INVESTIGATING THE IMPACT OF HYPERGLYCAEMIA ON EARLY EMBRYO ENVIRONMENT AND DEVELOPMENT

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Abstract

The development of metabolic conditions has been associated with suboptimal nutrient provision to the embryo during pregnancy. Metabolic disorders have also been linked to subfertility; obese women produce fewer and smaller oocytes that are metabolically distinct from the oocytes of non-obese women. However, the impact of metabolic conditions on the secretions of the oviduct has not been extensively investigated. The work presented in this thesis has used an established *in vitro* model of the oviduct to determine the extent to which hyperglycaemia in the presence or absence of insulin modified oviduct secretions. These secretions were used to examine whether embryo survival and metabolism were also affected. Bovine oviduct epithelial cells (BOECs) were cultured under conditions designed to model acute and chronic hyperglycaemia, and in the presence or absence of physiologically relevant insulin concentrations. The fluid secreted by BOECs was analysed using ultramicrofluorometric assays and high-performance liquid chromatography, and expression of key genes was also assessed. Chronic treatment of BOECs under the above conditions modified cell secretions, since the concentrations of glucose, alanine, glycine and glutamine were significantly altered. The data on concentrations of substrates was then used to inform the composition of embryo culture medium, using 1mM, 3.6mM and 8.9mM glucose for the '8.5', '7.3' / '11+', and '11-' environments respectively, and modifying lactate, pyruvate and amino acid concentrations accordingly. The modified embryo environments did not affect preimplantation embryo cleavage and blastocyst rates; however, they modified the rates of pyruvate and glycine consumption, as well as arginine consumption/production. In summary, hyperglycaemia in the presence or absence of insulin modifies oviduct secretions, which in turn leads to altered embryo metabolism. Further experiments may elucidate whether a combination of molecules implicated in metabolic conditions exert a more pronounced effect on the composition of oviduct-derived fluid, as well as embryo survival and metabolism.

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Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

Chapter 1

Introduction

The success of mammalian reproduction can be measured by the delivery of healthy live young. However, there are many factors that may influence this, not least the health status of the mother. Indeed, compelling data now exists to show that events occurring prior to, and during pregnancy can influence the life-long health of the offspring. Such a paradigm is best described by the Developmental Origins and Health and Disease hypothesis, proposed initially by David Barker (Barker, 2004). Clear examples of a prenatal exposure leading adverse health outcomes in the offspring include diseases such as spina bifida, which can result through an absence of dietary folic acid during early pregnancy, foetal alcohol syndrome disorders, which manifests as physical and behavioural problems and is caused by heavy maternal alcohol intake during pregnancy, and the so-called 'thalidomide syndrome' that was caused by ingestion of the drug thalidomide to combat morning sickness, and led to phocomelia in the offspring.

Examples such as these indicate clearly the sensitivity of the developing offspring to events during pregnancy. The severe examples above result in a clear phenotype in the offspring, however data are now revealing that more subtle modifications to offspring health can arise in response to maternal health and fitness. Numerous studies on animals have indicated that dietary challenge, especially during the first few days of embryo development can lead to lifelong health impacts in the offspring. The embryo is very sensitive to environmental changes at the preimplantation stage, such as those induced by an imbalanced maternal diet (reviewed by (Watkins, Papenbrock and Fleming, 2008)). Embryo development in a low-nutrient environment promotes embryonic developmental plasticity to ensure embryo survival and growth, however, these mechanisms of plasticity may be associated with the development of metabolic conditions in the adult life of the offspring (Watkins *et al.*, 2008).

One of the challenges of understanding the links between maternal physiology during pregnancy and offspring health is the relative difficulty of sampling the environment in which early development usually takes place. Indeed, we have very little knowledge of how maternal diet and health affects the composition of the environment in which early development occurs. These limitations in accessing the environment of the pre-implantation embryo, combined with ethical implications associated with the use of

human tissue, have supported the use of animal models, and, in many cases, the bovine as a model organism for the study of reproductive tract pathologies.

This thesis has set out to use an *in vitro* experimental model to examine how maternal hyperglycaemia affects the volume and the composition of the bovine oviduct epithelial cell secretions, as well as the impact of a hyperglycaemia-modified environment on embryo survival and metabolism. In order to fully introduce the work undertaken, it is necessary to summarise the key aspects of female reproductive physiology, and then to review the approaches that have been used to examine the links between maternal physiology and early development.

1.1. Oestrous cycle

The female reproductive tract provides the environment in which fundamental reproductive processes such as gamete transportation, fertilization and embryo development occur. The female tract facilitates the change from supporting gamete maturation to sustaining the first stage of a pregnancy. This cyclicity is hormonally regulated and may be thought of as having two components; the uterine cycle and the ovarian cycle, which differ functionally, but occur in parallel.

1.1.1. Ovarian cycle

In most mammals, the external manifestation of the internal cyclicity of the ovarian cycle is termed oestrous. The oestrous lasts for a short period, and coincides with ovulation. Oestrous is apparent through behavioural changes (Johnson, 2007), the most clear sign of sexual receptivity being the acceptance of the female of being mounted by the male (Orihuela, 2000). In the bovine, the oestrous cycle lasts between 18-24 days and consists of two distinct phases, the follicular and the luteal phase. The follicular phase, which lasts about 4-6 days in the bovine, involves the final maturation of the oocyte and ovulation to the lumen of the oviduct. The luteal phase, which lasts approximately 14-18 days in the bovine, follows ovulation and involves the formation of the corpus luteum from the remaining cells of the ovarian follicle (Forde *et al.*, 2011).

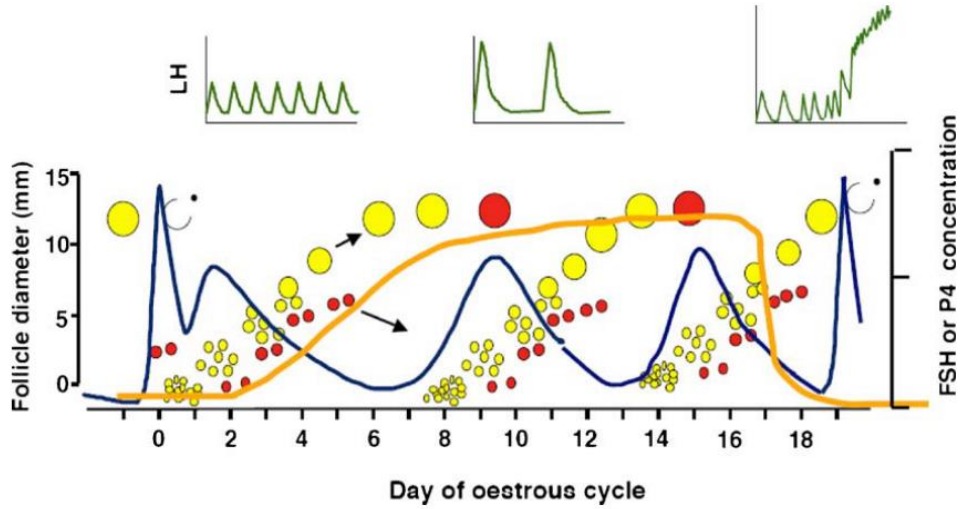
In humans and higher primates, ovarian cyclicity is exhibited through the menstrual cycle, rather than the oestrous cycle. There are key differences between the oestrous and the menstrual cycle. Firstly, at the end of luteal phase of the menstrual cycle, the endometrium is shed through a process called menstruation*;* in the oestrous cycle, the endometrium is re-absorbed. Additionally, in oestrous cycles*,* females are only receptive to males at oestrous and not during the luteal phase; on the contrary, in humans, females are receptive to males throughout the cycle. Furthermore, the menstrual cycle typically lasts about 28 days, whereas the oestrous cycle typically lasts about 21 days, although this can vary according to species. Moreover, in the menstrual cycle, day 1 is the first day of menstruation, as opposed to oestrous, where day 1 is the first day of oestrous (Johnson, 2007).

As reviewed by Mwaanga and Janowski, there is another state, called anoestrus, during which animals do not exhibit normal oestrous cycle or manifestations of heat, a period physiologically occurring before puberty, as well as during and after pregnancy and parturition (Mwaanga and Janowski, 2000).

