excitation of blue and green fluorophores, respectively (Figure 6.1). For each morula, a z-stack of 20 µm (1 µm interval) was consistently taken at the lower mid equatorial level. To evaluate the lipid droplet content, images were further analysed using Volocity 6.0.1 software. Droplets were considered as relevant from a size of 0.5 µm³ onwards. This minimal threshold was set to overcome false positive counting of background pixels. The number of lipid droplets, their
mean size (in µm³) and total volume of lipid droplet content (in µm³) were calculated by the software and expressed as ‘total number of lipid droplets per cell’ and ‘total volume of lipid droplets per cell’.

**Figure 6.1.** Fluorescent confocal image (400x magnification) of a morula (150 h p.i.) stained with DAPI and BODIPY 493/503 visualized with 405 and 488 nm diode lasers for excitation of blue and green fluorophores, respectively. Green fluorescence indicates lipid droplets. Blue represents chromatin material.

**Cryotolerance evaluation**

Blastocysts (normal or expanded) were vitrified using the cryologic solid surface vitrification technique, described by Vajta *et al.*, (1998). After washing in 800 µl PBS plus 5 mg/ml BSA, blastocysts (7.5 d p.i.) were equilibrated in PBS medium supplemented with 10% ethylene glycol and 10% DMSO for 1 min. Subsequently, embryos were washed in a 600 µl sucrose medium (1 M) plus 20% ethylene glycol and 20% DMSO. Thereafter, 4-6 embryos were transferred within 20 seconds in drops (3 µl) of wash medium and loaded onto the fine hook at the end of a plastic fibreplug. As described by Aerts *et al.* (2008), the droplets were vitrified by contact with the surface of a metal cube (CVM1, Linde Gas Cryoservices, The Netherlands), precooled by partial immersion in liquid
nitrogen (LN₂). The fibreplugs were subsequently inserted into prechilled straws with a built-in sealant, and placed in storage holes in the CVM1 cube. After vitrification of all embryos, the straws were collected in a holding container and plunged in LN₂. To warm the embryos, fibreplugs were removed from the straws and warmed in a series of sucrose solutions (1 M, and 0.5 M) and PBS plus 5 mg/ml BSA as the final medium for 5 min each, all at ambient temperature. Finally, warmed blastocysts were transferred to the embryo culture medium and incubated in a modular incubator as described above. Following a 24 h and a 48 h culture period, survival rates (re-expansion and hatching) were assessed.

**Statistical analyses**

Data are expressed as means ± SEM. Differences were considered as significant at $P \leq 0.05$ and as tendencies at $P < 0.1$. Relative transcript abundance generated through microarray was analysed with FlexArray: a statistical data analysis software for gene expression microarrays. Specifically, raw data were corrected by background subtraction, then normalized within and between each array (Loess and quantile, respectively). Statistical comparison between treatments was done with the Limma algorithm. Relative transcript abundance generated through qRT-PCR was analysed using the SigmaStat (Jandel Scientific) software package using one-way ANOVA with multiple pairwise comparisons using the Student–Newman–Keuls method post hoc. Other statistical procedures were carried out with PASW Data Collection 5.6 (for Windows, Chicago, IL, USA). To analyse developmental competence and vitrification data, survival rates were compared between the three treatments using a binary logistic regression model taking replicate, treatment, and the interaction term into account. Where no significant interaction was present, the term was omitted from the final model. GSH levels and lipid droplet content data were analysed using a mixed model ANOVA, taking treatment as fixed factor and replicate as random factor. Replicate, treatment, and the interaction term were taken into account. If no significant interaction was present, the term was omitted from the final model. No data transformations were necessary for inequality of variance between groups or for achieving normality for any data.
Results
Developmental competence of oocytes matured in the presence of elevated NEFA

Supplementing maturation medium with a combination of elevated SA, PA and OA concentrations (HIGH COMBI) significantly reduced cleavage and blastocyst rates at 48 h.p.i. and at day 7.5 p.i., respectively ($P < 0.05$) compared to control medium. The HIGH COMBI treatment also tends to reduce the number of cleaved zygotes reaching the blastocyst stage compared with the control and HIGH SA group ($P < 0.1$). The data of the effects of HIGH SA and HIGH COMBI exposure during maturation on developmental competence are presented in Table 6.2.

Table 6.2. Developmental competence of NEFA-exposed oocytes.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>control</th>
<th>HIGH SA</th>
<th>HIGH COMBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes</td>
<td>574</td>
<td>583</td>
<td>590</td>
</tr>
<tr>
<td>Cleaved</td>
<td>396 (69.0) \textsuperscript{a}</td>
<td>377 (64.7) \textsuperscript{ab}</td>
<td>372 (63.0) \textsuperscript{b}</td>
</tr>
<tr>
<td>Blastocysts from oocytes matured</td>
<td>157 (27.4) \textsuperscript{a}</td>
<td>152 (26.1) \textsuperscript{a}</td>
<td>108 (18.3) \textsuperscript{b}</td>
</tr>
<tr>
<td>Blastocysts from cleaved zygotes</td>
<td>157 (39.6) \textsuperscript{a}</td>
<td>152 (40.3) \textsuperscript{a}</td>
<td>108 (29.0) \textsuperscript{b}</td>
</tr>
</tbody>
</table>

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7.5 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Oocytes (n = 1747; 7 replicates) were matured under the following conditions: 1) control (72 µM total, physiological, NEFA), 2) HIGH SA (75 µM SA), and 3) HIGH COMBI (425 µM total, elevated, NEFA). Data marked with a and b superscripts per row are significantly different ($P < 0.06$).

Whole genome expression in blastocysts originating from oocytes matured in the presence of elevated NEFA

Transcriptomic comparison between blastocysts originating from oocytes matured under HIGH COMBI conditions compared to controls revealed that 190 genes were differently expressed of which 85 up- and 105 down-regulated ($P < 0.05$, fold change 1.5, Figure 6.2).
Figure 6.2. Volcano plot of the microarray results in blastocysts originating from oocytes matured under HIGH COMBI conditions (425 μM total, elevated, NEFA) versus blastocysts originating from oocytes matured under control conditions (72 μM total, physiological, NEFA). The dots which are located at the left upper side of the plot are the statistically significantly down-regulated genes, whereas the dots located at the right upper side of the plot are those which are statistically significantly up-regulated ($P < 0.05$ and fold change 1.5).

Major pathways affected in the HIGH COMBI embryos were related to lipid and carbohydrate metabolism (Figure 6.3).
Figure 6.3. Results of pathway analyses with the Ingenuity software for a microarray. The major pathways affected (with $P < 0.05$) in blastocysts originating from oocytes matured under HIGH COMBI conditions (425 µM total, elevated, NEFA) versus blastocysts originating from oocytes matured under control conditions (72 µM total, physiological, NEFA). Pathways with the highest significance of involvement are ranked on top.
In the HIGH SA blastocysts, 38 genes displayed a different transcript abundance compared to the control group, of which 14 were up- and 24 were down-regulated ($P < 0.05$, fold change 1.5, Figure 6.4).

**Figure 6.4.** Volcano plot of the microarray results in blastocysts originating from oocytes matured under HIGH SA conditions (75 µM SA) versus blastocysts originating from oocytes matured under control conditions (72 µM total, physiological, NEFA). The dots which are located at the left upper side of the plot are the statistically significantly down-regulated genes, whereas the dots located at the right upper side of the plot are those which are statistically significantly up-regulated ($P < 0.05$ and fold change 1.5).

Major pathways affected in the HIGH SA embryos were related to lipid metabolism and cell death (Figure 6.5).

Figure 6.6 provides an overview of specific affected genes mapped into main pathways influenced in blastocyst exposed to elevated NEFA concentrations.
Figure 6.5. Results of pathway analyses with the Ingenuity software for a microarray. The major pathways affected (with \( P < 0.05 \)) in blastocysts originating from oocytes matured under HIGH SA conditions (75 µM SA) versus blastocysts originating from oocytes matured under control conditions (72 µM total, physiological, NEFA). Pathways with the highest significance of involvement are ranked on top.
Figure 6.6. Major pathways affected in blastocysts originating from oocytes matured under HIGH COMBI or HIGH SA conditions. In the boxes genes are listed that are differently expressed (DOWN- or UP-regulated) in the HIGH COMBI group compared to control counterparts and in the HIGH SA group compared to control counterparts. HIGH COMBI embryos originate from oocytes matured under HIGH COMBI conditions (425 µM total, elevated, NEFA). HIGH SA embryos originate from oocytes matured under HIGH SA conditions (75 µM SA). Control embryos originate from oocytes matured under control conditions (72 µM total, physiological, NEFA).
Re-evaluation of microarray results through qRT-PCR, with particular focus on genes of interest, in blastocysts originating from oocytes matured in the presence of elevated NEFA

Six of the nine genes analysed were differentially expressed between the treatment groups (Figure 6.7). Multiple of the re-evaluated genes followed the same direction trend as the microarray data. Four major genes were differently expressed both in the data generated through both microarray and qRT-PCR methods. **S100A11**: stress-responsive protein is involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. **MSMO1**: sterol-C4-methyl oxidase-like enzyme is localized in the endoplasmic reticulum membrane and plays a role in cholesterol biosynthesis. **GPCPD1**: glycerophosphocholine phosphodiesterase 1 plays an indispensable role for peroxisomes in the total process of ether lipid synthesis. **SCP2**: sterol carrier protein 2 is an intracellular lipid transfer protein. This gene is highly expressed in organs involved in lipid metabolism.

![Graph](image-url)

**Figure 6.7.** Comparison of relative transcript abundance in blastocysts. Blastocysts originate from oocytes matured under the following conditions: 1) control (72 µM total, physiological, NEFA), 2) HIGH SA (75 µM SA), and 3) HIGH COMBI (425 µM total, elevated, NEFA). The squares designate the validated genes. Quantification was normalized to the endogenous control H2AFZ. Bars marked with a and b superscripts are significantly different (P < 0.05).
Glutathione (GSH) content in oocytes matured in the presence of elevated NEFA

Oocyte maturation under HIGH COMBI conditions resulted in a trend for increased GSH content compared to HIGH SA oocytes (10.3 ± 0.38 vs. 8.78 ± 0.55 pmol/oocyte; *P* < 0.1). No significant difference in GSH content was observed in control oocytes (9.4 ± 1.17 pmol/oocyte) compared to oocytes matured under high NEFA conditions.

Lipid droplet content in morulae originating from oocytes matured in the presence of elevated NEFA

As shown in Table 6.3, oocyte maturation under HIGH COMBI conditions resulted in morulae with an increased total lipid droplet content per cell compared to HIGH SA counterparts (3482.89 ± 491.13 µm³ vs. 1927.59 ± 234.57 µm³; *P* < 0.05). The total number of lipid droplets per cell in the HIGH COMBI and control morulae was higher compared to the HIGH SA counterparts (185.07 ± 19.39 and 178.48 ± 14.84 vs. 113.10 ± 5.76; *P* < 0.05). The mean lipid droplet size was not affected by treatment. No significant difference in lipid droplet content was observed in control morulae compared to morulae originating from both high NEFA treatments.

Table 6.3. Lipid droplet content of morulae originating from oocytes matured under elevated NEFA conditions.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>HIGH SA</th>
<th>HIGH COMBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume lipid droplets per cell (µm³)</td>
<td>2440.23 ± 236.52^a^</td>
<td>1927.59 ± 234.57^a^</td>
<td>3482.89 ± 491.13^b^</td>
</tr>
<tr>
<td>Size lipid droplets (µm³)</td>
<td>14.33 ± 1.23</td>
<td>16.68 ± 1.66</td>
<td>17.56 ± 1.92</td>
</tr>
<tr>
<td>Number of lipid droplets per cell</td>
<td>178.48 ± 14.84^a^</td>
<td>113.10 ± 5.76^b^</td>
<td>185.07 ± 19.39^a^</td>
</tr>
</tbody>
</table>

Lipid droplet content (mean ± SEM) in morulae 150 h p.i. (n = 74; 3 replicates) did originate from oocytes matured under the following conditions: 1) control (72 µM total, physiological, NEFA), 2) HIGH SA (75 µM SA), and 3) HIGH COMBI (425 µM total, elevated, NEFA). Data marked with a and b superscripts per row are significantly different (*P* < 0.05).
Cryotolerance of blastocysts originating from oocytes matured in the presence of elevated NEFA

No differences in re-expansion rates at 24 h post warming could be found between treatments. Embryos originating from HIGH SA-exposed oocytes showed a tendency for lower re-expansion rate at 48 h post warming compared with controls (38.6 % vs. 54.0 %; $P < 0.1$).

Discussion

Elevated NEFAs have been implicated as key factor in the association between metabolic imbalances, cellular dysfunction and related pathologies (Wu et al., 2000; McGarry et al., 2002; Lu et al., 2003; Ulloth et al., 2003). Exposing bovine oocytes to elevated NEFA concentrations not only reduces the oocyte’s development (Jorritsma et al., 2004; Leroy et al., 2005; Aardema et al., 2011), but also impacts on the resultant blastocyst physiology (Van Hoeck et al., 2011; 2013). In this context, we previously showed that embryos arising from fertilized, NEFA-exposed oocytes have a significantly lower cell number, increased apoptotic cell index, aberrant transcriptional activities, altered amino acid turnover, and altered metabolism, with particular reference to glucose intolerance and a reduced oxidative activity (Van Hoeck et al., 2011; 2013), all indicators for a lower embryo quality and viability. By performing microarray analyses using the Bovine EmbryoGENE network, we were able to identify pathways affected in blastocysts originating from NEFA exposure during final oocyte maturation. Connecting these gene expression data to functional/biochemical information at different stages (GSH at oocyte level, lipid droplet content at morula level and cryotolerance at blastocyst level) helped to reveal the potential mechanisms through which elevated NEFA concentrations can impact.

Follicular conditions under which the oocyte completes final maturation are recognized to influence gene expression patterns in subsequent embryos (Latham and Sapienza, 1998; Watson et al., 1999). Our data confirm this, as final oocyte maturation under high NEFA conditions resulted in altered gene expression profiles of the resultant day 7.5 blastocysts. In this study, we have built upon our previous findings (Van Hoeck et al., 2011; 2013) to describe a possible mechanism for the
manner in which suboptimal metabolic conditions, more specifically up-regulated lipolysis, during the time of ovulation and early conception can have a profound effect on the phenotype and gene expression profile of the early pre-implantation embryo. Transcriptional events need to be validated, but are highly relevant to gain information about mechanistic events going on at the cellular level. NEFAs are substrates for pathways that use fatty acids for I) energy production, II) ceramide generation, III) complex lipogenic pathways, or IV) for other processes (for example omega-oxidation in the endoplasmic reticulum). As shown in Figure 6.6, the expression of genes related to the oxidative pathway (Pathway I) and the lipid synthesis pathway (Pathway III) was predominantly influenced in blastocysts originating from high NEFA-exposed oocytes. These pathways will be discussed more into depth and will be linked to the functional data available.