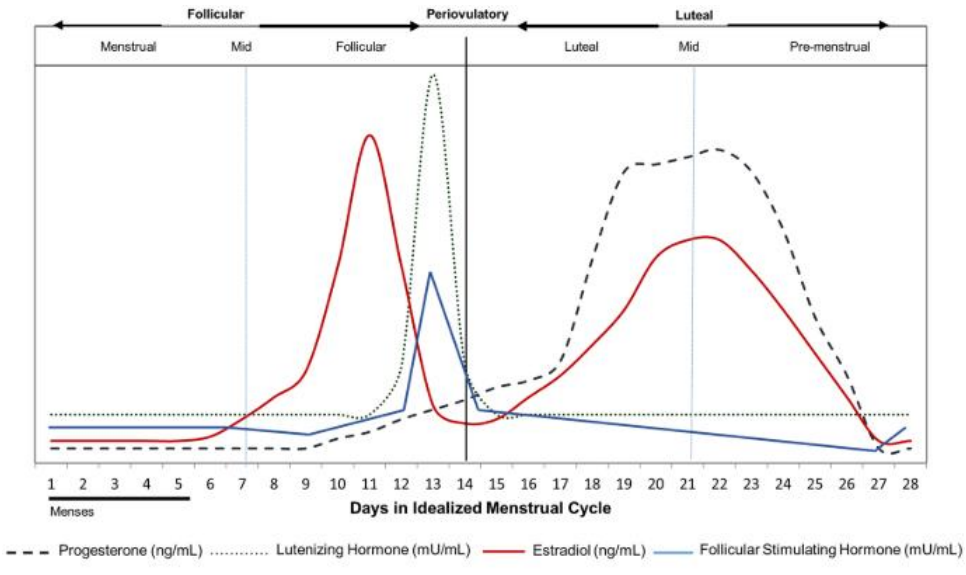
In spite of the differences between the oestrous and the menstrual cycle, there are fundamental similarities between the two. The hormonal regulation shows notable similarities, even though the duration of the secretion of each hormone varies. Additionally, key biological processes throughout the cycle are induced by the same events, for example ovulation, described later in more detail, is induced by the LH surge in both cases.

1.1.2. Endocrine regulation of the oestrous cycle

A schematic representation summarising the cyclicity of the oestrous cycle is shown in Figure 1.1.



a)



b)

Figure 1.1.: a) Schematic representation of hormonal secretions during the oestrous cycle in the bovine and association with the pattern of ovarian follicle growth. The blue line represents FSH, which increases transiently before follicular growth, while LH pulsatile secretion patterns are modified in terms of amplitude and frequency according to the stage of the cycle (green line in inserts above). Day 0 of the cycle indicates the beginning of the early luteal phase, progressing to the mid-luteal phase and the to the follicular phase. Day 21 of the present graph is the day of ovulation. Progesterone levels (orange line), increase after day 2 of the cycle, almost plateauing on day 8 until day 17, when they drop significantly before ovulation. Red circles represent atretic follicles, whereas yellow circles are healthy follicles (Forde *et al.*, 2011). b) Schematic representation of the endocrinological profile of the menstrual cycle (Draper *et al.*, 2018). As discussed in the text, day 1 of the menstrual cycle is the day when menstruation begins. The hormonal profile of the two cycles is similar, with high levels of oestrogen preceding ovulation, high levels of progesterone following it and a surge of LH just before oocyte ovulation.

The gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH) are secreted by the anterior pituitary gland. This secretion of FSH and LH is regulated by the gonadotrophin-releasing hormone (GnRH), which is a decapeptide secreted in the hypothalamus (Schally *et al.*, 1971). Similar to gonadotrophin secretion, GnRH secretion is also pulsatile (Forde *et al.*, 2011). As a response to GnRH pulse, FSH and LH are released within a few minutes (Johnson, 2007), a process mediated by binding of the GnRH on the GnRH receptors in the pituitary gland (Kakar, Rahe and Neill, 1993).

Each peak in the peripheral LH coincides with a GnRH pulse, and the amplitudes of LH pulses are determined by the frequency of GnRH pulses (Clarke and Cummins, 1985). This pulsatile secretion of GnRH and the pre-ovulatory surge of the hormone from the hypothalamus prevents the gonadotrophs in the anterior pituitary gland from becoming desensitised (Forde *et al.*, 2011). FSH and LH can be stored in the same secretory granules, but their secretion is not synchronous, suggesting that secretion of the two gonadotrophs is differentially controlled and led by separate exocytosis pathways (Farnworth, 1995).

As will be discussed in more detail in section 1.2, FSH and LH are crucial for ovarian follicular development, follicles being involved in mechanisms of positive and negative feedback of the hypothalamic – pituitary – gonadal axis (Johnson, 2007)(Forde *et al.*, 2011). In brief, LH acts on theca cells in the ovary to trigger androgen synthesis. FSH acts on granulosa cells in the ovary which convert androgens into oestrogens. Androgens and oestrogens are steroid hormones, derived from a common precursor molecule, cholesterol (Johnson, 2007). In the human ovarian cycle, the levels of oestrogens are highest before ovulation and are relatively low during the time when fertilisation occurs (Winuthayanon *et al.*, 2015), following the broad profile of LH. The levels of progesterone follow the opposite pattern, being highest after ovulation and around the time of fertilisation (Johnson, 2007). Oestradiol is known to have a dual function in the regulation of gonadotrophin secretion. At low levels, it has an inhibitory role in the secretion of gonadotrophins through a negative feedback mechanism; conversely, at high levels, it induces a positive feedback mechanism, therefore inducing a surge of FSH and LH, necessary to trigger ovulation. On the other hand, high levels of progesterone, similar to those observed during the luteal phase of the cycle, exert a negative feedback

mechanism, thus maintaining low levels of FSH and LH. Additionally, high levels of progesterone block the positive feedback effect of oestradiol (Johnson, 2007).

During the follicular phase of the ovarian cycle, the levels of oestrogen are low. Steadily, the levels of oestrogen rise, as the androgen synthesised by the thecal cells causes the granulosa cells to proliferate (Johnson, 2007). These increased levels of oestrogen, in turn, exert a negative feedback effect, thus reducing the FSH levels, whereas LH levels plateau (Hillier, 1994). At the time of selection of the dominant follicle, there is a further increase in oestrogen levels a switch from inhibin-B to inhibin-A occurs. This oestrogen surge exerts positive feedback over gonadotrophins, reflected by a surge in the levels of LH and FSH. At this time the follicle gets ovulated and directed into the oviduct (Johnson, 2007). The FSH levels in the circulation are then reduced again due to oestrogen secretion from the dominant follicle, and the remaining follicles become atretic (Hillier, 1994).

The luteal phase of the cycle is dominated by the corpus luteum, which forms from the remnants of the ovulated follicle and by increased systemic levels of progesterone (Forde *et al.*, 2011). Progesterone suppresses the levels of the gonadotrophins by negative feedback, and the levels of androgens also become suppressed. Even though oestrogen levels appear to rise during the luteal phase, there is no gonadotrophin surge, as progesterone suppresses that feedback mechanism (Johnson, 2007). If pregnancy is not established, then, in the bovine, prostaglandin F is detected in the uterine fluids, suggesting that luteolysis (Lamothe, Bousquet and Guay, 1977), defined as the regression of the corpus luteum (McCracken, Custer and Lamsa, 2019),has occurred (Lamothe, Bousquet and Guay, 1977). This results in reduced levels of progesterone in circulation, increasing levels of oestrogen and GnRH stimulation, for the beginning of a new oestrous cycle (Forde *et al.*, 2011).

1.1.3. Uterine cycle

In parallel to the ovarian cycle, the uterus also undergoes cyclical changes. The follicular phase of the ovarian cycle corresponds to the proliferative phase of the uterine cycle. During this phase, the endometrium thickens and re-epithelises in readiness for a pregnancy, should conception occur. During this phase of the cycle, oestradiol 17β is the

dominant hormone. The luteal phase of the ovarian cycle corresponds to the secretory phase of the uterine cycle, when the uterus increases its secretions in readiness to support a pregnancy; this phase of the cycle is progesterone-dominated (Johnson, 2007).

The hormonal fluctuation observed during the cycle regulates the action of the female reproductive tract and the ovary, which will be described in the next section. Previous work in the laboratory suggests that addition of oestrogen, progesterone, or pathologically high or low concentrations of androgens result in altered secretions of the oviduct epithelium (Simintiras *et al.*, 2016). The importance of these findings has led us to explore the impact of other pathophysiological conditions, such as the ones described in this thesis, on the composition of the pre-implantation environment.