Gene expression related to oxidative metabolism was influenced in blastocysts originating from HIGH SA- and HIGH COMBI-exposed oocytes compared to control blastocysts (Figure 6.6), which is consistent with the findings from our previous studies (Van Hoeck et al., 2011; 2013a). Bovine oocytes oxidize fatty acids during oocyte maturation (Ferguson and Leese, 1999; Paczkowski et al., 2012). When fatty acid supply is plentiful, there might be up-regulation of mitochondrial activity (Iossa et al., 2002), which results in elevated levels of reactive oxygen species (ROS) (Burton et al., 2003). Functional analyses showed that, after maturation, HIGH SA-exposed oocytes tend to contain less GSH compared to HIGH COMBI-exposed oocytes. We hypothesize that this reduced GSH level in HIGH SA matured oocytes might be the result of more GSH depletion in oocytes, matured under HIGH SA conditions, in order to neutralize excessive ROS levels. However, one can also assume that HIGH SA oocytes were not properly provided with GSH by their corresponding cumulus cells as we previously documented on a significant down-regulated expression of GPX1 in HIGH SA-exposed cumulus cells (Van Hoeck et al., 2013). In this context, it is obvious that cumulus cells are vital for the oocyte to complete successful maturation and sustain further development (Tanghe et al., 2002). They play a crucial role in down-regulating oxidative stress levels homeostasis by providing GSH to the oocyte (Geshi et al., 2000). Low intracellular GSH may be related to, or responsible for, lower
developmental competence in *in vitro* matured oocytes (Sutton-McDowell *et al.*, 2004) as GSH plays a major antioxidant role during pre-implantation embryonic development (Cagnone *et al.*, 2012). Interestingly, also a significantly increased expression of *GADH* has been described in HIGH COMBI oocytes (Van Hoeck *et al.*, 2013). The *GADH* enzyme will spare NAD(P)H, which is essential for the recycling of GSH. Such profound alterations at gene expression level might also become apparent at functional level.

As shown in Figure 6.6, blastocysts originating from HIGH COMBI exposed oocytes displayed a significant increase in RNA encoding enzymes involved in lipid synthesis pathways; for example *MSMO1*, *GPCPD1*, and *SCP2*. *MSMO1* plays an important role in cholesterol biosynthesis and *GPCPD1* in the process of ether lipid synthesis. *SCP2* increases fatty acid uptake and targets fatty acids to unique lipid pools. Apparently, the expression of *AGPAT* was up-regulated in HIGH COMBI blastocysts compared to HIGH SA counterparts. *AGPAT* plays a role in the formation of two important types of lipids: glycerophospholipids and triacylglycerols. The fact that our previous study (Van Hoeck *et al.*, 2013) documented on a parallel increase in the mRNA abundance of *ACSL1* and *ACCA* is highly suggestive of an overall increase in lipogenetic activities in such embryos compared to embryos originating from HIGH SA-treated oocytes. In this context, functional analyses revealed that morulae originating from HIGH COMBI-exposed oocytes contain a larger volume of lipid droplets per cell. We endeavoured to visualize lipid droplets at the blastocyst stage as well, but fluorescence distraction, caused by the blastocoel, makes it difficult to correctly interpret the resultant data. However, only the blastocysts from the HIGH SA treatment tended to display a lower cryotolerance compared to the control blastocysts, whereas the freezing capacity of the HIGH COMBI blastocysts was unaffected. The latter is somehow surprising, because embryos with large amounts of vesicles and high lipid content are assumed to be more sensitive to cryopreservation procedures (Mohr and Trounson, 1981; Leibo and Loskutoff, 1993). The ability to store fatty acids in lipid droplets (mostly containing triacylglycerol) in the HIGH COMBI embryos might have a protective function to avoid conversion of the acyl-CoA to other toxic down-stream lipid metabolites, such as reactive oxygen species.
(Cnop et al., 2001; Burton et al., 2003; Li et al., 2010). Stored within cells in lipid droplets, triacylglycerol itself is probably harmless, but both the lipolysis of intracellular triacylglycerol stores and the pathway of triacylglycerol synthesis can produce fatty acids and lipid intermediates (as shown in Figure 6.6; Pathway II) that have direct deleterious properties, for example ceramides (Shimabukuro et al., 1997; Shimabukuro et al., 1998; Maedler et al., 2001; Lu et al., 2003). The fact that expression of PLIN3, a protein that facilitates lipase access to the droplet under lipolytically stimulating conditions (Souza et al., 1998; 2002; Brasaemle et al., 2000) was significantly down-regulated in the HIGH COMBI blastocysts compared to their control counterparts, might corroborate this ‘protective’ theory of a lipogenetic shift in the HIGH COMBI embryos.

It is important to consider that the embryos analysed in this study (and our previous studies; Van Hoeck et al., 2011; 2013), are those that made it to the blastocyst stage and therefore have survived the negative effects (at least till day 7.5 p.i.). Whether the genes are differently expressed due to compensatory mechanisms in order to ‘cope’ with the stress or whether these are ‘disease’-indicative genes remains to be elucidated. In this context, the generation of functional data at oocyte and morula level, which also included information from oocytes and zygotes with compromised development, was vital. Yet, the question whether the activated pathways towards lipid storage provide protective/compensatory mechanisms or rather activate other toxicity-related pathways remains to be clarified. Both options may have an important impact during further embryonic and fetal development, an impact that might not become apparent until post-implantation or even the post-natal stage (Watkins et al., 2008; Jungheim et al., 2011). Remarkably, in somatic cell lines, excessive accumulation of lipids in peripheral tissues has been closely associated with the pathological state of insulin resistance in type II diabetes (Zhang and Zhang, 2012).

In conclusion, transcription of genes related to oxidative stress responses was altered in blastocysts originating from HIGH SA- and HIGH COMBI-exposed oocytes. Moreover, the expression of genes related to fatty acid synthesis was up-regulated in blastocysts originating from the HIGH
COMBI-exposed oocytes. The pathways affected by NEFA-treatments during *in vitro* maturation are highly dependent on the specific types of NEFA used in each treatment. Changing the ratio of specific NEFA types in the *in vitro* maturation medium can jeopardize development and even cellular processes of resultant embryos, an issue that extends to human fertility. Appropriate regulation of metabolism is seen as an increasingly important aspect of disease progression, and the present study, in conjunction with our previous studies (Van Hoeck *et al.*, 2011; 2013), supports this notion in reproductive medicine and more specifically in early development.
List of References


CHAPTER 7

Elevated concentrations of non-esterified fatty acids during bovine embryo culture compromise pre-implantation embryo development and influence gene transcription.


Abstract

Elevated non-esterified fatty acid (NEFA) concentrations represent a common feature of a distorted maternal metabolic health, typically present in obesity, type II diabetes patients or during a period of negative energy balance (NEB). These metabolic disorders are associated with decreased fertility and there is growing evidence that elevated NEFA concentrations play a key role in this process. Reports about the consequences of NEFA exposure during final oocyte maturation are available yet. However, little is known about the direct effects of elevated NEFA concentrations during pre-implantation embryo development. Therefore, bovine oocytes were matured and fertilized in vitro following standard procedures. Resulting zygotes were cultivated for 6.5 days in medium containing 1) physiological NEFA concentrations = control, 2) elevated stearic acid (SA) concentrations = HIGH SA and 3) elevated NEFA concentrations = HIGH COMBI. A reduced developmental potential was observed for embryos exposed to the HIGH SA and HIGH COMBI treatment compared to control embryos. Nevertheless, HIGH COMBI embryos had a higher capacity to develop into blastocysts when compared to HIGH SA embryos. Elevated NEFA concentrations during
embryo culture influenced gene expression patterns in subsequent blastocysts through multiple pathways, mainly related to oxidative metabolism and ceramide formation. In conclusion, embryonic exposure to elevated NEFA concentrations impairs embryo development and affects gene expression patterns in resultant blastocysts. The extent of the impact seems to depend on the type of NEFA applied. These data show that elevated NEFA concentrations in the micro-environment of the developing zygote can be responsible for the high rates of embryonic loss observed in females suffering lipolytic disorders.

Introduction
Reduced oocyte and embryo quality are recognized as major factors in the problem of disappointing fertility in modern dairy cows (Leroy et al., 2008; 2012), but also in women suffering metabolic conditions related to lipolysis, such as obesity and type II diabetes (Pasquali et al., 2003). Maternal metabolic disorders, linked to lipolytic conditions, are characterized by the break-down of own lipid stores, leading to elevated non-esterified fatty acid (NEFA) concentrations in the blood and follicular fluid, which in turn jeopardize follicle cell viability and affect the oocyte’s developmental capacity (Jorritsma et al., 2004, Leroy et al., 2005a; Aardema et al., 2011). However, a hampered oocyte development is not the only concern in females with a distorted maternal metabolism. Maternal lipolytic conditions are a risk factor for early embryo mortality (Santos et al., 2004). Embryo survival is significantly reduced in high yielding dairy cows suffering negative energy balance (Dunne et al., 2000; Lucy, 2001; Santos et al., 2004; Diskin and Morris, 2008). In this context, Satori et al. (2002) and Leroy et al. (2005b) showed that the proportion of viable bovine embryos was much lower in lactating cows compared to non-lactating cows. More than 40% of the dairy cow conceptuses are lost within two weeks post insemination, suggesting that oocyte and early embryo quality is compromised (Leroy et al., 2008). A considerable proportion of embryo loss may be attributable to inadequate circulating progesterone concentrations (Lonergan, 2011), however additional factors are involved as well.
Also clinical centers, providing human assisted reproductive technology services, report that human oocytes from obese patients have reduced oocyte developmental potential and that obese women experience higher abortion rates during early pregnancy (Zaadstra et al., 1993; Fedorcsak et al., 2000; Metwally et al., 2007; Bellver et al., 2010). Efforts to clarify these high rates of embryonic loss focused on the effect of maternal metabolic health on the oocyte during its final maturation period. Using a bovine in vitro maturation model, we could demonstrate that elevated NEFA concentrations during final oocyte maturation can cause carry-over effects towards the day 7.5 blastocyst stage reflected by a reduced embryonic quality, lower viability and distorted oxidative metabolism (Van Hoeck et al., 2011; 2013a; 2013b). However, the newly formed embryo is also highly sensitive to changes in its micro-environment (Rizos et al., 2003; Leese et al., 2008). Recently, Maillo et al. (2012) emphasized this by showing that the reproductive tracts of negative energy balance (NEB) cows have a compromised ability to support early embryo development compared to non-lactating cows. This further stresses the importance of the micro-environment in optimal development of young embryos.

The embryonic pre-implantation stage is a critical time in the growth of an organism marked by increased energy demands and sensitivity to availability of maternal nutrients (Tsujii et al., 2009). As such, embryos respond to changes in their in vitro environment with altered metabolic (Reis et al., 2003) and transcriptional activities (Edirisigne et al., 1984; Johnson and Nasr-Esfahani, 1994; Barnett and Bavister, 1996). For example, ovine embryos cultured in a medium supplemented with human serum contain abundant lipid droplets and develop into lambs with a higher birth weight than those cultured in serum-free medium (Thompson et al., 1995). We previously reported that culture of pre-implantation embryos to dietary induced hyperlipidemic conditions reduces embryo development and quality (Leroy et al., 2010). The mechanistic insights for these findings are lacking so far, as the supplemented sera contain lipid fractions that are significantly different compared to those of control serum. Focussing on saturated NEFAs, Jungheim et al. (2011) demonstrated that blastocyst exposure to elevated concentrations of palmitic acid (PA) affects murine embryo quality. In
addition, Nonogaki et al. (1994) documented that elevated PA and polyunsaturated fatty acids (PUFA) concentrations, including linoleic acid (LA), inhibit embryo development in mice. This could be overcome by the addition of antioxidants, which shows that increased oxidative stress levels may be, at least partially, responsible for the reduced development. Information on possible direct consequences of pathophysiological NEFA concentrations during bovine early embryo development is not available so far. We hypothesized that elevated NEFA concentrations may be mechanistically involved in the high rates of embryonic mortality observed in females suffering lipolytic conditions. Therefore, the present study aims to:

- examine the effect of elevated NEFA concentration during 6.5 days of bovine embryo culture on further embryo development and morphology.
- unravel relevant metabolic pathways by measuring the relative RNA abundance of candidate genes in blastocysts originating from embryos exposed to elevated NEFA concentrations during culture.

The combination of the new transcriptomic data and the developmental data, should improve our current understanding on the possible mechanisms through which specific lipolytic disorders can impact on embryo survival rates.

**Materials and Methods**

**Preparation of NEFA treatments**

All chemicals were purchased from Sigma (Bornem, Belgium), unless otherwise stated. Stearic acid (SA, C18:0), palmitic acid (PA, C16:0), and oleic acid (OA, C18:1) were dissolved in a stock solution of pure ethanol at concentrations of 25, 150 and 200 mM, respectively. These ethanol stock solutions were vortex-mixed for 4 min and diluted in final solutions to obtain the desired final concentration in the culture medium. The final medium is supplemented with 0.75% BSA free of fatty in order to improve NEFA solubility. All treatments were vigorously sonificated for 4 h and filter-sterilized under aseptic conditions.
In vitro embryo production

In vitro embryo production procedures were performed as previously described (Van Hoeck et al., 2011; 2013a), using immature oocytes retrieved from bovine ovaries collected within 2 h of slaughter. Briefly, unexpanded cumulus oocyte complexes (COCs) surrounded by five or more cumulus cell layers (quality grade I) were matured in vitro. Therefore, COCs were washed in 500 ml maturation medium and matured in groups of 50-60 COCs in 500 ml serum-free maturation medium containing TCM199 supplemented with 0.4 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 mg/mL Gentamycin and murine epidermal growth factor (mEGF, 20 ng/ml) in four-well plates (Nunc, Langenselbold, Germany) for 24 h in humidified air with 5% CO₂ at 38.5°C. After in vitro maturation, COCs were co-incubated in groups of 100-120 with spermatozoa at a final concentration of 10⁶ sperm cells/ml for 20 h at 38.5°C in 500 ml fertilisation medium (containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM Na₂HPO₄, 2.1 mM CaCl₂·2H₂O, 0.4 mM MgCl₂·6H₂O, 25 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, 2 µL/mL phenol red, 6 mg/mL BSA, 50 µg/mL Gentamycin and 10 µL/mL heparin) in a humidified 5% CO₂ incubator. Frozen semen from a bull of proven in vitro fertility (Van Hoeck et al., 2011; 2013a; 2013b) was thawed and live spermatozoa were selected by centrifugation on a discontinuous Percoll gradient (90 and 45%, Biosciences, Amersham, UK). The final sperm-egg ratio was adjusted to 5000:1. After co-incubation with spermatozoa, the presumptive zygotes were vortex-mixed for 3.5 min to remove excess sperm and cumulus cells. Subsequently, zygotes were rinsed twice more in the 4 well dish containing the treatment-specific culture medium and were incubated per 20-25 in reduced surface wells of a 96 well dish containing 75 µl medium without mineral oil overlay (modular incubator: 38.5°C, 5% CO₂, 5% O₂ and 90% N₂) during 6.5 days. The basic culture medium used in this study is synthetic oviductal fluid (SOF) medium (AD; 0.34 mM Na Citrate; 2.78 mM Myoinositol; 0.108 mM NaCl; 7.16 mM KCl; 1.19 mM KH₂PO₄; 0.822 mM MgSO₄·7H₂O; 0.06% v/v Na DL-Lactate; 25 mM NaHCO₃; 0.0266 mM Phenol Red; 0.727 mM Na Pyruvate; 1.78 mM CaCl₂·2H₂O; 3% v/v BME 50x; 1% v/v MEM 100x; 0.4 mM glutamine; 50 µg/ml Gentamycin) enriched with 5% serum and 0.75% BSA free of fatty acids.
Experimental design
The types and concentrations of free fatty acids used in this study are based on bovine in vivo serum studies (Leroy et al., 2005a) and are pathophysiologically appropriate. As in our previous studies (Leroy et al., 2005a; Van Hoeck et al., 2011) SA was the most toxic fatty acid in terms of disrupting final oocyte maturation and as the chemical structure of SA (C18:0) is mostly reliant to the structure of the mono-unsaturated OA (C18:1), we optioned to implement SA as additional saturated NEFA treatment. We have tried to distinguish effects induced by saturated NEFAs (SA) versus effects induced by a combination of saturated (SA and PA) and mono-unsaturated (OA) NEFAs. In order to improve the relevance of our in vitro model, we used a culture medium supplemented with physiological NEFA concentrations as control medium (Van Hoeck et al., 2011; 2013) and added 5% foetal calf serum (FCS) (to mimic dynamic interactions between the supplemented NEFAs and the other lipid fractions present in the serum).

Experiment 1. The developmental consequences of embryonic exposure to elevated NEFA concentrations:
In order to evaluate the effect of elevated NEFA exposure on embryo development, embryos were exposed to the following NEFA treatments during 6.5 days:
1. control: physiological NEFA concentrations (72 µM total NEFA containing 28 µM SA, 23 µM PA, and 21 µM OA).
2. HIGH SA: elevated SA concentrations as under high lipolytic conditions (280 µM SA).
3. HIGH COMBI: combination of elevated NEFA concentrations as under high lipolytic conditions (720 µM total NEFA, containing 280 µM SA, 230 µM PA, and 210 µM OA).
Each treatment was tested for its effect on cleavage rate (48 h post insemination (p.i.)) and blastocyst yield (7.5 days p.i.). In total, 780 bovine compact COCs were matured, fertilized and cultured (divided over 3 replicates).
Experiment 2. The developmental consequences of embryonic exposure to SA - a dose response curve:
Exposure of embryos during 6.5 days of culture to the HIGH SA condition (described in experiment 1) was lethal. However, in order to generate embryos to gain mechanistic insights in potential pathways affected in blastocysts originating from HIGH SA-exposed embryos, the dose of SA applied during culture must not be lethal, but toxic. To identify such a dose that allowed blastocyst formation to a certain extent, we determined a dose response curve of developmental effects when SA was applied during embryo culture.
Therefore, embryos were exposed for 6.5 days to the following NEFA treatments:
1. 0 µM SA.
2. 75 µM SA.
3. 150 µM SA.
4. 300 µM SA.
Each treatment was tested for its effect on cleavage rate (48 h p.i.) and blastocyst yield (7.5 days p.i.). In total, 823 bovine compact COCs were matured, fertilized and cultured in this experiment (divided over 3 replicates).

Experiment 3. Production of embryos for whole transcriptome analyses in resultant day 7.5 blastocysts:
To generate embryos to perform microarray analyses in resultant day 7.5 blastocysts, embryos were exposed to adapted NEFA treatments, based on the findings in experiment 2, for 6.5 days:
1. control: physiological NEFA concentrations (72 µM total NEFA containing 28 µM SA, 23 µM PA, and 21 µM OA).
2. HIGH SA: elevated SA concentrations (100 µM SA).
3. HIGH COMBI: combination of elevated NEFA concentrations (720 µM total NEFA, containing 280 µM SA, 230 µM PA, and 210 µM OA).
Large-scale analysis of gene transcription profiles was performed using the microarray technique (972 oocytes were matured in 4 replicates) and results were validated using the quantitative real-time PCR (qRT-PCR) method.
Embryo developmental competence assessment

Cleavage (48 h p.i.), and blastocyst rates (7.5 days p.i.) were defined as the number of cleaved zygotes or formed blastocysts per oocyte matured, respectively. The number of blastocysts from cleaved zygotes was also evaluated.

Quantification of gene transcription through microarray technology

Gene expression analyses using the bovine EmbryoGENE microarray slides were performed as previously described (Cagnone et al., 2012). Total RNA from each replicate was extracted and purified using the PicoPure RNA Isolation Kit (Life Science, Ottawa, Ontario). After DNase digestion (Qiagen, Toronto, Canada), quality and concentration of extracted RNA were analysed by a bioanalyzer (Agilent, Diegem, Belgium). All extracted samples showed good quality with an RNA integrity number > 7.5. For microarray purposes, purified RNA was amplified by in vitro transcription by T7 RNA amplification using the RiboAmp HSPlus RNA Amplification Kit (Life Science, Ottawa, Ontario) and labelled with Cy3 and Cy5 using the ULS Fluorescent Labelling Kit (Kreatech, Durham, North America), and antisense RNA (825 ng per replicate) was hybridized on the Agilent-manufactured EmbryoGENE slides in a two-colour dye-swap design. Following 17 h of hybridization at 65°C, microarray slides were washed for 1 min in gene Expression Wash Buffer 1 (room temperature), 3 min in gene Expression Wash Buffer 2 (42°C), 10 sec in 100% acetonitrile (room temperature) and 30 sec in Stabilization and Drying Solution (Agilent, Diegem, Belgium). Slides were scanned with PowerScanner (Tecan, Männedorf, Switzerland) and features extraction was done with Array-pro6.3 (MediaCybernetics, Warrendale, Pennsylvania). Data were analysed through the use of Ingenuity Pathways Analysis (IPA; Ingenuity Systems, www.ingenuity.com), which served to identify pathways that were differentially expressed between treatments. A fold change cutoff of 1.5 with a P value < 0.05 was set to identify genes whose expression was significantly differentially regulated.

Re-evaluation of data generated with microarray through qRT-PCR

Re-evaluation of microarray results was done by qRT-PCR. Poly(A) RNA of day 7.5 blastocysts was extracted using a Dynabead mRNA Direct
Extraction KIT (Dynal Biotech, Madrid, Spain) according to the manufacturer’s instructions, with minor modifications. Immediately after extraction, the RT reaction was carried out following the manufacturer’s instructions (Bioline, London, UK), using poly(T) primer, random primers, and the MMLV reverse transcriptase enzyme, in a total volume of 40 µl, in order to prime the RT reaction and to synthesize cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and the RT reaction was completed with the addition of 100 units of reverse transcriptase. The mixture was incubated at 42°C for 60 min to allow the RT of RNA, followed by 70°C for 10 min to denature the RT enzyme. Four groups of cDNA per experimental group were used with three replicates for all genes of interest. Experiments were conducted to compare relative levels of each transcript and histone H2AFZ in every sample. The PCR was performed by adding a 2 µl aliquot of each sample to the PCR mix, containing the specific primers, to amplify the genes of interest. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 7.1. Quantification was performed as described previously (Bermejo-Alvarez et al., 2010). PCR conditions were optimized to achieve efficiencies close to 1 and the comparative cycle threshold (CT) method used to quantify expression levels as described by Schmittgen and Livak (2008). Quantification was normalized to the endogenous control H2AFZ. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the control gene (H2AFZ) CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2-ΔΔCT.
Table 7.1. Details for primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Fragment Size (bp)</th>
<th>Gene Bank Accession No.</th>
</tr>
</thead>
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<tr>
<td>H2AFZ</td>
<td>AGGAGCAGTAAGCCATGCGATGCTG CACCCACACGACATGGCCGCCCTTGG</td>
<td>209</td>
<td>NM_174809.2</td>
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<tr>
<td>RAB34</td>
<td>TGGCTGAGCTGGAGAAACCG GCTCTGGGGGAGCAGTGGG</td>
<td>262</td>
<td>NM_001033612.1</td>
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<tr>
<td>CNN3</td>
<td>CCAAGGCAAGCAAGTGACAC CCAGTGTCCAGGAGTG</td>
<td>203</td>
<td>NM_001038179.1</td>
</tr>
<tr>
<td>CNN1</td>
<td>TACAGGGTTACGCCGCTGGGG AAAGTGGCTGGGTCAGAAC</td>
<td>348</td>
<td>BT030714.1</td>
</tr>
<tr>
<td>FADS</td>
<td>GGTTGCGAGGGACATTTCG ACATTGCGCACGAAACTGAG</td>
<td>281</td>
<td>NM_001083691.2</td>
</tr>
<tr>
<td>SIRT2</td>
<td>CCCGAGAATCTCCACAC CTCGTCGCCACGAGAATG</td>
<td>262</td>
<td>EU562194.1</td>
</tr>
<tr>
<td>IDH</td>
<td>CGAGGGCCTTTCTCCAGTT CAGCAGAGCTGAGG</td>
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<td>ERK6</td>
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<td>RASG</td>
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<td>281</td>
<td>NM_001193020.1</td>
</tr>
</tbody>
</table>

**Statistical analyses**

Data are expressed as means ± SEM. Differences were considered as significant at $P < 0.05$ and as tendencies at $P < 0.1$. Relative transcript abundance generated through microarray was analysed with FlexArray: a statistical data analysis software for gene expression microarrays. Specifically, raw data were corrected by background subtraction, then normalized within and between each array (Loess and quantile, respectively). Statistical comparison between treatments was done with the Limma algorithm. Relative transcript abundance generated through qRT-PCR was analysed using the SigmaStat (Jandel Scientific) software package using one-way ANOVA with multiple pairwise comparisons using the Student–Newman–Keuls method post hoc. Other statistical procedures were carried out with PASW Data Collection 5.6 (for Windows, Chicago, IL, USA). To analyse developmental competence data, a binary logistic regression model was used taking replicate, treatment and the interaction term into account. Where no significant interaction was present, the term was omitted from the final model. No data
transformations were necessary for inequality of variance between groups or for achieving normality for any data.

Results

Experiment 1. The developmental consequence of embryonic exposure to pathophysiologically relevant, elevated NEFA concentrations

Cleavage rates for zygotes cultured under HIGH SA conditions (60.85%) were significantly lower compared to controls (72.93%) ($P < 0.01$). Exposing zygotes to HIGH SA or HIGH COMBI conditions resulted in a significant reduction of the number of oocytes reaching the blastocyst stage at day 7.5 p.i. (1.55% and 12.11%, respectively) compared to control zygotes (23.31%) ($P < 0.001$). A significantly better development was observed for cleaved zygotes from the HIGH COMBI group to develop into day 7.5 blastocysts when compared to the HIGH SA zygotes ($P < 0.001$). The capacity of cleaved zygotes to become blastocysts was also drastically lower in the HIGH SA and HIGH COMBI group (2.55% and 18.67%, respectively) compared to control zygotes (31.96%) ($P < 0.01$). The developmental competence data are presented in Table 7.2.

Morphological appearance of blastocysts originating from high NEFA-exposed embryos: the opacity of embryos exposed to the HIGH COMBI treatment, was lower compared to the control and HIGH SA-exposed embryos, which is suggestive of an increased intracellular lipid content of the blastomeres (Figure 7.1).
Table 7.2. Developmental competence of bovine embryos exposed to elevated NEFA concentrations during 6.5 days of *in vitro* culture.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>control</th>
<th>HIGH SA</th>
<th>HIGH COMBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes</td>
<td>266</td>
<td>258</td>
<td>256</td>
</tr>
<tr>
<td>Cleaved</td>
<td>194 (72.93) (^a)</td>
<td>157 (60.85) (^b)</td>
<td>166 (64.84) (^{ab})</td>
</tr>
<tr>
<td>Blastocysts from oocytes matured</td>
<td>62 (23.31) (^a)</td>
<td>4 (1.55) (^b)</td>
<td>31 (12.11) (^c)</td>
</tr>
<tr>
<td>Blastocysts from zygotes</td>
<td>62 (31.96) (^a)</td>
<td>4 (2.55) (^b)</td>
<td>31 (18.67) (^c)</td>
</tr>
</tbody>
</table>

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7.5 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Embryos were cultured under the following conditions: 1) control (72 µM total, physiological, NEFA), 2) HIGH SA (280 µM SA), and 3) HIGH COMBI (720 µM total, elevated, NEFA). Data marked with a and b superscripts per row are significantly different \((P < 0.05)\).

Figure 7.1. Conventional microscopic images (100x magnification) of embryos originating from zygotes cultured under: A) control (72 µM physiological NEFA); B) HIGH SA (280 µM SA); and C) HIGH COMBI (720 µM elevated NEFA).

Experiment 2. The developmental consequences of embryonic exposure to SA: a dose response curve

Cleavage rates of zygotes cultured in 150 µM SA (57.21%) and 300 µM SA (35.43%) were significantly lower compared to controls (69.18%) \((P < 0.01)\). Furthermore, cleavage rates of zygotes cultured in the presence of the highest SA concentration (300 µM) (35.43%) were significantly lower compared to control zygotes (69.18%) and zygotes cultured in 75 µM SA (62.67%) and 150 µM SA (57.21%) \((P < 0.001)\). Exposing zygotes to 150 µM and 300 µM SA resulted in a significant reduction of the number of oocytes reaching the blastocyst stage at day 7.5 p.i. (5.24% and 0%, respectively) when compared to control zygotes (26.03%) and zygotes exposed to 75 µM (20%) \((P < 0.001)\). The capacity of cleaved zygotes to
become blastocysts showed the same significant differences: cleaved zygotes exposed to 150 µM (9.16%) and 300 µM SA (0%) had a significant reduction of their developmental competence compared to controls (37.62%) and cleaved zygotes exposed to 75 µM SA (31.91%) ($P < 0.001$). Developmental competence data are presented in Table 7.3.

**Table 7.3.** Developmental competence of bovine embryos exposed to different SA concentrations during 6.5 days of *in vitro* culture.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>0 µM SA</th>
<th>75 µM SA</th>
<th>150 µM SA</th>
<th>300 µM SA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146</td>
<td>225</td>
<td>229</td>
<td>223</td>
<td></td>
</tr>
<tr>
<td><strong>Cleaved</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>101 (69.67)</td>
<td>141 (62.67)</td>
<td>131 (57.21)</td>
<td>79 (35.43)</td>
<td></td>
</tr>
<tr>
<td><strong>Blastocysts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from oocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38 (26.03)</td>
<td>45 (20)</td>
<td>12 (5.24)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><strong>Blastocysts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from zygotes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38 (37.62)</td>
<td>45 (31.91)</td>
<td>12 (9.16)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7.5 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Embryos were cultured under the following conditions: 1) 0 µM SA, 2) 75 µM SA, 3) 150 µM SA, 4) 300 µM SA. Data marked with a and b superscripts per row are significantly different ($P < 0.05$).

**Experiment 3. Whole genome expression in blastocysts originating from embryos cultured in the presence of elevated NEFA**

Based on the dose response curve for SA, 100 µM SA was identified as toxic dose that allowed appropriate blastocyst formation to perform microarray analyses. The developmental consequence of embryonic exposure to elevated NEFA concentrations with adapted HIGH SA treatment are presented in Table 7.4.
Table 7.4. Developmental competence of bovine embryos exposed to adapted NEFA treatments during 6.5 days of in vitro culture.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>control</th>
<th>HIGH SA</th>
<th>HIGH COMBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes</td>
<td>319</td>
<td>326</td>
<td>327</td>
</tr>
<tr>
<td>Cleaved</td>
<td>234 (73)(^a)</td>
<td>221 (68)(^{ab})</td>
<td>211 (65)(^b)</td>
</tr>
<tr>
<td>Blastocysts from oocytes matured</td>
<td>93 (29)(^a)</td>
<td>93 (29)(^a)</td>
<td>52 (16)(^b)</td>
</tr>
<tr>
<td>Blastocysts from zygotes</td>
<td>93 (40)(^a)</td>
<td>93 (42)(^a)</td>
<td>52 (25)(^b)</td>
</tr>
</tbody>
</table>

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7.5 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Embryos were cultured under the following conditions: 1) control (72 µM total, physiological, NEFA), 2) HIGH SA (100 µM SA), and 3) HIGH COMBI (720 µM total, elevated, NEFA). Data marked with a and b superscripts per row are significantly different ($P < 0.05$).

Transcriptomic comparison between blastocysts originating from embryos cultivated under HIGH COMBI conditions compared to controls revealed that 311 genes were differently expressed of which 206 up- and 105 down-regulated ($P < 0.05$ and fold change 1.5; Figure 7.2). Top networks affected were related to i) cell morphology, cell-to-cell signalling and interaction, hematological system development and function; ii) lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism; iii) lipid metabolism, small molecule biochemistry, molecular transport.
Figure 7.2. Volcano plot of the microarray results in blastocysts originating from embryos cultured under HIGH COMBI conditions (720 µM NEFA) versus blastocysts originating from embryos cultured under control conditions (72 µM total, physiological, NEFA). The dots which are located at the left upper side of the plot are the statistically significantly down-regulated genes, whereas the dots located at the right upper side of the plot are those which are statistically significantly up-regulated ($P < 0.05$ and fold change 1.5).