1.2. Oocyte development

The ovary is the site of gametogenesis in females. It is formed from a bipotential precursor, a non-differentiated genital ridge, during foetal life (Lucas-Herald and Bashamboo, 2014)., the coelomic epithelium that resides in the primitive gonad condenses and surrounds the primordial germ cells (Ungewitte and Yao, 2013).. This action forms the primordial follicle, and the germ cells cease mitotic proliferation and enter meiosis (Ungewitte and Yao, 2013) , which they arrest at the prophase stage (Sarma, Findlay and Hutt, 2019). Follicles can remain metabolically quiescent at the first meiotic prophase, at an arrested state, for up to 40 (Coticchio *et al.*, 2014) to 50 years (Gosden and Lee, 2010), until the initiation of menstrual or oestrous activity at puberty (Jaffe and Egbert, 2018) . Follicular development is a very slow process, since, in the human, a primordial follicle may have been recruited approximately a year before it became dominant (Conti and Chang, 2016).

At the stage of the primordial follicle, the primary oocytes are surrounded by the granulosa cells of the ovary (Jaffe and Egbert, 2018), which are flattened mesenchymal cells in the ovary. (Figure 1.2) As a primordial follicle progresses to form a pre-antral follicle, the oocyte grows significantly in size, approximately 100-fold, reaching a final size of approximately 120µM in the human (Gosden and Lee, 2010). At that stage, organelles, structural components and soluble components can be detected in abundance in the follicle (reviewed by (Gosden and Lee, 2010)). Extensive RNA synthesis occurs (Johnson, 2007)(Gosden and Lee, 2010), which will be used in subsequent stages of oocyte maturation (Johnson, 2007) and in the first stages of embryo development, if fertilisation occurs (Coticchio *et al.*, 2014). Also, during this phase, the oocyte secretes glycoproteins, which will eventually form the zona pellucida, separating the oocyte from the surrounding granulosa cells (Johnson, 2007). Moreover, at this stage, theca cells of follicle are formed from ovarian stromal precursor cells (Magoffin, 2005), and they surround the granulosa cells (Johnson, 2007).

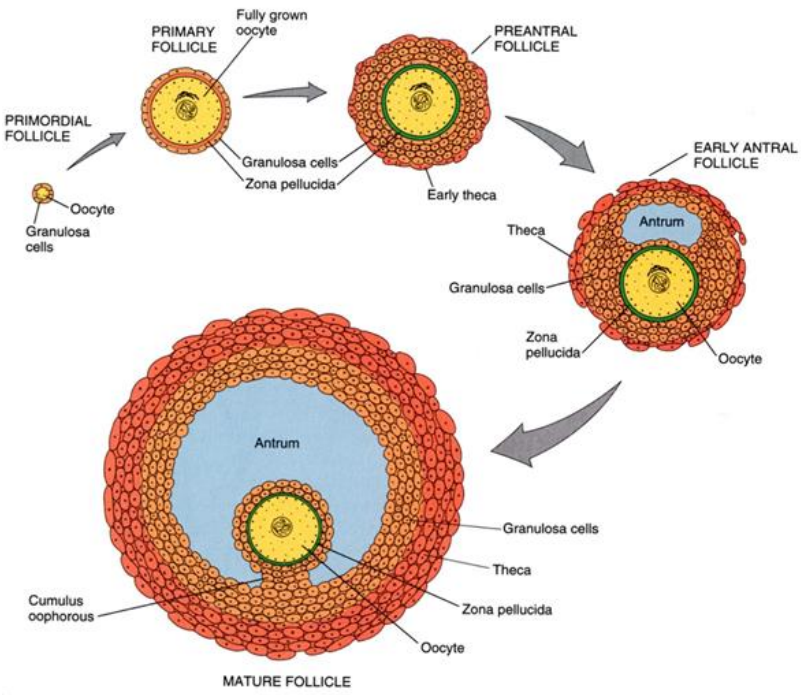


Figure 1.2.: Oocyte maturation (figure from (Widmaier, Raff and Strang, 2006)). At the prenatal stage, primordial germ cells migrate to the foetal ovary and undergo mitotic divisions, followed by entry into meiosis and arrest at the prophase. At birth, they are at the primordial follicle stage and, at puberty, they resume the first meiotic division. Follicular growth occurs in parallel with entry into the second meiotic division, until the follicles reach the preovulatory stage and meiotic metaphase II. The oocyte will then get ovulated and will complete the second meiotic division post-fertilisation (Gosden and Lee, 2010)

Follicular progression is known to progress relatively quickly at the antral stage, over 40 to 50 days in the human (Conti and Chang, 2016). During the transition of pre-antral to antral follicles, the cells of theca layer are further differentiated to cells of the theca interna and theca externa (Magoffin, 2005). The cells of the theca interna are highly vascularised cells, associated with steroidogenesis, whereas the cells of the theca externa are more loosely organised, do not produce steroid hormones and reside between the cells of the theca interna and the interfollicular stromal area of the ovary (Magoffin, 2005). The granulosa cells secrete follicular fluid, which, in the end of that phase, will have formed the follicular antrum. The increase of the volume of follicular fluid produced and, consequently, the overall size of the follicle, results in the oocyte remaining in suspension in the follicular fluid (Johnson, 2007). The cells surrounding the oocyte are differentiated granulosa cells (Matzuk *et al.*, 2002), called the cumulus oophorus, and are connected to the rest of the granulosa cells with a thin stalk of granulosa cells (Johnson, 2007), whereas the granulosa cells lining the wall of the follicle are the mural granulosa cells (Matzuk *et al.*, 2002). At this stage, the androgens and the

oestrogens produced by theca and granulosa cells are closely linked to follicular growth and maturation. Androgens are produced in the theca cells then become aromatised to oestrogen in the granulosa cells (reviewed by (Forde *et al.*, 2011)). The androgens act synergistically with FSH to promote granulosa cell proliferation. At the same time, oestrogens can also promote granulosa cell proliferation, therefore activating a system of positive feedback. Consequently, the levels of oestrogen surge just before ovulation (Johnson, 2007).

The selection of the dominant follicle is associated with the responsiveness of granulosa cells of follicles to FSH, which at this time increases and induces aromatase synthesis in the granulosa cells. This follicle will be the first one to secret oestrogen (Hillier, 1994), whose levels increase. Increased levels of oestrogen and inhibin-A synthesis, rather than inhibin-B, indicate the development of the dominant follicle (Welt *et al.*, 2001)which is the only follicle that remains viable and secretes oestrogen toward the end of the follicular phase (Hillier, 1994). If the LH surge, described in section 1.1.2, coincides with the appearance of receptors of LH in both the outer granulosa cells and the theca cells, then the follicle becomes dominant (Xu *et al.*, 1995).

After the surge of LH, significant endocrine changes occur in the follicle, mainly attributed to the granulosa cells, which, instead of aromatising androgens to oestrogens, start synthesizing progesterone (Johnson, 2007). Just before ovulation, the oocyte completes the first meiotic division. Even though the oocytes are characterised by radial symmetry, with the exception of the nucleus, which is positioned slightly eccentrically, the meiotic spindle migrates close to the oocyte cortex. Consequently, the first polar body is formed (Maro and Verlhac, 2002; Gosden and Lee, 2010), a process which enables chromosome segregation without loss of any of the products stored in the oocyte that will be of use after fertilisation (Maro and Verlhac, 2002). The second meiotic division is then initiated, where it arrests at the metaphase stage. This is accompanied by a simultaneous maturation of the cytoplasm; the oocyte detaches slightly from its surrounding granulosa cells, and the cortical granules are formed by its Golgi apparatus, which move to the surface to enable cortical reaction if fertilisation occurs (Johnson, 2007).

Ovulation completes the follicular phase of the cycle, which can vary across species. At ovulation, the follicle ruptures and follicular fluid enters the oviduct, along with the oocyte, surrounded by the cumulus cells (Johnson, 2007).

During the luteal phase of the cycle, the corpus luteum is formed from conversion of the granulosa cells and inner theca cells to large and small luteal cells, respectively, and high levels of LH are associated with this conversion. Luteal cells secrete inhibin A, oxytocin and, in humans and greater apes, oestradiol (Johnson, 2007). The role of the corpus luteum is to produce adequate levels of progesterone to support a potential pregnancy and endometrial receptivity (Forde *et al.*, 2011). As mentioned in section 1.1.2, if this is not established, luteolysis occurs, during which lutein cells collapse and progressive cellular death can be observed, which results in the formation of scar tissue, called corpus albicans (McCracken, Custer and Lamsa, 2019).