In the HIGH SA blastocysts, 358 genes displayed a different transcript abundance compared to the control group, of which 292 up- and 66 down-regulated ($P < 0.05$ and fold change 1.5; Figure 7.3). Major pathways affected in the HIGH SA embryos were related to lipid metabolism and cell death. Top networks affected were related to i) amino acid metabolism, post-translational modification, small molecule biochemistry; ii) cellular assembly and organization, cellular compromise, cancer; iii) organ morphology, cell-to-cell signaling and interaction, cellular growth and proliferation. IPA identified the NRF2-mediated oxidative stress response as the top canonical pathway in the HIGH SA embryos compared to control embryos.
Figure 7.3. Volcano plot of the microarray results in blastocysts originating from embryos cultured under HIGH SA conditions (100 µM SA) versus blastocysts originating from embryos cultured under control conditions (72 µM total, physiological, NEFA). The dots which are located at the left upper side of the plot are the statistically significantly down-regulated genes, whereas the dots located at the right upper side of the plot are those which are statistically significantly up-regulated ($P < 0.05$ and fold change 1.5).

Figure 7.4 provides an overview of the major genes that were mapped into main pathways influenced in blastocyst exposed to elevated NEFA concentrations.
Figure 7.4. Major pathways affected in blastocysts originating from embryos cultured under HIGH COMBI or HIGH SA conditions. The boxes highlight genes that are differently expressed (DOWN- or UP-regulated) in the HIGH COMBI and HIGH SA group compared to control counterparts. HIGH COMBI embryos originate from embryos cultured under HIGH COMBI conditions (720 µM total, elevated, NEFA). HIGH SA embryos originate from embryos cultured under HIGH SA conditions (100 µM SA). Control embryos originate from oocytes matured under control conditions (72 µM total, physiological, NEFA).
Re-evaluation of microarray results through qRT-PCR, with particular focus on genes of interest, in blastocysts originating from embryos cultivated in the presence of elevated NEFA.

Several re-evaluated genes (6 out of 9) followed the same expression direction trend as the microarray data.

**RAB34** activates several pathways, of which the mitogen-activated protein (MAP) kinase cascade has been well-studied. This cascade transmits signals down-stream and results in the transcription of genes involved in cell growth and division.

**DHR** (dehydrogenase/reductase): constitutes a large family of NAD(P)(H)-dependent oxidoreductases, sharing sequence motifs and displaying similar mechanisms. SDR enzymes have critical roles in lipid, amino acid, carbohydrate, cofactor, hormone and xenobiotic metabolism as well as in redox sensor mechanisms.

**CNN3** (calponin-related gene): involved in negative regulation of ATPase activity.

**INSIG1** (insulin induced gene): plays an important role in the SREBP-mediated regulation of cholesterol biosynthesis: by binding to the sterol-sensing domain of SCAP (SREBP cleavage activating protein) it makes the SCAP/SREBP complex stay longer in the ER, thus prohibiting SCAP from carrying activated SREBP to the golgi complex.

**SIRT** (sirtuin): a class of proteins that possess either mono-ribosyltransferase, or deacylase activity, including deacetylase, desuccinylase, demalonylase, demyristoylase and depalmitoylase activity.

**FADS** (fatty acid desaturase): catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids. The principal product of SCD is oleic acid, which is formed by desaturation of stearic acid.

However, only 2 out of the 9 genes have been statistically validated (*RAB34* and *DHR*).
Discussion

Extensive research revealed that the oocyte and embryo are of questionable quality in females suffering metabolic disorders linked with the break-down of own lipid stores. While there is growing evidence that metabolic unfavourable lipolytic conditions, with particular reference to elevated NEFA concentrations, during oocyte maturation influence oocyte development and resultant embryo quality (Leroy et al., 2005a, Van Hoeck et al., 2011; 2013a; 2013b), little was known about direct consequences of high NEFA levels for the developing pre-implantation embryos. In this study, we hypothesized that the high rates of embryonic loss, observed in females suffering lipolytic disorders, could be related to elevated NEFA concentrations, not only through impairment of the development and quality of the maturing oocyte, but also through direct effects on early embryo development.

Our data substantiated this hypothesis, as the presence of elevated NEFA concentrations, during bovine pre-implantation embryo growth, jeopardized subsequent development and influenced the transcriptome profile in resultant day 7.5 blastocysts. The extent to which pre-implantation embryo development becomes restricted, was highly dependent on the quantity and type of NEFAs added. The mono-unsaturated OA seemed to compensate for the effects induced by the saturated NEFAs (SA and PA). In this context, embryonic exposure to 280 µM of saturated NEFAs (SA) was lethal. By contrast, exposure to a combination of 280 µM of SA together with elevated concentrations of PA and OA was not as toxic as the 280 µM SA alone. OA (C18:1) is a mono-unsaturated fatty acid found in high concentrations in follicular, oviducal and uterine fluids (Tsujii et al., 2001). Interestingly, Haggarty et al. (2006) measured the fatty acid composition of human pre-implantation embryos and found that those embryos that survived beyond the 4-cell stage tended to have a higher ratio of unsaturated to saturated fatty acids. In this context, but at earlier stages, Aardema et al. (2011) showed that OA had a positive effect on oocyte maturation and subsequent embryo development in the cow, overcoming the adverse effects of the other major saturated PA and SA.

Elevated NEFA conditions can affect gene expression pattern of resultant blastocysts, with particular focus on critical oxidative metabolism- and
ceramide-related gene function as identified by the IPA software. The following mechanistic pathways are potentially involved.

Pathway I. The gene expression pattern of the oxidative metabolism pathway was affected in blastocysts originating from both HIGH SA- and HIGH COMBI-exposed embryos. Fatty acids have a functional role at several physiological levels: oocytes accumulate fatty acids from their environment, potentially changing their lipid content (Kim et al., 2001; Adamiak et al., 2004). Even though a zygote mainly relies on glycolysis for its ATP generation (Sutton-McDowall et al., 2010), inhibition of β-oxidation during pre-implantation mouse embryo development reduces the subsequent embryonic developmental potential (Hewitson et al., 1996), which illustrates their possibility to oxidize fatty acid sources during embryo development. Production of ROS is a feature of aerobic respiration and causes damage to cellular macromolecules including DNA, protein and lipid (Stadtman and Levine, 2000; Takahashi et al., 2000). Excessive ROS levels cause DNA damage in bovine embryos (Takahashi et al., 2000; Sturmy et al., 2009) and induce fragmentation and apoptosis in human embryos (Yang et al., 1998). Oxidative stress is also a major cause of developmental arrest at the 2-cell stage in mice (Johnson and Nasresfahani, 1994; Favetta et al., 2007). In this context, Nonogaki et al. (1994) reported that the addition of antioxidants could counteract the inhibited embryo development induced by elevated PA and LA concentrations.

One common feature in HIGH SA- and HIGH COMBI-exposed embryos was the affected expression of genes related to pathways priming insulin resistance, e.g. the Protein Kinase C (PKC) pathway. Indeed, increased ROS production can activate several pathways involved in the pathogenesis of insulin resistance. The PKC pathway related gene expression is particularly up-regulated in HIGH SA embryos compared to controls and is recognized as a marker for the cellular REDOX state. PKC-directed phosphorylation of Nuclear factor-like 2 (NRF2) is a critical event for its nuclear translocation in response to oxidative stress and this NRF2 pathway popped up (after IPA analysis) as the most significant toxicity pathway affected in blastocysts originating from HIGH SA-exposed embryos during culture.
Pathway II. Another pathway regularly affected, in somatic cell types, due to high NEFA exposure is the ceramide pathway leading to apoptosis (Shimabukuro et al., 1998, Maedler et al., 2001, Lu et al., 2003). Interestingly, in HIGH COMBI blastocysts, expression of the serine palmitoyl transferase gene was clearly down-regulated. Watson et al. (2009) showed that inhibition of serine palmitoyl transferase reduces ceramide biosynthesis and subsequent ceramide accumulation in muscle cells (Bilan et al., 2009). As this serine palmitoyl transferase gene was not down-regulated in the embryos exposed to the saturated HIGH SA treatment, we suggest that the mono-unsaturated OA (as present in the HIGH COMBI treatment) activates protective pathways to reduce ceramide formation in the HIGH COMBI embryos.

Pathway III. The opacity of the blastocysts, originating from the embryos exposed to the HIGH COMBI treatment, seemed to be lower compared to the control and SA-exposed embryos. The presence of intracellular lipids causes a reduction in light passing through the embryo (Leroy et al., 2005b), which could suggest that these blastocysts contain more intracellular lipids. This assumption is in line with findings of Aardema et al. (2011) and also with the observation in our previous studies (Van Hoeck et al., 2013a; 2013b) in NEFA-exposed oocytes. Thus, this led the authors/us hypothesize that the expression of genes related to lipid synthesis would be influenced in high NEFA-exposed blastocysts. Somehow surprising, gene expression related to fatty acid synthesis was not drastically influenced. Interestingly, the expression of another gene involved in lipid storage was significantly up-regulated in HIGH COMBI-exposed embryos, the LDLR receptor. It is tempting to hypothesize that the combination of this affected expression and the fact that we supplemented 5% FCS to the culture medium, might explain the darker color of the HIGH COMBI embryos. In this context, also the altered expression of the stearoyl-CoA desaturase genes in the high NEFA-exposed embryos might be relevant (Sinclair et al., 2010). This assumption needs to be further clarified. Nevertheless, this finding is important as an increased lipid content in embryos can have long term repercussions, such as premature blastulation (Walker et al., 1992) or even a higher birth weight (Thompson et al., 1995).
Pathway IV. It is difficult to interpret the altered expression of genes related to the endoplasmic reticulum (ER) stress response pathway as there was no consistency in the transcription profiles of the affiliated genes. Previously, Yang et al. (2011) proposed that high NEFA exposure can result in ER stress, whereas we have no indications that this pathway is drastically affected in bovine embryos exposed to high NEFA levels. It is the first time that, direct effects of pathophysiological relevant NEFA concentrations on pre-implantation embryo development were studied. were investigated and this during 6.5 days of in vitro culture. The gene expression data in blastocysts, originating from embryos exposed to the HIGH COMBI condition during culture, reveal that some crucial pathways in the resultant HIGH COMBI blastocysts are ‘affected’. To which extent such ‘toxic’ or ‘compensating’ mechanisms are sufficient to sustain further embryonic development, post day 7.5, remains an intriguing issue for debate.

The lipid profile of the oocyte is dynamic and largely dictated by the environment in which it develops (McKeegan and Sturmey, 2012) and also developing embryos are capable of internalizing and metabolizing fatty acids present in their micro-environment (Haggarty et al., 2005; Sturmey et al., 2009). Tsujii et al. (2009) emphasized that serum and oviduct fluids play important roles in the development of blastocysts. Exchange of nutrients will pass through the endothelial cells of the blood vessel, the basal membrane of the oviduct and the oviductal epithelium. Preliminary bovine research performed at our laboratory revealed that physiological NEFA concentrations in the oviductal fluid (290 µM) are not significantly different from the basal NEFA concentrations in the serum (320 µM) (unpublished results). Work of Childs et al. (2008) showed that PUFA feeding affects the fatty acid composition of the genital tract. The weight of lambs from embryos cultured in serum-containing medium, which possessed abundant cytoplasmatic lipid droplets, was significantly heavier than that of lambs from embryos cultured in serum-free medium (Thompson et al., 1995). Additionally, mean gestation length was longer in cows receiving embryos cultured in culture medium enriched with serum compared to embryos cultured under serum-free conditions (Thompson et al., 1995). The mechanistic insights for these findings are limited so far.
The micro-environment of the very early developmental stages can affect gene expression patterns through epigenetic regulation and any impairment at that level might induce pertinent changes that can persist or become evident at later stages: after birth, during puberty or even at adulthood (Latham, 1999; Sinclair and Singh, 2007). As such, maternal metabolic lipolytic conditions 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, first described by Barker et al. (1989). In this context, our transcriptome data showed that HIGH SA embryos display a down-regulated expression of histone-cluster genes (HIST1H1C, HIST1H2BN). This made us assume that embryonic exposure to elevated NEFA induces differences in histone, methylation and acetylation patterns in day 7.5 blastocysts, possibly predictive for a deviating development or even post-natal abnormalities. Further research is warranted.

In conclusion, the presence of elevated NEFA concentrations during bovine pre-implantation embryo development clearly jeopardizes the capacity of an embryo to reach the blastocyst stage. Furthermore, the effect on development and gene transcription seems to be determined by the dose and types of NEFAs administered. Transcriptome analysis showed that multiple pathways are involved, be it mainly related to oxidative metabolism and ceramide formation. This study further supports the belief that elevated NEFA concentrations can be responsible for the high rates of embryonic loss observed in females suffering lipolytic disorders.
List of References


concentrations during bovine oocyte maturation compromise early embryo physiology. *PLOS One* 6: 23183.


CHAPTER 8

General Discussion.

1. Mismatch Between Metabolism and Fertility: the central hypothesis.

A normal ‘metabolic health’ status of the mother is essential to safeguard successful ovulation, conception and further embryo development. Female fertility is particularly compromised under conditions of lipolysis-linked maternal metabolic disorders such as obesity, type II diabetes and negative energy balance (Opsomer et al., 1998; Sebire et al., 2001; Butler, 2005; Lash and Armstrong, 2009). The break-down of own lipid stores leads to elevated blood concentrations of non-esterified fatty acids (NEFAs). Such elevated NEFA concentrations jeopardize follicle cell viability and hamper oocyte developmental capacity, i.e. the chance of an oocyte to be successfully fertilized and the odds to develop until the blastocyst stage (Jorritsma et al., 2004, Leroy et al., 2005a; Aardema et al., 2011). However, a hampered oocyte development is not the only concern in females suffering lipolytic disorders; also the risk for embryonic mortality is drastically higher (Clark et al., 1998; Fedorcsak et al., 2000; Dunne et al., 2000; Lucy, 2001; Santos et al., 2004; Metwally et al., 2007; Diskin and Morris, 2008; Bellver et al., 2010).

In-depth mechanisms linking lipolytic conditions to these high rates of embryonic loss have not been provided so far. In addition, besides the direct effect of lipolytic conditions on oocyte maturation, embryo physiology might be affected as well. More specifically, we hypothesized that elevated NEFA concentrations are partly responsible for the questionable oocyte and embryo quality in females suffering lipolytic disorders through the following actions.

A. Problems induced by elevated NEFA exposure during final oocyte maturation.

Since the earliest stages of embryo development are primarily controlled by the oocyte (Watson et al., 1999; Wrenzycki et al., 2007), it is likely that a suboptimal environment within the ovary can account for poor reproductive outcomes. This implies that metabolic perturbations,
induced in the oocyte, may not simply result in suboptimal conception rates, but can also induce (phenotypic) changes that only become visible at later stages. Compromised oocytes result in poorly developed embryos that might be less capable to cross-talk with the endometrial epithelial cells (Santos et al., 2004). The hypothesis that elevated NEFA concentrations during the time-window of final oocyte maturation can perturb subsequent embryo development and/or phenotype, needed to be explored.

B. Problems induced by elevated NEFA exposure during early embryo development.

Most of the research efforts focused on the effect of maternal metabolic health on the oocyte during its final maturation period. However, from in vitro research it is known that the newly formed embryo is also highly sensitive to changes in its micro-environment (Rizos et al., 2003; Leese et al., 2008). Information on possible direct consequences of NEFA exposure during early embryo development was not available. In 2005b, Leroy et al. already suggested that the ability of the reproductive tract to support normal embryo development may be impaired in lactating cows due to elevated NEFA concentrations. Recently, Rizos et al. (2010) and Maillo et al. (2012) emphasized this theory by showing that the oviduct of the lactating dairy cow is compromised in its ability to support early embryo development compared to that of non-lactating cows. This further stresses the significance of the micro-environment in the optimal development of young embryos.