There has been extensive research on the effect of external stimuli on the female reproductive tract and the ovary in particular, some of which will be discussed later in section 1.8. However, there are relatively limited data on the impact that such stimuli may exert on the environment where fertilisation and early embryo development will occur. The environment in which these events occur, the oviduct, will be discussed in the next section.

1.3. The oviduct

1.3.1. Oviduct structure

The oviduct, known as the Fallopian tube or “tube” in the woman, , has been described as “a muscular tube with a mucous lining” by Leese (Leese, 1988). It is a tube-shaped structure, comprised of a single columnar epithelium, surrounded and supported by stromal cells and muscle cells, as well as a serosa (Winuthayanon *et al.*, 2015). The oviduct can be anatomically divided in four regions: the utero-tubal junction, the isthmus, the ampulla and the infundibulum, which has fimbriae that open towards the peritoneal cavity (Figure 1.3), (Leese, 1988). The thickness of the oviduct increases from the infundibulum towards the utero-tubal junction (Hunter, 2012). The infundibulum is often termed the “pre-ampulla”, and the utero-tubal junction is referred to as the “intramural” or “interstitial” portion (Leese, 1988).

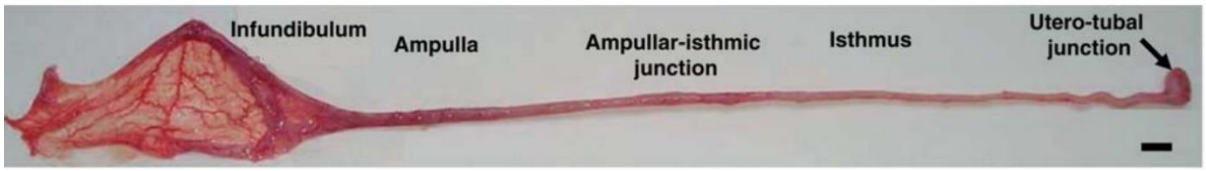


Figure 1.3.: Structure of the oviduct. The infundibulum, ampulla, isthmus and utero-tubal junction are shown. The ampullary-isthmic junction is the site of fertilisation. Figure from (Aviles, Gutierrez-Adan and Coy, 2010)

The subepithelial part of the oviduct consists of mesothelial cells, fibroblasts, collagen fibrils and inner and outer smooth muscle tissue (Clyman, 1966), (Figure 1.4). The mucosa of the oviduct has folds with a typical tree branch-like structure, a structure creating a variety of microenvironments and helping prevent a large number of spermatozoa from reaching the oocyte (Coy, Avile and Monde, 2012).

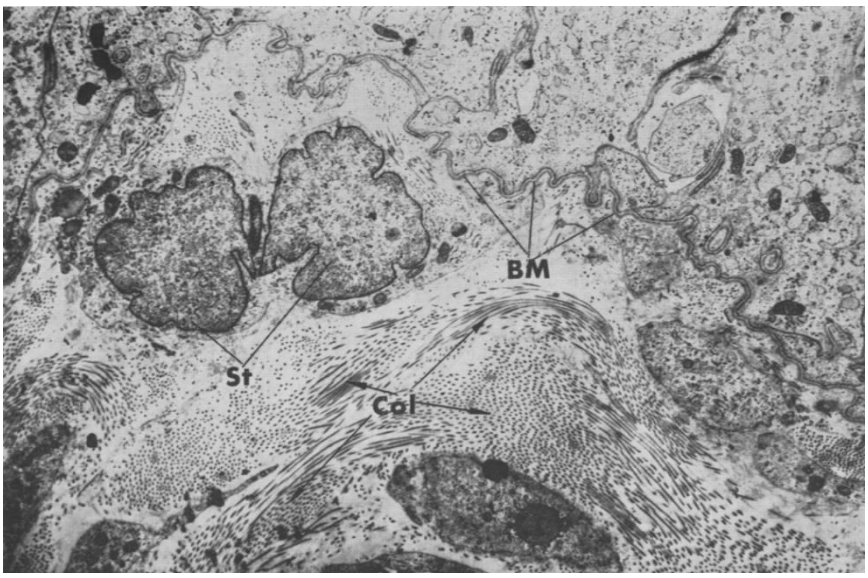


Figure 1.4.: Electron microscopy picture of the sub-epithelial area of the oviduct. BM = basal membrane, St= stromal cells, Col=collagen (Clyman, 1966)

1.3.1.1. Oviduct epithelium

The isthmus and ampulla of the oviduct contain two epithelial cell types; secretory cells and ciliated cells (Joshi, 1995) (Figure 1.5). The ratio of ciliated to secretory cells decreases from the infundibulum to the utero-tubal junction (Leese, 1983, 1988). Secretory cells are involved in the production of the oviduct fluid, containing among others oviduct-specific glycoproteins. In contrast, ciliated cells are implicated in gamete transport to the site of fertilization, embryo transportation (Comer, Leese and Southgate, 1998), and sperm binding involved in the formation of a sperm reservoir, discussed later in section 1.4 (Comer, Leese and Southgate, 1998; Coy *et al.*, 2012). Indeed, it has been found that the movement of a cumulus-oocyte complex into the oviduct is facilitated by interaction between the matrix of the cumulus-oocyte complex and the ciliary crowns of the oviduct epithelial cells (Lam *et al.*, 2000).

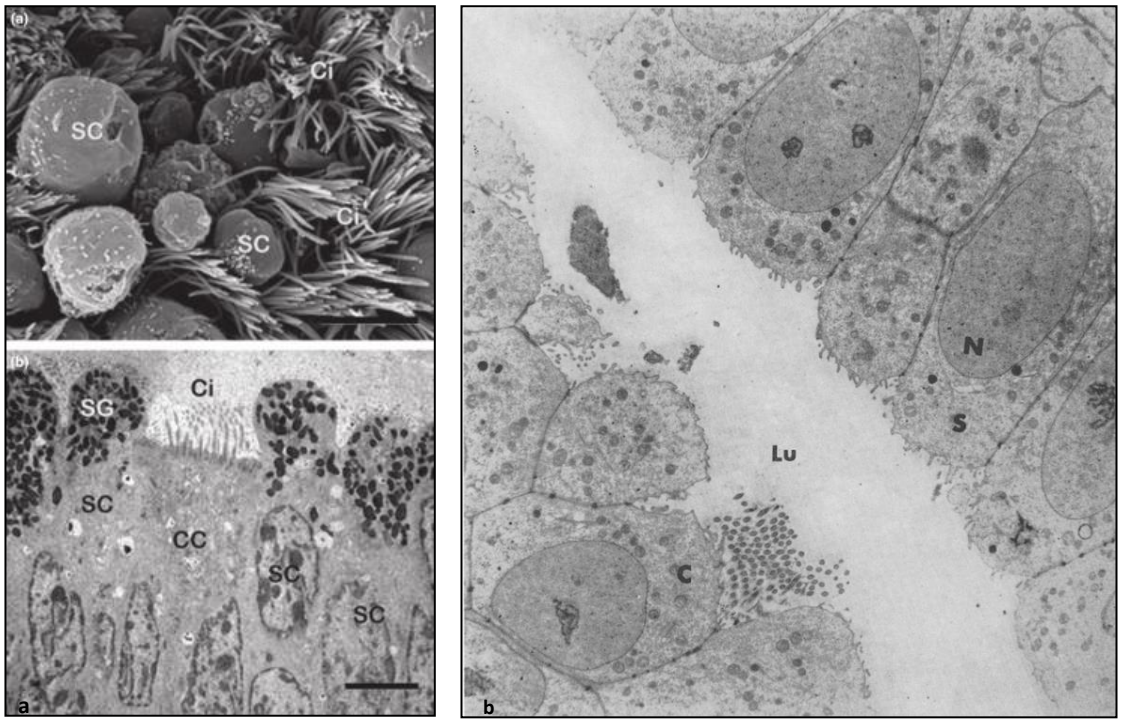


Figure 1.5.: a) Oviduct epithelial cells, distinguished in ciliated (CC, Ci=cilia) and secretory cells (SC). (Coy, Avile and Monde, 2012), b) Electron microscopy picture of the fallopian tube. Ciliated and Secretory Epithelial cells can be observed. The lumen can be clearly seen. Lu=lumen, S=secretory epithelial cells, C=ciliated epithelial cells, N=nucleus (Clyman, 1966).

1.3.2. Role of the oviduct

The oviduct plays a number of roles in facilitating early development. It is the site for final gamete maturation, fertilisation and the first stages of early embryo development, which is referred to as the preimplantation stage.