As overviewed in Figure 8.1, the main goal of this PhD study was to clarify the role of elevated NEFA concentrations in the micro-environment of the oocyte and early pre-implantation embryo. We hypothesized that elevated NEFA concentrations are important metabolic clues linking maternal lipolytic disorders to high rates of embryonic loss seen in these mothers. We have investigated ‘whether and how’ these elevated NEFA concentrations during final oocyte maturation and early embryo development affect further embryonic growth and embryonic physiology, using the cow as a model.
Figure 8.1. Presentation of the research work performed in this PhD study.
2. Major findings: effects of elevated NEFA concentrations during oocyte maturation and early embryo development.

Using a bovine model, we learned that oocyte maturation under elevated NEFA concentrations results in blastocysts with a significantly lower cell number and an increased apoptotic cell ratio. Reduced blastocyst cell numbers might delay the timing of gastrulation and morphogenesis later on, and can be responsible for increased rates of pregnancy loss, as has been documented in mice (Tam, 1988; Power and Tam, 1993; Hishinuma et al., 1996). In addition, blastocysts originating from high NEFA-exposed oocytes displayed a less active oxidative metabolism, reflected by reduced oxygen, pyruvate and glucose consumption, up-regulated lactate consumption and higher amino acid metabolism, indicative of up-regulation of repair mechanisms and a higher incidence of DNA damage (Sturmey et al., 2010). Thus, inappropriate supplementation of NEFAs in the maturation environment has a significant negative impact on the metabolism of resulting embryos post fertilisation (Van Hoeck et al. 2011; 2013a). Also gene expression patterns were influenced in embryos originating from high NEFA-exposed oocytes. We have found that, when β-oxidation was inhibited during oocyte maturation, the reduced developmental competence of oocytes matured under high NEFA conditions could be rescued to a certain extent. This intriguing finding suggests that the metabolic strategy in terms of substrate utilization adopted by the mitochondria plays a crucial role in dictating oocyte development and that the oocyte is exquisitely sensitive to metabolic perturbation, i.e. sublethal effects of which can persist post fertilisation. Optimal mitochondrial function is crucial for further embryo development and implantation. Disabled mitochondrial activity has been associated with suboptimal fetal and placental development (Wakefield et al., 2011). In Table 8.1, the major findings resulting from final oocyte maturation under high NEFA conditions are summarized. The micro-environment of the oviduct is important to support fertilisation and the first stages of embryo development as well. Significant lower developmental competence results were obtained for embryos cultured during 6.5 days under high NEFA conditions compared to controls, which might substantiate the findings of Rizos et al. (2010). The latter researchers
showed that the oviducts of dairy cows under metabolic pressure are less capable to support the early stages of embryo growth. In addition, transcriptomic comparison through microarray analyses between blastocysts originating from embryos cultured under high NEFA conditions revealed that many genes were differently expressed compared to control embryos. Embryo development and embryonic phenotype were differently affected dependent on the timing of NEFA exposure, i.e. during in vitro oocyte maturation or embryo culture. Furthermore, different results were observed in response to either a saturated NEFA treatment (HIGH SA) or a combination NEFA treatment (HIGH COMBI), containing both saturated (SA and PA) and mono-unsaturated NEFAs (OA). The concentration of the SA in the HIGH SA treatment was equal to the concentration of the SA present in the HIGH COMBI treatment, which means that the effect induced by the SA alone (HIGH SA), can be to some extent, more pronounced than the effect of the same concentration SA in combination with the mono-unsaturated OA and saturated PA (HIGH COMBI). For example, HIGH COMBI exposure particularly impacted on the oocyte’s developmental potential when applied during oocyte maturation, whereas the HIGH SA treatment rather impacted on resultant embryo quality (reflected by an increased apoptotic cell ratio). One explanation for this might be the fact that mono-unsaturated fatty acids, which is in our case OA in the HIGH COMBI treatment, have been reported to prevent saturated NEFA-induced apoptosis by the induction of BCl-2 and the prevention of mitochondrial release of cytochrome c, or by the competition between fatty acids for transport into the cell or for cell metabolism (Cnop et al., 2001; Maedler et al., 2001; Listenberger et al., 2003; Mishra et al., 2005). Also the metabolomic results are different when comparing blastocysts originating from HIGH SA-exposed oocytes to HIGH COMBI-exposed oocytes. This suggests that resulting blastocysts are dealing with different metabolic strategies in response to the different NEFA types and mixtures. Further in-depth study, by means of transcriptome analyses and functional experiments, was necessary to gain mechanistic insights into the diverse effects sorted by the different NEFA types and treatments.
Table 8.1. The effects of elevated NEFAs during oocyte maturation or embryo culture.

### High NEFA EXPOSURE DURING BOVINE OOCYTE MATURATION (24 h *in vitro* maturation)

<table>
<thead>
<tr>
<th></th>
<th>OOCYTE exposed to</th>
<th>ZYGOTE originating from oocyte exposed to</th>
<th>MORULA originating from oocyte exposed to</th>
<th>BLASTOCYST originating from oocyte exposed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIGH COMBI</td>
<td>HIGH SA</td>
<td>HIGH COMBI</td>
<td>HIGH SA</td>
</tr>
</tbody>
</table>

**CHAPTER 4**

**DEVELOPMENT**
- cell number
- apoptotic cell index

**QUALITY**
- oxygen consumption
- glucose consumption
- pyruvate consumption
- lactate consumption

**METABOLISM**
- amino acid turn over

**VIABILITY**
- rescued development when β-oxidation blocked

**CHAPTER 5**

**METABOLISM**
- oxygen consumption
- mitochondrial membrane potential

**CHAPTER 6**

**PATHWAY ANALYSIS**
- glutathione content
- lipid content

**QUALITY**
- cryotolerance

### High NEFA EXPOSURE DURING BOVINE EMBRYO DEVELOPMENT (6.5 days *in vitro* culture)

<table>
<thead>
<tr>
<th></th>
<th>ZYGOTE exposed to</th>
<th>MORULA originating from embryo exposed to</th>
<th>BLASTOCYST originating from embryo exposed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIGH COMBI</td>
<td>HIGH SA</td>
<td>HIGH COMBI</td>
</tr>
</tbody>
</table>

**CHAPTER 7**

**DEVELOPMENT**
- = no difference compared to control

**QUALITY**
- morphology

**PATHWAY ANALYSIS**
- oxidative metabolism

**NEFA** = non-esterified fatty acid

HIGH COMBI = elevated concentrations of saturated and mono-unsaturated NEFAs | HIGH SA = elevated concentrations of saturated NEFAs

↓ significantly reduced compared to control | ↑ trend for reduction compared to control

= no difference compared to control

↑ significantly increased compared to control | ↑↑ trend for increase compared to control
3. The difference between HIGH SA and HIGH COMBI induced effects: are these differences physiologically relevant in life and how do we have to interpret this?

Most studies performed so far in somatic cell lines focused on the effects of one specific NEFA type, which results in very explicit observations. However, the entire NEFA profile as present in the serum needs to be considered when extrapolating data generated in vitro to the in vivo situation. We should not only focus on the consequences of the increased saturated NEFA fraction, but on the entire NEFA profile. Each type of NEFA seems to have its specific effects on the early stages in life, but also seems to be able, at least to some extent, to counteract effects of other NEFA types. However, to which extent such counteracting mechanisms are sufficient in order to sustain further development of the gamete (and subsequent embryo physiology) remains an intriguing issue (and will be discussed in the sections below). Nevertheless, increased saturated-to-unsaturated NEFA ratios jeopardize the processes of final oocyte maturation and early embryo culture. In addition to the NEFA profile, also the quantity of the NEFA supplement is crucial. As explained above, the concentrations of albumin and NEFA used in our research sections (Chapter 4, 5, 6 and 7) are based on bovine in vivo serum and follicular fluid analyses under pathophysiological conditions (Leroy et al., 2005a).

4. Major pathways unraveled.

By means of genome wide transcriptome profiling in blastoycts, we were able to design schemes in which the major genes with altered expression were linked to the pathways potentially involved in NEFA-toxicity. Using functional experiments, we verified the relevance of the altered expression of several key genes (Van Hoeck et al., 2011; 2013a; 2013b, 2013c). Being primarily based on transcriptome information, the data presented in this section should be interpreted with caution. It is not clear whether all transcripts will be translated and/or even post-translationally modified (Robert et al., 2010). However, combination of transcriptome and phenotypic data generated at the early stages in life evidenced that NEFA exposure during final oocyte maturation and early embryo culture
affect similar pathways. Comparison of the transcriptome data from the HIGH COMBI blastocysts (originating from HIGH COMBI-exposed oocytes or embryos) with the HIGH SA blastocysts (originating from HIGH SA-exposed oocytes or embryos) pointed to some major, but also very specific pathways being affected in response to the specific NEFA types (Figure 8.2). The mechanistic insights will be discussed below.
Figure 8.2. Major pathways affected in blastocysts originating from oocyte matured and embryos cultured under high NEFA conditions. The boxes highlight genes that are differently expressed (DOWN- or UP-regulated) in the blastocysts originating from HIGH COMBI exposed oocytes (IVM) or embryos (IVC) compared to HIGH SA counterparts.
PATHWAY I. One common threat observed after HIGH SA or HIGH COMBI exposure during in vitro maturation (IVM) and in vitro culture (IVC) was the altered expression of genes related to oxidative metabolism. Up-regulation of the fatty acid oxidation pathway results in increased reactive oxygen species (ROS) (Burton et al., 2003). As the mitochondrial genome possesses limited DNA repair mechanisms compared to nuclear DNA (Clayton, 1991), mitochondria are highly sensitive to increased ROS concentrations (Wallace, 1987; Tarin, 1995). Resultant perturbations in mitochondrial function are critical for oocyte development and have been related to reduced embryo quality (Van Blerkom, 2004). This is in line with findings in somatic cell lines, showing that an excess in saturated NEFAs (such as SA and PA) triggers fatty acid oxidation and results in more ROS. For example, in muscle cells, 0.5 mM PA elevated ROS production more than 2-fold (Duval et al., 2007). Excessive ROS levels activate cell death pathways, although the mechanisms involved are still poorly understood (Listenberger et al., 2001; Itani et al., 2002; Li et al., 2006; Schonfeld and Wojtczak, 2008).

Interestingly, whole transcriptome analyses revealed that blastocysts originating from HIGH SA-exposed embryos, display more differences in expression of oxidative metabolism related genes compared to blastocysts originating from HIGH SA-exposed oocytes. The underlying reason for this observation might be the fact that the bovine oocytes rely on lipid oxidation during oocyte maturation (Ferguson and Leese, 1999; Paczkowski et al., 2012). In vitro, the amount of triglycerides present in the oocyte decreases during maturation, and, at least in the pig, there is a corresponding rise in oxygen consumption sufficient to account for this depletion (Sturmey and Leese, 2003). In addition, Cetica et al. (2002) reported a significant rise in lipase activity in bovine oocytes during maturation, suggesting that fatty acid oxidation occurs. By contrast, pre-implantation embryos are relatively quiescent (Leese, 1991; 1995; 2003). In these embryos, pyruvate and glucose are preferentially consumed, much of it being converted, at least in vitro, to lactate via ‘aerobic glycolysis’, which yields a modest amount of ATP (Leese, 1995). Therefore, we assume that NEFAs added during embryo culture will not be recycled as energy sources and thus immediately exceed physiological levels.
PATHWAY II. Another pathway regularly affected in response to NEFA exposure in somatic cell types is the ceramide pathway leading to apoptosis (Shimabukuro et al., 1998, Maedler et al., 2001, Lu et al., 2003). We could not validate this ceramide pathway as causative clue for NEFA toxicity, neither during final oocyte maturation nor during early embryo culture. However, ceramide related gene expression was influenced, but rather in a down-regulated direction. This was somehow unexpected and particularly visible when NEFA treatments were applied during in vitro embryo culture for 6.5 days.

How can we explain the fact that the HIGH COMBI treatment, containing SA, PA and OA, seems to be somehow ‘less toxic’ compared to the HIGH SA treatment. On a molar basis, unsaturated NEFAs are less prone to conversion into lipid metabolites (Bilan et al., 2009). Holland et al. (2007) and Watson et al. (2009) have put forward the hypothesis that PA-induced insulin resistance results from changes in the lipid metabolite ceramide (Bilan et al., 2009). Watson et al. (2009) further showed that inhibition of serine palmitoyl transferase reduces ceramide biosynthesis and accumulation in muscle cells (Bilan et al., 2009). This is particularly interesting as this pathway appeared to be down-regulated in embryos cultured under HIGH COMBI conditions. Studies in muscle cells demonstrate that PA is shunted towards ceramide and diacylglycerol accumulation but not triglyceride accumulation, whereas the polyunsaturated fatty acid LA (C18:2) and the mono-unsaturated fatty acid OA are preferably stored as triglycerides (Lee et al., 2006; Sabin et al., 2007).

PATHWAY III. The lipogenesis pathway is known to be triggered in response to NEFA exposure. In high NEFA-exposed oocytes Aardema et al. (2011) noted considerable alterations in lipid droplet size and quantity. Similarly, in blastocysts originating from HIGH COMBI-exposed oocytes, we observed an increase in mRNA encoding enzymes involved in fatty acid synthesis pathways, i.e. suggestive of up-regulated lipogenic activities in such embryos and indicative of sequestration of fatty acids as triglycerides into lipid droplets. This effect was obvious at gene expression level in blastocysts originating from HIGH COMBI-exposed oocytes, but less obvious in the HIGH COMBI blastocysts originating from the NEFA exposure during embryo culture. In this context, functional
analyses validated that morulae originating from HIGH COMBI-exposed oocytes contain an increased lipid droplet content per blastomere. Though, only the blastocysts from the HIGH SA treatment tended to display a lower cryotolerance compared to the control blastocysts, whereas freezing capacity of the HIGH COMBI blastocysts was unaffected (Van Hoeck et al., 2013b). In line with this, Nagano et al. (2006) reported that lipid-rich bovine oocytes display an improved development (Nagano et al., 2006). The ability to efficiently store esterified fatty acids in lipid droplets is assumed to be ‘protective’ as those fatty acids stored in lipid droplets will not be shunted towards other lipotoxic pathways (Aardema et al., 2011).

PATHWAY IV. It is difficult to interpret the altered expression of genes regulating other pathways such as the ‘endoplasmic reticulum stress response’ pathway as there was no consistency in the expression direction of the affiliated genes. Previously, Yang et al. (2011) proposed that NEFA exposure can induce endoplasmic reticulum (ER) stress. Furthermore, fatty acid rich human follicular fluid, originating from women suffering from hyperlipolytic associated metabolic diseases, was added to mouse maturation medium, and led to altered oocyte maturation and more ER stress (Robker et al., 2011).


Glucose intolerance. One intriguing finding was the drastic up-regulated expression of the maternally derived IGF2R imprinted gene in blastocysts originating from HIGH COMBI-exposed oocytes compared to control counterparts. This, in combination with the finding that the same HIGH COMBI embryos displayed an up-regulated expression of the GLUT1 glucose receptor, whereas metabolic studies clearly revealed that the latter embryos did not succeed in consuming glucose, seems to indicate that such embryos become/are glucose intolerant. Increased ROS production can activate several pathways involved in the pathogenesis of insulin resistance. What is the relevance of insulin resistance in terms of embryos? The primary metabolic function of insulin is to rapidly increase glucose uptake in the target tissues. How do blastocysts internalize glucose? Insulin-stimulated glucose uptake is thought to be unique to
In Figure 8.3, one of the major pathways associated with insulin resistance is presented, the Protein Kinase C (PKC) pathway. To find out whether this pathway was affected, we mapped several key genes, of which the expression was significantly influenced in response to HIGH COMBI or HIGH SA exposure during embryo culture. Mechanisms involved in the onset of insulin resistance were initiated, at least at the transcriptome level, after embryonic culture in the presence of both high NEFA treatments. The PKC pathway related gene expression was particularly up-regulated in HIGH SA embryos compared to controls and is recognized as a marker for the cellular REDOX state. PKC-directed phosphorylation of NRF2 is a critical event for its nuclear translocation in response to oxidative stress and this NRF2 pathway turned out to be the most significant toxicity pathway (according to the IPA analysis) affected in blastocysts originating from HIGH SA exposed embryos during culture (Van Hoeck et al., 2013c). In this context, in cultured aortic endothelial cells, saturated NEFAs have also been reported to stimulate the de novo diacylglycerol synthesis and PKC activity (Kim and Cho, 1996). In smooth muscle cells (Kobayashi et al., 2002) similar mechanisms were initiated as well in response to saturated NEFAs, resulting in an increased ROS production via PKC-dependent activation of NAD(P)H oxidase (Keller et al., 1993; Kang et al., 2005).

It has been previously suggested that high levels of saturated and unsaturated NEFAs produce insulin resistance through different mechanisms. These different pathways have been described using a somatic cell study. PA led to accumulation of ceramide and interfered with insulin action only down-stream of the insulin receptor, at the level of PKB/Akt phosphorylation, while OA and LA blunted insulin action at the level of IRS-1/PI-3-kinase (Schmitz-Peiffer et al., 1999; see Figure 8.3). Saturated NEFAs induce elevated levels of diacylglycerol and ceramide that correlate positively with insulin resistance (Lee et al., 2006; Todd et al., 2007; Kuda et al., 2009) and provoke insulin resistance of glucose uptake in muscle cells, without affecting the basal rate of glucose uptake (Perdomo et al., 2004; Powell et al., 2004; Sinha et al., 2004; Duval et al., 2005).
2007; Pimenta et al., 2008; Hommelberg et al., 2009). In this context, when oocytes were exposed to the HIGH COMBI treatment during oocyte maturation, resultant blastocysts did fail to consume glucose, whereas blastocysts originating from HIGH SA-exposed oocytes, consumed glucose, though less than control embryos. We could not explain these observations so far, but the latter paragraph might provide a basis to gain further insight.

Another general threat observed after high NEFA exposure during final oocyte maturation, but also in response to high NEFA exposure during embryo culture (as shown in Figure 8.4), was the altered expression of genes related to Ca\textsuperscript{2+} homeostasis. The microarray data, comparing gene expression in blastocysts of both high NEFA treatments with control blastocysts, show that expression of genes related to Ca\textsuperscript{2+} homeostasis was affected. One explanation might be that ROS can directly stimulate the opening of the mitochondrial membrane transition pore and thereby initiate mitochondrial cytochrome c release (Brookes et al., 2000; Kang et al., 2002). Subsequently, ROS induces the release of Ca\textsuperscript{2+} from mitochondria, which in turn stimulates Ca\textsuperscript{2+}-dependent enzymes such as Ca\textsuperscript{2+}-dependent proteases, nucleases, and phospholipases, initiators of apoptosis. In Figure 8.4, we have linked several genes that were differently expressed, in HIGH COMBI en HIGH SA blastocysts, to this apoptosis cascade. Once Ca\textsuperscript{2+} efflux has been triggered, a series of common pathways of apoptosis are initiated, each of which may be sufficient to destroy the cell (Kang et al., 2005). Furthermore, we saw several genes related to the cytoskeletal architecture (for example actin) that were differently expressed in response to NEFA exposure. Interference of intracellular Ca\textsuperscript{2+} homeostasis appears to be related with a disruption of the cytoskeleton. A rise in Ca\textsuperscript{2+} concentration by ROS can promote dissociation of actin microfilaments and also activate certain proteases (e.g. calpains, of which the expression was clearly influenced in high NEFA blastocysts originating from NEFA exposure during in vitro oocyte maturation and in vitro embryo culture) that cleave actin-binding proteins. This might result in the loss of anchorage to the cytoskeleton complex (Dourdin et al., 2001; Kang et al., 2005).
Figure 8.3. Presentation of genes that were differently expressed in high NEFA-exposed embryos and linked to the insulin resistance related Protein Kinase C pathway. The boxes highlight genes that are differently expressed (DOWN- or UP-regulated) in the blastocysts originating from HIGH COMBI exposed or HIGH SA exposed embryos compared to control counterparts.
Figure 8.4. Presentation of genes that were differently expressed in high NEFA-exposed embryos and linked pathways associated with Ca\(^{2+}\) homeostasis. The boxes highlight genes that are differently expressed (DOWN- or UP-regulated) in the blastocysts originating from HIGH COMBI exposed or HIGH SA exposed embryos compared to control counterparts.
5. How profound are NEFA-induced deviations: is the embryo insulted or is the embryo adapting to cope with the insult?

It is clear that, both during final oocyte maturation and during early embryo development, high NEFA exposure can influence resultant embryo development and physiology. Do the observed deviations provide answers to cope with stresses, or do they indicate NEFA-induced toxicity and subsequent cell dysfunction? It is necessary to bear in mind the considerable ‘autonomy’ of the early embryo, which enables it to grow in a variety of environments (Leese, 2003). The concern is to which degree the compensatory mechanisms will cope with the stress due to the changed micro-environment and whether the adaptations are sufficient to ensure a successful embryo implantation and full-term pregnancy. Several ‘effects’ may not become apparent until relatively late in embryonic life.

5.1. Embryos can be affected.

Embryo mortality between day 14 and day 20 after fertilisation is a well-described problem in metabolically compromised women (Clark et al., 1998; Fedorcsak et al., 2000; Metwally et al., 2007; Bellver et al., 2010) and cows (Dunne et al., 2000; Lucy, 2001; Santos et al., 2004; Diskin and Morris, 2008). This timing coincides with important processes such as cell lineage specialization, embryo elongation and embryonic disc formation. It is not known whether elevated NEFA concentrations during oocyte maturation or embryo culture can disrupt these vital steps beyond the blastocyst stage. We showed that day 7.5 blastocysts, originating from oocytes matured under HIGH SA and HIGH COMBI conditions, have a lower cell number compared to control blastocyst. In mice, reduced blastocyst cell numbers have been linked to a delayed timing of gastrulation and morphogenesis and possible increased rates of pregnancy loss (Tam, 1988; Power and Tam, 1993; Hishinuma et al., 1996). However, during later gestation, increased rates of proliferation in embryos that survive can compensate for deficient numbers of pre-implantation blastomeres so that fetal size may be restored to the normal level (Power and Tam, 1993; Hishinuma et al., 1996; Kwong et al., 2000).
5.2. Embryos can be flexible.
We showed that embryos originating from HIGH COMBI-exposed oocytes display a distorted oxidative metabolism and impaired mitochondrial function. One can hypothesize that the embryo adapts its metabolism to use alternative metabolic pathways (Lane and Gardner, 2000). However, optimal mitochondrial function is crucial for embryo development and implantation (Van Blerkom, 2004). Even more, disabled mitochondrial activity has been associated with suboptimal fetal and placental development (Wakefield et al., 2011). Furthermore, HIGH COMBI blastocysts display up-regulated gene expression profiles of lipid synthesis pathways, which might be a protective/coping mechanism in order to channel fatty acids away from lipotoxic pathways (Cnop et al., 2001; Aardema et al., 2011). However, excessive accumulation of lipids in peripheral tissues is also closely associated with insulin resistant related pathologies (Zhang and Zhang, 2012).

Expression of several genes involved in epigenetic regulating mechanisms were changed in high NEFA blastocysts (Chapter 5, 6 and 7). For example, an up-regulated expression of a DNA methylation gene (DNMT3A) and histone cluster (HIST1H2BN) associated gene was observed in blastocysts originating from oocytes exposed to HIGH COMBI treatment. Also after NEFA exposure during in vitro culture, microarray data revealed that HIGH SA embryos displayed a down-regulated expression of histone-cluster genes (HIST1H1C, HIST1H2BN). This might be predictive for a deviating development or even post-natal abnormalities (as will be discussed below).

6. A bovine model to study the effect of elevated NEFA concentrations on oocytes and embryos.
The oocyte and embryo are of questionable quality in females suffering lipolytic disorders. Satori et al. (2002) and Leroy et al. (2005b) showed that the proportion of viable embryos is drastically reduced in lactating cows compared to non-lactating cows or heifers. More than 40% of the dairy cow conceptuses are lost within two weeks post-insemination, suggesting that oocyte and early embryo quality may be compromised (Leroy et al., 2008). Obese women undergoing assisted reproductive
treatment have a significantly lower chance for a top quality embryo in vitro and a lower chance to establish pregnancy compared to control women (Pasquali et al., 2003). Using a dietary induced obese mouse model, Igoscheva et al. (2010) reported an increased prevalence of embryonic death in these mice due to fragmented embryos. In line with this, rat diabetes models show that the total blastocyst cell numbers are reduced in females suffering diabetic conditions (Lea et al., 1996; Pampfer et al., 1997). The latter data are based on in vivo experiments, which makes it impossible to distinguish whether the observed effect is exerted at the level the oocyte, zygote, embryo or sperm cell. Furthermore, we do not know whether the latter problems are caused through lipolytic related, increased NEFA levels, or rather through other factors altered in the blood of mothers suffering metabolic disorders associated with lipolysis. Thus, there was a need for a valid animal model to investigate the effects of metabolic stress, with particular focus on elevated NEFA concentrations, around the period of conception. In this PhD thesis, experiments were performed using a bovine in vitro embryo production model, which allowed us to define the stage (oocyte versus embryo level) at which elevated NEFA concentrations impact. The support of a bovine in vitro reproduction model to investigate oocyte and pre-implantation embryo physiology has steadily grown over the last decade, parallel to the more traditional murine model. There are substantial similarities between human and bovine ovarian function and oocyte characteristics (Campbell et al., 2003; Adjaye et al., 2007) and gametes are remarkably similar with respect to microtubule formation during fertilisation, the timing of genome activation, metabolic requirements, interactions with the culture medium and duration of pre-implantation development (Navara et al., 1995; Anderiesz et al., 2000; Ménézo et al., 2002; Campbell et al., 2003).

The types and concentrations of NEFAs used in the present thesis are based on bovine in vivo studies (Leroy et al., 2005a) and are pathophysiologically relevant. Circulating free fatty acid concentrations in women suffering lipolysis-linked metabolic disorders, including obesity (Reaven et al., 1988; Stolba et al., 1993), are very similar to NEFA concentrations detected in cows during an episode of up-regulated lipolysis (Leroy et al., 2005a). Using in vitro maturation models to mimic
lipolytic disorders, NEFA concentrations should be based on intrafollicular rather than on serum concentrations of SA, PA and OA (Leroy et al., 2005a). However, when studying the effect of elevated NEFA exposure during early embryo development, serum concentrations should be used as there is a close interaction between the oviductal fluid and serum. Preliminary bovine research performed at our laboratory revealed that physiological NEFA concentrations in the oviductal fluid (290 µM) are not significantly different from the basal NEFA concentrations in the serum (320 µM) (unpublished results).

Standard serum-free in vitro production systems are devoid of fatty acids, although the physiological environment, in which the oocyte matures in vivo, contains physiological concentrations of NEFAs (Leroy et al., 2005a). In order to improve the relevance of our in vitro model, we therefore used a maturation and culture medium supplemented with basal NEFA concentrations as control medium. Furthermore, methods used to determine in vitro NEFA cytotoxicity, should mimic the in vivo situation as much as possible. In the blood, the majority of serum NEFA is found to be associated with albumin, a carrier protein (Chung et al., 1995; Richieri and Kleinfelt, 1995; McArthur et al., 1999). Albumin supplementation to the in vitro maturation and culture media is not only required to avoid aggregation and precipitation of the NEFAs (Ulloth et al., 2003), but also fulfills a crucial 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 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, such as ratios > 5, should be avoided because they represent conditions in which NEFAs cannot be released (Cnop et al., 2001). This may influence the interpretation of the data.

Whether our in vitro model is suitable to withdraw/extrapolate conclusions towards the in vivo problem of the increased incidence in
embryonic loss remains a matter for debate. Is the knowhow, generated so far, using bovine in vitro studies, transferable to the in vivo situation? ✓ To some extent yes. The work of Maillo et al., 2012 reported problems at the embryonic level due to negative energy balance conditions and hinted to elevated NEFA concentrations as responsible metabolites. ✓ To some extent not. The study of Matoba et al. (2012) could not reveal differences in oocyte quality of cows suffering lipolytic disorders. Therefore, it remains to be elucidated why we can relate our in vitro data related to NEFA exposure during embryo culture to the in vivo data about embryo quality in cows suffering lipolytic disorders, while this is not possible for our data generated when NEFA exposure was performed at the level of the oocyte. Is this the result of serum inclusion during our in vitro embryo culture set-up to safeguard blastocyst growth, in contrast to the maturation set up where we decided to work serum-free? Exclusive ‘focus’ on elevated NEFA concentrations (and not on changes of other lipid fractions) obliges us to work with a defined medium. When NEFAs are applied in the absence of the other lipid fractions, they may behave differently as compared to the in vivo situation. For example, in a serum-free medium, NEFAs cannot be ‘captured’ by other lipid fractions, which might result in an increased ‘toxicity effect’ and thus exacerbate potential consequences of elevated NEFA concentrations in vitro compared to in vivo. By contrast, the inclusion of other lipid fractions would imply that we cannot discern whether possible effects result effectively from the NEFA fraction. 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 matter for debate. For example, we should also take into account the evidence that in vitro-matured oocytes are less metabolically active than those matured in vivo, which additionally compromises proper extrapolation of our in vitro data towards the in vivo situation.
7. Several concerns remaining unaddressed: perspectives for future research.

7.1. Long term NEFA exposure.
Before entering the oviduct, the oocyte has spent several months or years in the ovary. Most of the in vitro studies performed so far, only describe effects during the final oocyte maturation period in which the oocytes were exposed to elevated concentrations for only 24 h. Under in vivo conditions, however, oocytes are exposed to such levels for weeks. This potential carry-over effect of adverse conditions during oocyte growth and maturation on further fertility outcome was first proposed by Britt (1992). Britt hypothesized that the developmental competence of the oocyte and steroidogenic capacity of the follicle in high yielding dairy cows is determined by their biochemical environment during the months of follicular growth before ovulation. Therefore, primary follicles exposed to adverse conditions associated with the metabolically challenging period of NEB may be less capable of producing adequate amounts of oestradiol and progesterone (post-ovulation) (Britt, 1992; Roth et al., 2001b). Moreover, such follicles are likely to contain an inferior oocyte, which will then be ovulated around 60-80 days post-partum. So far, these long-term carry-over effects have only been substantiated by research in heat-stressed dairy cows (Roth et al. 2001a; Morton et al., 2007). To check whether this hypothesis is also plausible in terms of lipolytic conditions (and devoid of all possible confounding factors typical in in vivo research), follicles should be cultivated under elevated NEFA conditions for several days or even weeks. Research in our laboratory revealed that murine pre-antral follicles, when exposed to high NEFA concentrations for 12 days, contain oocytes with a dramatically reduced developmental capacity (Valckx et al., 2012).

7.2. What about long-term consequences on embryo development and postnatal health in response to elevated NEFA exposure perconception?
We generated transcriptome data and phenotypic data from the oocyte stage till the day 7.5 blastocyst stage (overview in Table 8.1). Information on further pre-implantation embryo development and on the process of cellular differentiation are lacking. However, Degrelle et al. (2005)
showed that the step of early bovine embryo elongation is critical to guarantee further pregnancy. It remains to be investigated whether the altered embryo physiology, originating from a deviant micro-environment during bovine oocyte maturation, can persist into legacy effects in the resulting offspring. Using a mouse model, Jungheim et al. (2011) emphasized our concern by showing that elevated PA (a saturated NEFA) concentrations, applied during mouse embryo culture, not only jeopardized embryonic development, but also resulted in a deviant growth of the fetus and even in a distorted post-natal development (Jungheim et al., 2011).