The role of the oviduct in gamete activation is threefold: it mediates sperm capacitation and hyperactivation, which will be discussed later (section 1.4), it affects oocyte physiology and it guides oocyte-sperm interactions (Coy *et al.*, 2012). The oocyte gets retrieved into the oviduct through interactions between the extracellular matrix of the cumulus cells and the crowns of the ciliated cells in the infundibulum of the oviduct, an interaction which is transitory and of appropriate strength (Lam *et al.*, 2000). The passage of the cumulus-oocyte complex through the oviduct, to the site of fertilization, which is the ampullary-isthmic junction (Aguilar and Reyley, 2005) usually occurs in a few minutes (Croxatto, 2002), taking at most 15 minutes in rabbits and 45 minutes in pigs (Hunter, 2012). It then remains in this site for a few hours or days (Croxatto, 2002), until it starts moving towards the uterus (Hunter, 2012). In the bovine, the embryo will

remain in the oviduct until Day 4 of embryonic development (Maillo *et al.*, 2016). As reviewed by Coy *et al* (2012), on arrival to the ampulla, the cumulus-oocyte complex has been observed to be attached to the epithelial cells in the bovine (Coy *et al.*, 2012). In addition, in sheep and cows, at that time, the cumulus cells are removed from the complex, leaving a denuded oocyte. This suggests a quick response of the oviduct in releasing the sperm to fertilise the oocyte, before the oocytes become completely denuded (Coy *et al.*, 2012).

Additionally, the fluid secreted by the oviduct, which will be discussed in more detail later in this section, has the potential to promote embryo quality and development by protecting the embryos against adverse impacts of the environment on mitochondrial DNA (Lloyd *et al.*, 2009). Transport of the embryo towards the site of implantation occurs as a result of three processes: (a) contraction of the muscles around the oviduct, (b) beating of ciliated oviduct epithelial cells, which combine to produce (c) oviduct fluid flow (Li and Winuthayanon, 2017). The frequency of ciliary beating is regulated by oestrogen and progesterone, the latter inducing frequency reduction (Mahmood *et al.*, 1998)(Bylander *et al.*, 2010) and the former either increasing ciliary beating frequency (Li and Winuthayanon, 2017) or preventing the frequency reduction induced by progesterone (Mahmood *et al.*, 1998). Muscle contraction is due to the movement of the myosalpinx, which is stimulated by prostaglandins (Li and Winuthayanon, 2017) although it also responds to the ratio of oestradiol to progesterone (Hunter, 2012). The combined action of ciliary beating and muscular contraction results in stirring of the contents of the tube, therefore enabling contact of the components of the fluid with the gametes/embryo (Muglia and Motta, 2001; Lyons, Saridogan and Djahanbakhch, 2006).

Previous work has shown that there is extensive communication between the female reproductive tract and the gametes and/or the preimplantation embryo (Maillo *et al.*, 2016). The presence of gametes in the oviduct regulated the secretion of proteins associated with their survival, maturation and function, as well as the modification of the oviduct environment for the zygote, and most of these proteins were differentially regulated for spermatozoa and oocytes (Georgiou *et al.*, 2005, 2007). The presence of multiple embryos in the bovine oviduct has been associated with differential gene expression by oviduct epithelial cells, and, in particular, with downregulation of gene

expression for genes associated with the immune system. The presence of a single embryo failed to exert the same effects, potentially because these effects would be local and therefore undetectable (Maillo *et al.*, 2015). Study of the interactions described above can be performed with embryo co-culture with oviduct epithelial cells, oviduct fluid or oviduct extracellular vesicles (Maillo *et al.*, 2016).

In summary, the oviduct is much more than a tube in which the gametes are brought together and the embryo is transferred to the uterus; ; Abe referred to it as an "active organ that maintains and modulates a dynamic fluid-filled milieu" (Abe, 1996). Through the creation and secretion of fluid, it plays important roles in gamete activation, fertilization and early embryo development (Abe and Hoshi, 1997; Ulbrich *et al.*, 2010). The walls of the oviduct lumen are moist with oviduct fluid, regardless of the presence of gametes or embryos (Leese, 1988), secreted, in most cases, in small, rather than copious amounts by the oviduct epithelium (Tay, 2003). The oviduct fluid is a mixture of plasma-derived molecules and epithelium-derived secreted proteins (Leese, 1988; Walker *et al.*, 1996; Aguilar and Reyley, 2005). Its composition is termed dynamic, because it varies according to the presence of gametes or embryos as well as the stages of the cycle (Aviles, Gutierrez-Adan and Coy, 2010).

1.3.2.1. Oviduct fluid composition

Oviduct fluid contains a variety of substances, aiming to provide an optimum environment for gametes and preimplantation embryos (Aguilar and Reyley, 2005). Oviduct fluid composition has been described in humans, domestic animals and laboratory animals (Aguilar and Reyley, 2005), and a general overview of the fluid composition is described in the next sections, with reference to specific species where appropriate.

In general, non-electrolytes are transported into the lumen using three different mechanisms: direct transportation from the blood, with the molecules remaining unchanged, synthesis de novo by the oviduct cells and secretion in the lumen, and synthesis from the cumulus cells, present in the lumen. Other sources of non-electrolyte production, such as follicular, seminal and peritoneal fluids, appear to have a minor contribution to the final synthesis of the fluid (Leese, 1988).

Electrolytes

In situ collection of oviduct fluid has shown the presence of a variety of electrolytes in the oviduct fluid (Hugentobler, Morris, *et al.*, 2007). Potassium (Leese, 1988; Aguilar and Reyley, 2005) and bicarbonate (HCO3-) (Leese *et al.*, 2001) are present in the oviduct lumen in high concentrations relative to that seen in the serum (Aguilar and Reyley, 2005; Hugentobler, Morris, *et al.*, 2007), and this observation applies to all species studied (Aguilar and Reyley, 2005). The maintenance of an environment containing high levels of potassium and bicarbonate is thought to be important for the maintenance of appropriate pH (7.1-8.0 depending on the time of oestrous cycle) and osmolarity levels (Leese, 1988), as well as for developmental progression, which is claimed to be higher for 2-cell mouse embryos cultured in a higher potassium concentration (Roblero and Riffo, 1986). The importance of bicarbonate ions for embryo development is also observed in *in vitro* fertilisation experiments, where these ions are associated with the maintenance of appropriate extracellular pH (Swain, 2010). Chloride ions, which are very important for regulation of oviduct fluid secretion (section 1.3.4), are believed to move mainly towards the oviduct lumen, and this ion movement is dependent on adrenergic agents (Leese, 1988).

By contrast, the levels of calcium and magnesium differ across species and, for calcium, in different regions of the oviduct (Aguilar and Reyley, 2005), however calcium concentrations in the oviduct fluid are significantly lower than plasma concentrations (Leese, 1988). There is a positive correlation between the levels of magnesium and calcium in the oviduct fluid and their concentrations in serum, suggesting that their concentrations are dependent on blood concentrations (Hugentobler, Morris, *et al.*, 2007). In the early embryo, calcium has previously been associated with promoting the process of compaction at the morula stage (Miao and Williams, 2013), and the combined absence of calcium and magnesium in the medium used for the culture of early embryos has been found to facilitate blastomere removal for biopsy (Dumoulin *et al.*, 1998), suggesting a role for these ions in developmental progression. pH in the bovine oviduct has been measured to be approximately 7.6, which is higher than the pH of the uterus, measured to be about 6.96. These values are not within the pH range used for *in vitro* embryo experiments, which is between 7.1 and 7.4 for the cattle, however it can be

hypothesized that this range may be optimal for *in vitro* embryo development to the blastocyst stage, which would encompass transition from the oviduct to the uterus (Hugentobler *et al.*, 2004).

Energy substrates

The major energy substrates found in oviduct fluid are glucose, pyruvate, lactate and amino acids (Aguilar and Reyley, 2005). These are transported and accumulate at different rates in the lumen (Leese and Gray, 1985) although they tend to be present in the lumen of the ampulla at higher concentrations compared to the isthmus (Leese, 1983). Concentrations are also dependent on the stage of the oestrous cycle. For example, the levels of glucose in the oviduct fluid in pigs fall after ovulation, compared to their levels before ovulation (Nichol *et al.*, 1998). In cattle, glucose concentrations in the oviduct have been detected to be significantly lower compared to plasma glucose concentrations (Hugentobler *et al.*, 2008). Lactate concentrations follow the reverse pattern, being significantly higher than the respective plasma concentrations (Hugentobler *et al.*, 2008), and it has been suggested that lactate in the oviduct lumen is derived as a combination of metabolism of glucose in the circulation and lactate transport across the oviduct monolayer (Leese, 1988). Finally, pyruvate concentrations are higher than plasma concentrations in the first days of the oestrous cycle but are not different from the respective concentrations in the plasma on later days of the cycle (Hugentobler *et al.*, 2008). There is no association between the concentrations of these energy substrates and the systemic concentrations of oestrogen and progesterone (Hugentobler *et al.*, 2008). It is also reasonable to suppose that there exist specific microenvironments within the oviduct that cannot be reliably assayed using current methodologies.

Amino acids

The amino acids present in the oviduct fluid are detected in concentrations that vary depending on the stage of the cycle and the species studied, and are different from the respective concentrations in the circulation (Walker *et al.*, 1996; Tay *et al.*, 1997; Aguilar and Reyley, 2005). In the bovine, this variation according to the day of the cycle was not observed (Hugentobler, Diskin, *et al.*, 2007). Similar to glucose, lactate and pyruvate, the concentrations of amino acids in the oviduct lumen in the bovine were unaffected by

systemic levels of progesterone and oestradiol (Hugentobler, Diskin, *et al.*, 2007). Amino acids are crucial embryo substrates, and turnover of amino acids has been previously associated with embryo viability (Houghton *et al.*, 2002; Brison *et al.*, 2004; Sturmey, Brison and Leese, 2008). In agreement with the Quiet Embryo Hypothesis (Leese, 2002), viable embryos, showing lower DNA damage, had low amino acid turnover (Sturmey *et al.*, 2009). Addition of physiologically relevant levels of amino acids to embryo culture media increases the number of embryos reaching the blastocyst stage and improves the number of nuclei in the blastocysts and their morphology (Walker *et al.*, 1996).

Proteins and enzymes

The most abundant proteins in the oviduct fluid are albumin and immunoglobulin G (Oliphant *et al.*, 1978; Leese, 1988), which are derived from the circulation (Oliphant *et al.*, 1978). Apart from these, many non-plasma derived proteins have also been detected (Leese, 1988; Coy, Avile and Monde, 2012), of which the oviduct-specific glycoprotein, secreted by the oviduct epithelium (Aguilar and Reyley, 2005), is the best known and characterised. The oviduct-specific glycoprotein (OVGP1) has been identified in the oviduct fluid of several species examined (Leese *et al.*, 2001), at different rates in each region of the oviduct (Aguilar and Reyley, 2005). Species-specific differences have been observed both in mRNA expression of the *OVGP1* gene, in terms of gene expression across the oestrous cycle, and the amino acid length of the protein (reviewed by (Aviles, Gutierrez-Adan and Coy, 2010)). The levels of oviduct-specific glycoprotein in the fluid are highest during the periovulatory period, after which they decline. The precise role of OVGP1 remains elusive. The presence of oviduct-specific glycoprotein and heparin can reversibly interact with the zona pellucida of the oocyte, and mask sperm-binding sites, thus preventing polyspermy after fertilisation, as well as delaying proteolytic digestion of the zona pellucida by occupying pronase sites (Coy *et al.*, 2008). It has been also suggested that this protein plays a role in immunological protection of the embryo *in vivo* (Leese *et al.*, 2001). A role for the oviduct specific glycoprotein has been described in both the maintenance of sperm viability, motility and capacitation ((McNutt *et al.*, 1992), also reviewed by (Ghersevich, Massa and Zumoffen, 2015)),, and in zona pellucida hardening and modification for sperm-oocyte interactions, although species-specific differences suggest that its role is still unclear (reviewed by (Ghersevich, Massa and Zumoffen, 2015)).

Apart from the proteins described above, there are a variety of other proteins secreted by the oviduct epithelium in the bovine, including growth factors, cytokines, proteases, protease inhibitors, enzymes and other protein associated with gamete and embryo maturation and development (Pillai *et al.*, 2017). Growth factors regulate cell proliferation and differentiation, as well as cell invasiveness (Simmen and Simmen, 1991). They have a role in support of gamete and embryonic development (Aviles, Gutierrez-Adan and Coy, 2010) (Pillai *et al.*, 2017), and are also implicated in the regulation of embryo implantation (Simmen and Simmen, 1991). Cytokines are also associated with the recognition between the endometrium and the blastocyst-stage embryo, and together with growth factors, they promote implantation by facilitating contact between the embryo and the uterus (Simón, Gimeno and Frances, 1996), On the other hand, proteases and protease inhibitors are associated with the maintenance of homeostasis (Pillai *et al.*, 2017) and, in the rat, they have been linked to promotion of embryo development (Ichikawa *et al.*, 1985).

Glycosidases, which are hydrolytic enzymes acting on glycosyl compounds, have also been detected in bovine oviduct fluid, their concentrations varying in different stages of oestrus (Carrasco *et al.*, 2008). It has been hypothesized that these may play a role in the release of capacitated sperm into the oviduct fluid (Coy *et al.*, 2012). Moreover, the oviduct fluid has been shown to contain prostaglandins E and F (Aguilar and Reyley, 2005), which are of crucial importance to various reproductive processes, including induction of luteolysis (Ulbrich *et al.*, 2009), ovulation, fertilisation, implantation and decidualisation, through cyclooxygenase-2 – related pathways (Lim *et al.*, 1997). Steroid hormones have also been detected in oviduct fluid, whose concentration varies across species and according to the stage of the oestrous cycle (Aguilar and Reyley, 2005). In particular, progesterone is present in the oviduct fluid of the golden hamster (Libersky and Boatman, 1995) and both oestrogen and progesterone have been detected in the oviduct fluid of rabbits, at different stages of the oestrous cycle (Richardson and Oliphant, 1981). Oestrogen and progesterone receptors have also been previously found to be expressed in cells of the bovine epithelium (Rottmayer *et al.*, 2001; Simintiras *et al.*, 2016) and variable concentrations of these hormones modify bovine

oviduct epithelial cell secretions, to which the embryos are exposed (Simintiras *et al.*, 2016).

Extracellular vesicles

Importantly, extracellular vesicles secreted by bovine oviduct epithelial cells have been detected, which have been claimed to improve embryo quality and enhance cryoprotection in *in vitro* embryo cultures, in terms of trophoectoderm and total cell number and survival after vitrification. This data suggest a potential role of these vesicles in mediating the communication between the oviduct and the preimplantation embryo (Lopera-Vasquez *et al.*, 2016). Extracellular vesicles, in the forms of exosomes and microvesicles, have been previously detected in the follicular fluid and the uterus. In particular, microvesicles and exosomes containing microRNA have been detected in follicular fluid, where they may be involved in cell communication within the follicle (da Silveira *et al.*, 2012), as has been previously described to be the case in other cell types (Camussi *et al.*, 2010). Similarly, microRNA-containing exosomes and microvesicles have been identified in the uterus and in particular in the microenvironment where implantation will occur, where they are believed to contribute to the cross-talk between the embryo and the endometrium (Ng *et al.*, 2013).

Fatty acids

Stearic acid, linoleic acid and oleic acid are the most commonly encountered fatty acids in the oviduct fluid, followed by arachidonic acid, palmitic acid and eicosapentaenoic acid. Interestingly, arachidonic, eicosapentaenoic and dihomo-γ-linoleic acid were detected at higher concentrations than in the circulation (Drews *et al.*, 2018). Non-esterified fatty acids (NEFAs) are present in the oviduct at concentrations reflecting plasma NEFA concentrations, and are positively correlated with the levels of cholesterol and lactate and negatively correlated with triglyceride levels in the oviduct fluid (Jordaens, Van Hoeck, De Bie, *et al.*, 2017). NEFAs have also been shown to affect the metabolism and the barrier properties of the oviduct epithelium in a polarised manner. Apical-to-basal NEFA transport has been observed, potentially with the intention to shield the embryo from elevated NEFA levels in the oviduct lumen, whereas the reverse direction (basal to apical) of transport is limited, thus shielding the embryo from high NEFA levels in blood (Jordaens, Van Hoeck, Maillo, *et al.*, 2017).

Studies of embryo co-culture with bovine oviduct epithelial cells has shown a protective role of epithelial cells from toxic substances, such as ammonia, as well as providing embryotrophic support to the embryo (Ulbrich *et al.*, 2010), and improvement of blastocyst rates in the presence of oviduct epithelial cells (Gandolfi and Moor, 1987).

While we know much about the composition of the oviduct fluid, its dynamic nature and its potential to be modified according to the stage of the oestrous cycle and the presence of embryos and gametes, there is a need to deepen our understanding about how pathophysiological conditions of the mother affect this composition. In order to perform such studies, the establishment of good and representative models of the oviduct is necessary.