Bovine embryos may survive acute NEFA exposure probably due to an adaption in their metabolism (Van Hoeck et al., 2013a; 2013b) but this does not inevitably guarantee that the embryo will implant successfully and lead to a full-term pregnancy. As said above, several developmental problems may not become apparent until relatively late in embryonic life, day 7.5 after conception (Fischer-Brown et al., 2004). The micro-environment of the very early developmental stages can affect gene expression patterns through epigenetic regulation and any impairment at that level might induce pertinent changes that can persist or become evident at later stages: after birth, during puberty or even at adulthood (Latham, 1999; Sinclair and Singh, 2007). As such, maternal metabolic lipolytic conditions periconception and/or during early pregnancy might cause heritable changes, programming the offspring’s susceptibility to disease (Chavatte-Palmer et al., 2008). This concept is known as ‘Developmental Origins of adult Health and Disease’ or DOHAD, first described by Barker et al. (1989). Imprinted gene abnormalities have been implicated with deviating implantation, placentation, fetal growth and even post-natal health (such as the Beckwith-Wiedemann syndrome in human or the Large Offspring Syndrome in ruminants (Young et al., 1998). In this context, we already showed that expression of the maternally imprinted IGF2R gene was affected in blastocysts originating from HIGH COMBI-exposed oocytes (Van Hoeck et al., 2011). Rabbit and rodent models indicate that periconceptional overfeeding with low-protein diets led to intra-uterine growth retardation and post-natal defects (Watkins et al., 2008). In rodents, a maternal high-fat diet results in persistent lipid accumulation in adult offspring livers, even in the
absence of postweaning high-fat diet exposure (Cinti et al., 2005; Brophy et al., 2009), suggesting a more permanent programming effect by maternal health. Interestingly, in a mouse model of nutrient restriction, hepatic genes involved in lipid metabolism were again to naturally found to be increased in newborn offspring (Mortensen et al., 2010), suggesting that early regulatory events in utero are particularly sensitive to nutrient availability. Also IVF children are more prone to metabolic diseases compared conceived counterparts, solely by encountering a different micro-environment around conception (Ceelen et al., 2008). In humans, umbilical cord blood samples obtained from obese mothers showed increased index of fetal insulin resistance, which was associated with increased fetal adiposity and leptin levels relative to lean control mothers (Catalano et al., 2009).

We showed that oocyte maturation under elevated NEFA concentrations significantly up-regulated the expression of DNMT3A both in the matured cumulus oocyte complex and in the day 7.5 blastocysts after routine fertilisation and culture. The DNMT3A gene encodes a ‘de novo DNA methyltransferase’, whose regulation is essential for the proper establishment of epigenetic marks. We hypothesize that a deviating embryo metabolism, as described above, may furthermore affect the methylation process in this particular embryo phase. Ultimately, the best test to evaluate such long-term impact is to see whether embryos result in birth and result in a healthy offspring. In this context, preliminary work using a rabbit model (unpublished results) was already carried out. The latter rabbit model is highly convenient to investigate in vivo fetal development as rabbits have a short gestation period, easy in vitro manipulation properties, a precise timing of ovulation (Duranthon, 2009) and a hemochorial placental structure, which is similar to that of humans. Moreover, the rabbit lipid metabolism is closely related to that of humans (when compared to mice) (Picone et al., 2011).

7.3. Gender effects.
Erickson (1997) suggested that male pre-implantation embryos have a greater capacity to respond to the maternal environment. As a consequence, we need to reflect on the fact that male embryos exhibit altered responses to elevated NEFA exposure. In addition, male embryos
of different mammalian species proliferate to the blastocyst stage at a faster rate than female embryos (reviewed in Erickson, 1997) and following exposure to heat stress-induced ROS, female pre-implantation embryos showed to be more resistant than males (Pérez-Crespo et al., 2005). If programming initiates in the pre-implantation embryo, specific gender differences at this stage of development may also contribute. This should be taken into account when considering follow-up projects.

7.4. Species differences.

The effect sorted by elevated NEFA exposure not only depends on the quantity and degree of saturation, but also on the extent to which the supply of NEFA matches the species-specific nutrient requirements of the oocyte and the early embryo. As such, the species-specific levels of endogenous fat is crucial when considering effects of elevated NEFA exposure. For example: oocytes and embryos of mice are pale, and contain low levels of endogenous lipids (Loewenstein and Cohen, 1964), human oocytes contain an intermediate lipid level (Matorras et al., 1998) and an exceptionally high lipid content is observed in the pig oocyte (Sturmey et al., 2009). Especially in species with low lipid reserves in the gametes, such as the mouse (Downs et al., 2009), fatty acids are crucial for certain stages in development. Therefore, we hypothesize that human oocytes will rather suffer from an excess of NEFAs compared to mice ova. Indeed, Dunning et al. (2010) postulated that fatty acid metabolism is active in the early development of a mouse embryo. Quinn and Whittingham (1982) reported that exogenous OA and PA inhibited fertilisation of mouse embryos, but promoted blastocyst formation and hatching when added to 8-cell embryos. Also in rabbit zygotes, the rate of fatty acids oxidation is significantly constant in the 1- and 2-cell stages, but significantly increased in the 4-cell stage and continued to increase up to the blastocyst stage (Khandoker and Tsujii, 1998). Using our bovine model (as presented in this PhD thesis), we showed, that direct bovine embryonic exposure to elevated NEFA concentrations during the in vitro culture period reduces the chance to develop until the blastocyst stage. By contrast, our preliminary rabbit work (unpublished results) could not substantiate any detrimental effect of elevated NEFAs during embryo culture for rabbit pre-implantation embryo development. However,
fetuses, resulting from *in vitro* NEFA-exposed rabbit embryos, seemed to be smaller than controls. These data need further confirmation.

**7.5. Sperm effects.**

At the present time, the effects of elevated NEFA concentrations on sperm transport, viability and quality are poorly understood. Further research is needed to define the mechanisms controlling these processes.

**8. Back to the field.**

**8.1. Significance for the dairy manager. What can we learn to improve fertility?**

It is widely accepted that dairy cow fertility is rapidly declining and multidisciplinary actions are required to reverse this trend. Early embryo mortality is a well-described problem in metabolically compromised cows (Leroy *et al.*, 2008). Reproductive failure is a major reason for premature culling, affecting longevity of dairy cows and the sustainability of modern dairying. Multiple ‘symptomatic’ efforts have been done by designing dietary strategies to improve NEB condition of dairy cows. However, it is hard to come up with practical guidelines. Strategies should be based on alleviation of NEB, supporting follicle growth and optimizing oocyte and embryo quality, which somehow seems to be impossible in reality. It is generally accepted that the nutritional requirements for early resumption of ovarian activity and follicular growth are different from the nutritional conditions optimal for conception and early embryo growth. In this light, Garnsworthy *et al.* (2008) advised to not increase the lipid content over 5% of the dry matter intake to avoid a depression in circulating insulin concentrations during the first weeks post-partum. However, they deliberately added dietary fat to attenuate insulin concentrations during breeding in order to avoid overstimulation of the oocyte and zygote (Garnsworthy *et al.*, 2009). Fat feeding may alter the micro-environment of the growing and maturing oocyte, of the early and older embryo and thus may affect the reproductive outcome. We recently reported that dietary induced hyperlipidemic conditions can be harmful for embryo development and metabolism (Leroy *et al.*, 2010). The typical milk stimulating rations have been linked to reduced embryo quality as well
(McEvoy et al., 1997; Yaacub et al., 1999). Apart from rations supplemented merely with saturated or mono-unsaturated fatty acids (to increase energy intake), poly-unsaturated fatty acids are becoming increasingly popular; particularly as a way to increase milk concentrations of omega-3 fatty acids. Unfortunately, research results rarely provide a consensus in this perspective. The consequences of these fat feeding strategies on oocyte and embryo quality remain an intriguing issue.

8.2. Ethical concerns related to women with an unhealthy lifestyle.

Obesity is mainly the result of excessive food intake in combination with insufficient energy expenditure. Mutated genes may be responsible for an excess or deficiency in adipose tissue mass, but a genetic component is thought to play a minor role in overall energy homeostasis (Spiegelman and Flier, 2001). That means that most overweight women, that are trying to become pregnant, have no genetic ‘excuse’ for their distorted metabolic health. In this context, the concern of Hippocrates (410 B. C.) stating that “Fatness and flabbiness are to blame. People of such constitution cannot be prolific. The womb is unable to receive the semen and menstruate infrequently and little” is a matter for debate. Can we afford to neglect the signs of nature? When we cannot get pregnant spontaneously due to aberrant lifestyle factors (such as sedentary behavior, smoking, alcohol abuse), is it ethical to use the scientific knowhow to ‘solve’ this problem? And what do we think considering the studies reporting a twice as high a risk of a major birth defect in infants conceived with use of intracytoplasmic sperm injection or in vitro fertilization compared to naturally conceived infants (Hansen et al., 2002; 2005)? Such information should be made available to couples seeking artificial reproductive treatment before embarking upon actions. In highlighting the metabolic problems associated with lipolytic conditions and their potential relationship with subfertility, our findings are consistent with public health recommendations from the World Health Organization (WHO, 2004). It is stated in their Millennium Development Goals that the parents’ overall health and lifestyle choices before pregnancy can affect fertility, maternal health and the infant’s probability of developing chronic conditions later in life. Such choices include:
maintaining a healthy diet, being physically active and avoiding unhealthy behavior. Lifestyle modification is clearly a key component for the improvement of reproductive function in overweight women. Indeed, studies focusing on weight loss suggest that a weight reduction of 5-10% is already sufficient to significantly improve endocrine parameters and fertility (Kiddy et al., 1992; Clark et al., 1995). However, weight loss is a lipolytic process, so we could hypothesize that, when conception is aimed while weight loss is being acquired, the resultant blastocyst quality might be compromised. Apparently, our data result in the advice that weight loss should be achieved, although, not immediately before conception.
List of References


Lloyd et al. (1978) Hippocratic writings.


221


WHO (2004) World Health Assembly adopted a strategy for reproductive health to accelerate progress towards the attainment of international goals and targets, such as the Millennium Development Goals (MDGs).


CHAPTER 9

Conclusions.

It can be concluded that the oocyte and early embryo are very vulnerable to unfavourable, lipolytic-related, metabolic conditions in the mother. The combination of transcriptomic, metabolomic and functional data, collected during early developmental stages unraveled diverse mechanisms of metabolic dysregulation as a consequence of elevated NEFA conditions periconception, e.g. findings which can explain the higher rate of early pregnancy losses and miscarriages observed in females suffering lipolysis-linked metabolic disorders.

Overall, the addition of pathophysiologically relevant NEFA concentrations during bovine in vitro oocyte maturation and embryo culture reduced the proportion of zygotes that developed up to the blastocyst stage. Furthermore, high NEFA exposure during final oocyte maturation and during embryo culture affected the gene expression profile and physiology in the resultant day 7.5 blastocysts. Focusing on dairy cow fertility, pathological NEFA concentrations are proposed as a risk factor for disappointing reproductive performance. Our laboratory could substantiate this causative relationship. Many studies have reported a worrisome decrease in the reproductive performance of dairy cows and this problem appears to affect all countries benefiting from high yielding dairy herds. Reproductive failure is a major reason for premature culling, threatening longevity of dairy cows and the sustainability of modern dairying. Furthermore, only an optimal reproduction at herd level guarantees an acceptable environmental ecological footprint of milk production. However, many other factors need to be taken into account as well when addressing the loss in fertility in modern dairy cows.

In highlighting the metabolic problems associated with subfertility in women, our findings show that up-regulated lipolysis, which arises from obesity, type II diabetes or negative energy balance, results in reduced oocyte developmental competence with down-stream effects on the quality and physiology of the pre-implantation embryo. The observation
that a preovulatory oocyte and early embryonic environment, placed within the context of a metabolically compromised individual, produces oocytes and embryos with a severely impaired capacity to support normal embryo development is particularly alarming as rates of human obesity and type II diabetes in developed nations continue to increase.
Summary.

Maternal metabolic disorders, such as obesity, type II diabetes and negative energy balance, are known to affect female reproductive physiology. The rising incidence in metabolic disturbances affects reproduction, the biology of which remains partially undefined. Not only the physiological processes of normal follicular growth and ovulation are under pressure. The increasing incidence of embryonic and fetal losses suggest that the quality of the female gamete at the moment of ovulation, around conception and/or early post-conception, may be jeopardized as well. Years of expertise in in vitro production models contribute to the awareness about the influence of aberrant maternal metabolic conditions on oocyte and embryo quality. In Chapter 1, the association between maternal metabolic health and female fertility is critically approached.

Maternal metabolic disorders, linked to lipolytic conditions, are risk factors for reproductive failure. Reduced oocyte and embryo quality have been suggested as major contributors. These disorders are marked by increased NEFA concentrations in the blood. Elevated NEFA concentrations have been implicated as key factor in the association between metabolic imbalances, cellular dysfunction and related pathologies. Furthermore, high NEFA concentrations in the blood are known to alter the micro-environment of the maturing oocyte and early embryo. In vitro studies revealed that elevated NEFA concentrations are toxic for bovine and human follicle cell viability and even for bovine oocyte developmental competence. In Chapter 2, a review is provided about the impact of elevated NEFA concentrations on female fertility, with particular focus on the early stages of life.

In Chapter 3, the specific aims of the thesis are overviewed. Given the paucity of data on the effects of maternal metabolic status at the level of the oocyte and pre-implantation embryo, we have asked whether elevated NEFA exposure during bovine oocyte maturation and early embryo culture can influence subsequent embryo development and
physiology. Furthermore, we aimed to unravel the underlying molecular mechanisms through which NEFA exert negative effects.

In Chapter 4, we have asked whether elevated NEFA concentrations during final oocyte maturation affect the resultant blastocyst physiology, using the cow as a model. In our in vitro embryo production laboratory, bovine oocytes were exposed to pathophysiological NEFA conditions and subsequently fertilized. The zygotes were grown to blastocysts, which were evaluated for their quality in terms of cell number, apoptosis, gene expression patterns, amino acid turnover, and oxidative metabolism. Oocyte maturation under elevated NEFA concentrations resulted in blastocysts with significantly lower cell number and increased apoptotic cell ratio. In addition, the blastocysts displayed a reduced oxygen, pyruvate and glucose consumption, an up-regulated lactate consumption and more active amino acid metabolism, suggesting that these embryos alter their metabolism in response to suboptimal micro-environmental conditions.

In Chapter 5, we showed that elevated NEFA concentrations during in vitro oocyte maturation affect bovine oocyte developmental competence and the phenotype of the resulting embryos. In addition, we sought for basic insights into the precise etiological mechanisms. We hypothesized that negative effects of elevated NEFA exposure during oocyte in vitro maturation are linked to modifications in energy metabolism. More specifically, we hypothesized that mitochondrial (dys)function is involved in the mechanisms through which high NEFA exposure, during oocyte maturation, impacts on the oocyte’s developmental capacity and on resultant embryo quality, metabolism and viability. It was found that expression of genes related to REDOX maintenance was modified in NEFA-exposed oocytes, cumulus cells and resultant blastocysts. Moreover, the expression of genes related to fatty acid synthesis in embryos, that developed from high NEFA-exposed oocytes, was up-regulated. From a functional perspective, inhibition of fatty acid β-oxidation in maturing oocytes exposed to elevated NEFA concentrations restored developmental competence. There were no clear differences in mitochondrial morphology or oxygen consumption between treatments.
However, there was a trend for a higher mitochondrial membrane potential in zygotes derived from NEFA-exposed oocytes.

Using microarray techniques, we were able to screen multiple pathways affected at gene expression level. As such, in Chapter 6, microarray analyses in resultant blastocysts revealed highly valuable information about several pathways affected. The analyses showed a significant increase in RNA encoding enzymes involved in lipid synthesis pathways in the high NEFA group. The fact that our previous studies displayed a parallel increase in the mRNA abundance of genes related to fatty acid synthesis is highly indicative of increased lipogenic activities in such embryos compared to embryos originating from control oocytes. The ability to store NEFAs in lipid droplets might be protective by channeling fatty acids away from lipotoxicity pathways. Furthermore, these gene expression data in blastocysts have been linked to functional information by evaluating antioxidant levels, lipid droplet content and freezability in NEFA-treated oocytes, ensuing morulae and resultant embryos (respectively). The combination of the phenotypic data on individual embryo metabolism and the whole transcriptome analyses of the same embryos shed more light on the pathogenesis of the NEFA toxicity during oocyte maturation as well as on oocyte development and embryo quality.

We showed that deviating metabolic conditions in the oocyte’s micro-environment induced significant carry over effects on embryo quality and physiology up to 7.5 days after fertilisation. However, also the embryonic micro-environment is important to support fertilisation and the first stages of embryo development. Whether and how elevated NEFA concentrations may affect fertilisation and the first embryonic cell divisions is not known and needs further in depth study. Recent in vivo studies highlighted that the oviduct of dairy cows under metabolic pressure is less capable of supporting the early embryo growth stages and, in Chapter 7, we hypothesized that elevated NEFA concentrations may explain this finding. Therefore, we investigated whether elevated NEFA concentrations in the blood of the mother are a major metabolic key in the pathogenesis of subfertility, not only through impairment of the development and quality of the maturing oocyte, but also through
direct effects on the pre-implantation embryo. In order to better understand the possible mechanisms and pathways of toxicity of elevated NEFA concentrations, transcriptome analyses were performed. Data showed that embryos exposed to elevated NEFA concentrations during the culture period have a lower developmental potential. So far, transcriptome analyses showed that multiple pathways, mainly related to oxidative stress and lipogenesis, were affected in blastocysts originating from high NEFA-exposed embryos.

Finally, in Chapter 8, all results obtained from NEFA exposure during in vitro oocyte maturation and embryo culture are collectively discussed in the light of scientific knowledge. In this PhD study, we clearly showed that oocyte exposure to elevated NEFA concentrations during final maturation has implications for the embryo 7.5 days after exposure. Surprisingly, such embryos displayed glucose intolerant characteristics, were triggered by REDOX-regulating mechanisms and showed signs of mitochondrial dysfunction. Gene expression and functional data suggest that the resulting embryos have altered metabolic strategies, which might explain the aberrant energy metabolism and suggests a mechanism of metabolic dysregulation appearing in the pre-implantation embryo as a consequence of elevated NEFA concentrations periconception. Effects observed are highly dependent on the total fatty acid concentration and fatty acid profile applied. Furthermore, we highlighted that elevated NEFA concentrations in the blood of the mother are a major metabolic key in the pathogenesis of subfertility, not only though impairment of the development and quality of the maturing oocyte, but also through direct effects on the pre-implantation embryo. Elevated NEFA concentrations during embryo culture impact on further embryonic development and alter gene expression patterns in resultant day 7.5 blastocysts. Effects observed are highly dependent on the total fatty acid concentration and fatty acid profile applied.

It is the first time that direct effects of a realistic combination of pathologically elevated NEFA concentrations on the maturing oocyte and pre-implantation embryo were documented. In this PhD study, high-throughput technologies for the study of gene expression patterns were used, that allowed us to integrate and interpret data a posteriori and to
subsequently focus on the functional relevance of specific observations which resulted in down-stream interpretations. The combination of the new transcriptomic data and the data already available from our previous studies, together with the novel functional data, definitely improved our current understanding of the possible mechanisms through which specific NEFA exposure during oocyte maturation and early embryo culture affects resultant day 7.5 embryo physiology.

Our data confirmed that elevated NEFA concentrations are a scientifically intriguing and pathophysiologically important causative link between disturbed maternal metabolism and reduced oocyte and embryo quality. These findings might explain the higher rate of early pregnancy losses and miscarriages observed in women suffering lipolysis-linked metabolic disorders as this clearly shows that the oocyte and early embryo are very vulnerable to unfavourable metabolic conditions in the mother, leading to fertilisation failure or early pregnancy loss, and the latter being a major problem in the subfertility issue in dairy cows. Human medicine is also highly interested in these intriguing findings and public media reported these findings in more than 60 journals worldwide as our findings are consistent with public health recommendations which emphasize the importance of women being at healthy weight before starting a pregnancy.
Samenvatting.

Maternaal metabole problemen, zoals obesitas en type II diabetes bij de vrouw en negatieve energiebalans bij de koe, spelen een belangrijke rol in de pathogenese van de subfertilititeit. Zo hebben epidemiologische studies aangetoond dat vrouwen die lijden aan metabole stoornissen, gelinkt aan lipolyse, meer problemen ervaren om zwanger te geraken en bovendien een verhoogd risico lopen op embryonale sterfte. De pathogenese van deze vruchtbaarheidsproblemen is nog niet volledig opgehelderd. Enerzijds is geweten dat indirecte factoren, zoals een gewijzigde hormoonhuishouding, verantwoordelijk kunnen zijn voor veranderingen in de folliculaire groei en zelfs anovulatie. Anderzijds rapporteren recente studies dat ook directe metabole veranderingen in het micromilieu van de maturerende eicel en het jonge embryo nefaste gevolgen kunnen hebben voor de eicel en het embryo zelf. Jarenlang onderzoek met behulp van in-vitro-embryoproductiemodellen heeft ervoor gezorgd dat onze kennis over de gevolgen van metabole veranderingen op eicel- en embryokwaliteit drastisch is toegenomen. In Hoofdstuk 1 wordt met een kritische blik uitgelegd wat de impact kan zijn van metabole aandoeningen voor de vruchtbaarheid en worden de niveaus besproken waarop metabole wijzigingen in het bloed van de moeder kunnen leiden tot vruchtbaarheidsproblemen.

Vrouwen die lijden aan obesitas, type II diabetes en negatieve energiebalans vertonen een opvallende ‘wijziging’ van het maternale metabolisme, gekenmerkt door verhoogde niet veresterde vrije vetzuur (NEFA)-concentraties in het bloed. Zoals beschreven in Hoofdstuk 2, worden NEFAs steeds vaker erkend als belangrijke sleutelmoleculen in de pathogenese van bepaalde metabole stoornissen en gedaalde vruchtbaarheidsresultaten. Metabole veranderingen, zoals verhoogde NEFA-concentraties in het bloed, wijzigen de samenstelling van het micromilieu van de eicel en de granulosacellen. In-vitromodellen hebben aangetoond dat NEFAs nefast zijn voor de vitaliteit en steroidogene capaciteit van humane en boviene follikelcellen. Meer nog, boviene studies rapporteerden dat, wanneer de eicel wordt blootgesteld aan
verhoogde NEFA-concentraties, de eicelontwikkelingscompetentie wordt gereduceerd.

In Hoofdstuk 3 worden de verschillende doelstellingen van deze thesis toegelicht. Op basis van data die de cytotoxische eigenschappen van NEFAs beschrijven in tal van somatische celtypes, werd de vraag gesteld in welke mate verhoogde NEFA-concentraties ook gevolgen kunnen hebben voor de matuerende eicel en het jonge embryo. Bovendien wilden we nagaan in welke mate een verhoogde NEFA-blootstelling op eicelniveau en op jong embryonaal niveau verdere gevolgen kan hebben voor het embryo dat hieruit ontwikkelt. Hierbij poogden we de onderliggende biochemische en moleculaire mechanismen van de effecten, geïnduceerd door verhoogde NEFA-blootstelling gedurende eicelmaturatie en vroeg embryonale ontwikkeling, te karakteriseren.

Daarom werd in dit doctoraat gebruik gemaakt van een bovien in-vitro-eicelmaturatie- en embryocultuursysteem waarbij eicellen en embryo’s worden blootgesteld aan pathofysiologische NEFA-concentraties. De informatie die resulteerde uit deze modellen kan helpen bij ophelderen van de pathogenese van de gedaalde fertiliteit, en meer specifiek de verhoogde kans op embryonale sterfte, bij vrouwen die lijden aan metabolie stoornissen gelinkt aan lipolyse.

In Hoofdstuk 4 werd onderzocht in welke mate verhoogde NEFA-concentraties gedurende de finale eicelmaturatieperiode een overdraagbaar effect kunnen induceren op de kwaliteit, genexpressie, metabolisme en viabiliteit van het embryo dat er zich uit ontwikkelt. Met behulp van ons bovien in-vitromaturatiemodel konden we aantonen dat, wanneer de eicel wordt blootgesteld aan verhoogde NEFA-concentraties, de eicelontwikkelingscompetentie gereduceerd wordt. Bovendien stelden we vast dat niet alleen de eicelontwikkelingscompetentie door NEFA-blootstelling daalt, maar dat ook de embryo’s die zich uit deze eicellen ontwikkelen kwalitatief minderwaardig zijn ten opzichte van controle-embryo’s. Voorts zagen we dat dergelijke embryo’s, die zich ontwikkelen uit eicellen blootgesteld aan NEFAs, een aberrant metabolisme vertoonden. Ze consumeerden minder zuurstof, glucose en pyruvaat in
vergelijking met controle-embryo’s. In tegenstelling tot het gereduceerde energiemetabolisme, vertoonden de NEFA-embryo’s ook een opgereguleerd aminozuurmetabolisme, wat een lagere vitaliteit van deze embryo’s impliceert.

In Hoofdstuk 5 bestudeerden we de etiologische mechanismen achter de NEFA-toxiciteit. Hierbij werd gespeculeerd dat door een overmaat aan vetzuren, het metabolisme van de eicel stijgt, waardoor er een overmaat aan ROS geproduceerd wordt. Daarom werden er verschillende experimenten opgezet om te bepalen in welke mate NEFA-blootstelling gedurende eicelmaturatie de mitochondriale functie beïnvloedt. Genexpressieanalyses hebben aangetoond dat de expressie van oxidatieve stress gerelateerde genen wordt opgereguleerd, zowel in eicellen blootgesteld aan verhoogde NEFA-concentraties als in de omringende cumuluscellen. Ook functionele analyses, waarbij het mitochondriale metabolisme geïnhibeerd werd, toonden aan dat de mitochondriale functie betrokken is bij het induceren van het negatieve effect. In zygoten, afkomstig van eicellen gematureerd onder NEFA-condities, konden we echter geen significante verschillen aantonen in zuurstofconsumptie en enkel een trend tot gestegen mitochondriale membraanpolariteit vaststellen. Bovendien toonden genexpressieanalyses aan dat embryo’s resulterend uit dergelijke eicellen een verhoogde expressie vertoonden van genen gerelateerd aan vetzuursynthese, wat ons doet denken aan een shift van het metabolisme richting vetopslag.

In Hoofdstuk 6 werden met behulp van microarraytechnieken de genexpressieprofielen in kaart gebracht van de embryo’s afkomstig van eicellen blootgesteld aan verhoogde NEFA-concentraties gedurende de finale maturatieperiode. De analyses wezen op een op-gereguleerde expressie van genen betrokken in apoptoseprocessen en genen gerelateerd aan celproliferatie in de embryo’s afkomstig van eicellen blootgesteld aan hoge NEFA-concentraties ten opzichte van de controle-groep. Bovendien, werden er met behulp van deze microarraydata en de bijbehorende analysetechnieken verschillende mechanistische processen gekarakteriseerd. Zo werden er onder meer een gereduceerde expressie van genen betrokken in ceramideformatie en een opgereguleerde
expressie van genen betrokken in processen van triglyceridensynthese en pH regulatie waargenomen in embryo’s afkomstig van NEFA-eicellen ten opzichte van de controlegroep. Ook een gedaalde expressie van genen gerelateerd aan de processen van Ca\(^{2+}\) homeostase werd in kaart gebracht in de embryo’s afkomstig uit de hoge NEFA-groep ten opzichte van de controle-embryo’s. De relevantie van de pathways die op genexpressieniveau gewijzigd waren, werd grotendeels gevalideerd met behulp van glutathion kwantificatie in eicellen, kwantificatie van het vetdruppelgehalte in resulterende morulae en evaluatie van de invriesbaarheid van resulterende blastocysten.

Eicelmaturatie onder verhoogde NEFA-condities heeft duidelijk implicaties voor de embryo’s die er zich uit ontwikkelen. Nochtans is ook het milieu waarin het jonge embryo zich ontwikkelt belangrijk voor de verdere embryonale ontwikkeling. In hoeverre verhoogde NEFA-concentraties hierbij een directe invloed kunnen uitoefenen op deze jonge embryo’s werd onderzocht in **Hoofdstuk 7**. embryo’s werden daarvoor gedurende de cultuurperiode blootgesteld aan verhoogde NEFA-concentraties. Hierbij stelden we vast dat verhoogde NEFA-concentraties niet enkel schadelijk zijn op het niveau van de eicel, maar ook, wanneer aanwezig in het embryonale micromilieu, de ontwikkeling tot blastocyst kunnen verstoren. Om de etiologische processen te ontrafelen achter deze negatieve invloeden werd weerom met behulp van de microarrayanalysetechniek de genexpressieprofielen bepaald van embryo’s die blootgesteld werden aan verhoogde NEFA-concentraties. Dit leerde ons dat voornamelijk pathways met betrekking tot oxidatieve stress en triglyceridensynthese betrokken zijn in de effecten veroorzaakt door verhoogde NEFA-blootstelling gedurende de fase van vroeg-embryonale ontwikkeling.

Finaal, in **Hoofdstuk 8**, werden de resultaten van alle experimenten gebundeld en afgetoetst aan de informatie die reeds beschikbaar is in de literatuur. In dit doctoraatswerk hebben we duidelijk kunnen aantonen dat embryo’s afkomstig van eicellen blootgesteld aan verhoogde NEFA-concentraties een afwijkende fysiologie vertonen. Verhoogde NEFA-concentraties gedurende de finale eicelmaturatie resulteerden in
embryo’s met een gedaald cel aantal, verhoogde apoptose index, een gewijzigd metabolisme en gedaalde vitaliteit ten opzichte van controle-embryo’s. Opvallend hierbij was dat dergelijke embryo’s afkomstig van NEFA-eicellen glucose intolerante eigenschappen vertoonden en getekend waren door mechanismen geactiveerd door oxidatieve stress. De gevolgen van de NEFA-behandelingen waren afhankelijk van de hoeveelheid en type NEFAs waaraan de eicellen werden blootgesteld. Bovendien hebben we duidelijk kunnen aantonen dat verhoogde NEFA-concentraties in het bloed van de moeder niet alleen bepalend zijn voor de verdere ontwikkeling en kwaliteit van de eicel, maar ook directe gevolgen kunnen hebben voor het ontwikkelen van het jonge embryo. De effecten gesorteerd door de NEFA-behandelingen waren weerom afhankelijk van de hoeveelheid en type NEFAs waaraan de embryo’s werden blootgesteld.

De combinatie van nieuwe relevante fenotypische data samen met de transcriptoom-analyse heeft geholpen om het achterliggend mechanisme van directe embryonale NEFA-toxiciteit bloot te leggen. Onze data bevestigen het vermoeden dat verhoogde NEFA-concentraties gedurende de finale eicelmaturatie en de vroeg embryonale ontwikkeling een cruciale link vormen tussen maternale metabole stoornissen en de gedaalde eicel- en embryokwaliteit. Bovendien kunnen deze observaties het verhoogde risico op embryonale sterfte in moeders die lijden aan metabole stoornissen gelinkt met lipolyseprocessen verklaren. Al de bevindingen die reeds gegeven zijn voor dit onderzoek en de informatie die zal bekomen worden met het reeds ingeplande onderzoek, vormen de fundamentele basis voor toekomstig humaan onderzoek. De data kunnen bijdragen tot de opheldering van de etiologie van humane en bovine vruchtbaarheidsproblemen waarbij een gedaalde eicel- en embryokwaliteit centraal staan.
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