1.3.3. Modelling the female reproductive tract

The cow is a good model for investigating reproductive processes in the human since there are similarities in terms of genome structure (Elsik, Tellam and Worley, 2009), and the mechanisms required for oocyte maturation and regulation of the preimplantation embryo (Ménézo and Hérubel, 2002). The cow is considered a very useful model for studying *in vitro* embryo production experiments, as it pertains great similarities to the human with regards to the size of the oocyte to be used in *in vitro* experiments, timing to reach different developmental stages during preimplantation development, male gamete preparation protocols and embryo freezing procedures (Ménézo and Hérubel, 2002). Limitations of *in vivo* studies in the bovine are mainly associated with the small number of offspring, as usually one embryo only is produced, the difficulty in capturing interactions between the embryo and the mother, since the embryo is not easy to be localised in the female tract, and the difficulty determining whether pregnancy fails to occur because of lack of fertilisation or because of embryonic death. In addition, cattle raised in slaughterhouses are not representative of normal growth conditions and it is believed that up to 40% of sacrificed cows display low fertility (Ulbrich *et al.*, 2010).

Knowledge of the composition of oviduct fluid can been derived with various *in situ* and *in vitro* methods (Leese *et al.*, 2008). *In situ* methods include direct fluid retrieval from the oviduct and acute and chronic cannulation. As reviewed by Leese *et al* (2008) it is

possible to retrieve oviduct or uterine fluids directly, albeit in small amounts (Leese *et al.*, 2008). The most commonly used *in situ* method is cannulation (Leese *et al.*, 2008), either with the cannula directly inserted into the oviduct lumen of the anaesthetised animal or *in vitro*, using a preparation in which the ovarian artery is perfused (Aguilar and Reyley, 2005). Collection of oviduct fluid with cannulation is considered to provide a good representation of the *in vivo* oviduct environment (Aguilar and Reyley, 2005). However, chronic cannulation is associated with incidence of inflammation of the site of cannula insertion, hindrance of fluid flow to the peritoneal cavity and loss of fluid constituents due to the nature of long-term collection (Leese *et al.*, 2008). The issues are resolved with the application of acute cannulation on anaesthetised animals (Kenny *et al.*, 2002) for up to 3 hours (Hugentobler, Diskin, *et al.*, 2007; Hugentobler, Morris, *et al.*, 2007; Hugentobler *et al.*, 2008).

In vitro methods of fluid collection have been developed for reasons of ethical use of animal tissue, including vascular and luminal perfusion and excision and rapid sampling (Leese *et al.*, 2008). Vascular and luminal perfusion, described first by Leese and Gray for the rabbit oviduct (Leese and Gray, 1985), and also used by Gott et al (Gott *et al.*, 1988), has also been performed in the human, where fallopian tube excision and artery cannulation and perfusion enabled collection of the fallopian tube fluid and substrate analysis (Dickens *et al.*, 1995). Finally, excision of the reproductive tracts of animals after animal sacrifice can induce risks associated with post-mortem changes, especially due to cell necrosis and lack of a hypoxic environment, which may skew the results of the subsequent experiment. Rapid tissue excision, within a few minutes after sacrifice, ensures that post-mortem related changes are minimised (Leese *et al.*, 2008).

Three-dimensional culture of oviduct epithelial cells has been performed using a variety of techniques (Ferraz *et al.*, 2018), including organoids (Kessler *et al.*, 2015), perfusion culture (Reischl *et al.*, 1999), and air:liquid interface (Chen, Einspanier and Schoen, 2013; Gualtieri *et al.*, 2013). Adult stem cells have been detected in the fallopian tube, from which a monolayer of ciliated and secretory cells was produced, grown in a three-dimensional organoid *in vitro* (Kessler *et al.*, 2015). The perfusion system included cell growth on a perfusion chamber where the bovine oviduct epithelial cells were supplied continually with medium and, simultaneously, the metabolites secreted by the cells

were removed from cell culture (Reischl *et al.*, 1999). Transwells have been previously used in the laboratory (Figure 1.6) (Simintiras *et al.*, 2016). The first use of permeable supports for oviduct epithelial cell growth, where the same media was used in the apical and basal compartments, was performed on rabbit oviduct epithelium (Dickens, Southgate and Leese, 1993). Cells grown using this method have been shown to exhibit a negative charge in the apical side compared to the basal side (Dickens, Southgate and Leese, 1993; Dickens *et al.*, 1996; Leese *et al.*, 2001), providing evidence for the polarised nature of the cells in this preparation. This method was modified by Simintiras et al (Simintiras *et al.*, 2016), by retaining the basal medium but replacing the apical medium with moist air, enabling the creation of an air-liquid interface and the secretion of i*n vitro*-derived oviduct fluid, whose composition resembled the *in vivo* oviduct secretions (Simintiras *et al.*, 2016).

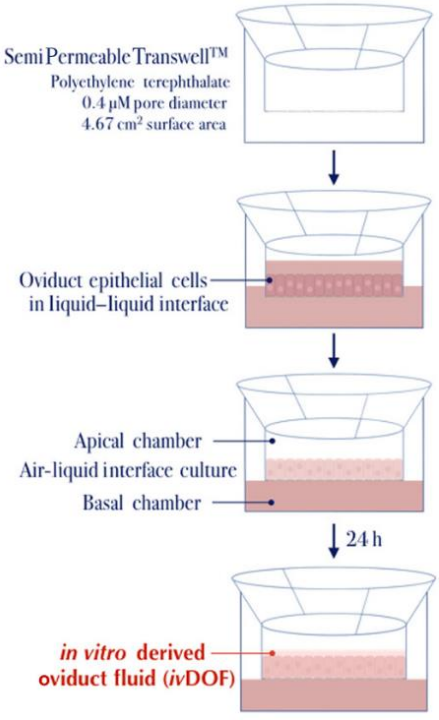


Figure 1.6.: *In vitro* model of the oviduct. (Simintiras et al., 2016)

Recently, a microfluidic model of the female reproductive tract was created (Figure 1.7), mimicking the hormonal environment encountered in the ovary, the oviduct, the uterus, the cervix and the liver (Xiao *et al.*, 2017). The system consisted of a donor module, where different experimental conditions were introduced, an acceptor module, where final products of the tissues were collected and five different tissue modules, representing each of the above tissues, and each module had pumps and valves, controlling fluid flow from one chamber to the next. In this model, cells for all *in vitro*

organs were harvested. Pressure and vacuum were applied to the valve and pump membranes therefore enabling fluid circulation within and between the modules involved. Before all five chambers were connected (Quintet-microfluidic platform, Quintet-MFP), a solo-MFP and a Duet-MFP were tested. This system was proved to be an efficient model of the female reproductive tract, by successfully supporting follicular development, maintaining cell viability, ciliary beating, and OVGP1 expression of fallopian tube epithelial cells, producing ectocervix tissue response, and hormonal control in the microfluidic culture. This system was successful in mimicking female hormone fluctuations at a pregnancy state, for a duration of approximately 100 days, due to the dynamic, compared to static, flow of media (Xiao *et al.*, 2017). Whether there are any limitations associated with the use of this device remains to be confirmed.

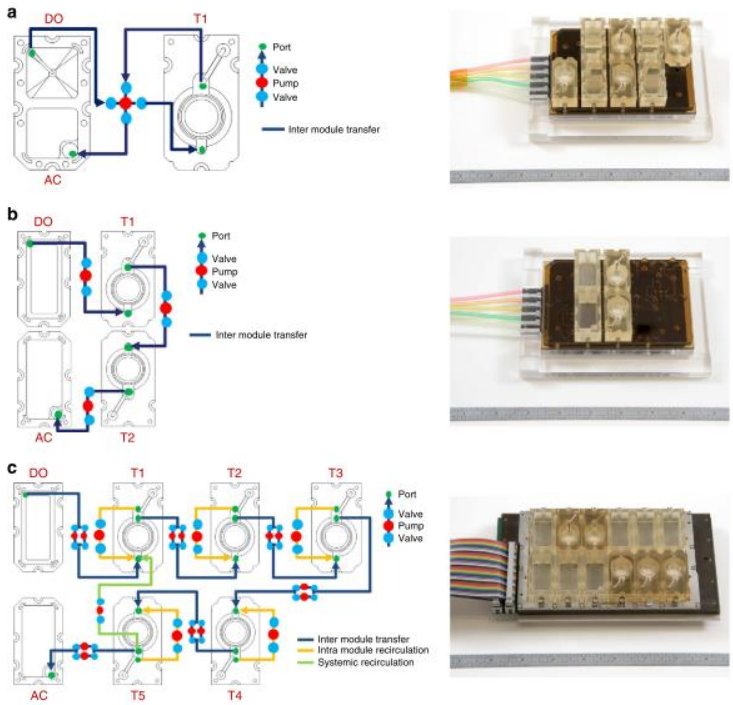


Figure 1.7: Microfluid platform model of the female reproductive tract. a) Mono-MFP, b) Dual-MFP, c) Quintet-MFP, DO: Donor module, AC: accepting module. Figure adapted from (Xiao *et al.*, 2017)

Finally, "oviduct-on-a-chip" has been recently created (Figure 1.8). Bovine oviduct epithelial cells were grown on a porous membrane which separated two compartments, a basal, representing circulation and an apical, which contained pillars where embryos and oocytes could be trapped. The design described in this experiment allowed continuous perfusion from an apical direction of the oviduct epithelial cells, which allowed them to remain differentiated. The device also allowed for live imaging, where epithelial cells, embryos and gametes could be observed on the apical side of the chip.

This model enabled interactions between the gametes or the embryos and the female reproductive tract to be studied, and the preparation supported embryo development as well as the determination of gene expression associated with methylation patterns in the early embryo. The authors claim this model will enable further research on molecules involved in pathophysiological pathways, related to potentially lethal diseases such as ovarian cancer. The authors suggest that their system, allowing observation of cell migration processes, such as the ones observed in ovarian cancer, will allow personalised treatment for this disease (Ferraz *et al.*, 2018). Whether this is indeed the case remains to be determined.

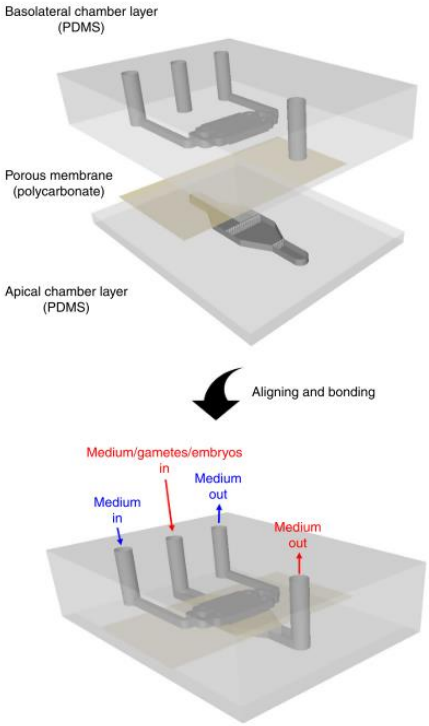


Figure 1.8.: Oviduct-on-a-chip model, containing a porous membrane, which separates the apical and basal compartments. The apical compartment contains pillars where oocytes and embryos may be retained. Figure adapted by (Ferraz *et al.*, 2018)

In summary, there are multiple methods used to study oviduct fluid, each of which with its own advantages and limitations, all of which attempt to mimic as closely as possible the *in vivo* environment. For the experiments described in this thesis, Transwells were used to mimic the formation of the bovine oviduct epithelial cell monolayer, using the method described by (Simintiras *et al.*, 2016), during which the cells were found to secrete a fluid whose composition is very similar to the *in vivo* derived fluid composition.

1.3.4. Mechanism of oviduct fluid secretion

A potential mechanism of fluid secretion was proposed by Leese *et al* (2001) , based on the processes of transport described by (Quinton, 1990) (Figure 1.9). Quinton (1990) describes a widely accepted mechanism of fluid secretion found in various epithelial tissues, based on the movement of ions, which drives water movement across the epithelial monolayer (Quinton, 1990). In this model, the role of chloride ions in fluid secretion is fundamental (Keating and Quinlan, 2008, 2012). Intracellular sodium (Na+) concentration is maintained at a low level, through the action of the Na^+/K^+-ATPase enzyme, which is located in the basolateral membrane and exports Na^+ from the cell in exchange for potassium ions. Cl^- flux is regulated via the $Na^+/K^+/2Cl^-$, which is an electroneutral carrier located in the basolateral membrane. Stimulation with a pharmacological agent or a neurotransmitter is shown to make the membranes permeable to Cl^-, and this switch induces a flux of Cl^- into the lumen, through the creation of an electrochemical gradient. The creation of this gradient, in turn, triggers a paracellular movement of Na^+ through the tight junctions and into the lumen. The high osmotic pressure created in the lumen by the ion movement is restored through water flow to the lumen, in the direction of the osmotic gradient, leading to fluid secretion (Quinton, 1990). Leese *et al* suggested that a similar mechanism might be applied to the secretion of oviduct fluid, which may be triggered by higher oestrogen secretion around the time of ovulation (Leese *et al.*, 2001).

In two ion transport studies, Keating and Quinlan suggest a purinergic regulation of ion transport across the oviduct epithelium, with a highly important role of extracellular ATP in transiently activating calcium-dependent chloride channels, and, consequently, chloride ion secretion. In addition, they suggest a role for potassium channels on the basolateral side of the oviduct epithelial cells, in providing a driving force for this extracellular ATP-induced increase in chloride ion secretion (Keating and Quinlan, 2012)(Keating and Quinlan, 2008). The observed increase in epithelial cell height during oestrus (Murray, 1995; Chen, Einspanier and Schoen, 2013) can be associated with higher rates of ion transfer and, consequently, higher volumes of fluid secreted (Leese *et al.*, 2001). Application of blockers of Cl^- channels had a significant effect on the cells (Downing *et al.*, 1997) suggesting the importance of this ion in fluid formation (Leese *et al.*, 2001).

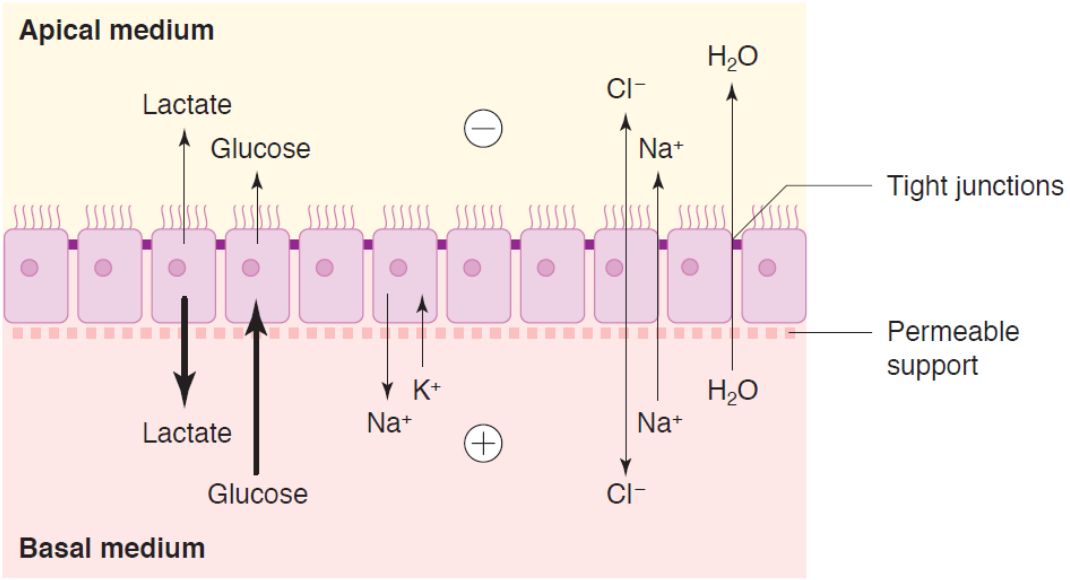


Figure 1.9: Mechanism of oviduct fluid secretion (see text for more details) (Leese *et al.*, 2001).

Other molecules, such as the nutrients lactate and glucose, detected in the oviduct fluid, are also known to be transferred across the epithelial cells (Leese *et al.*, 2001). In particular, Edwards and Leese, using the preparation for maintenance of the epithelial cells as a polarised monolayer in Transwells, showed that glucose is transported in the rabbit oviduct epithelium preferentially in a basal-to-apical, rather than an apical-to-basal direction, via facilitated diffusion (Edwards and Leese, 1993), consistent with the requirement of early embryos for a source glucose, especially with blastocyst formation. The same study reports that approximately 80% of the glucose is metabolised into lactate, which appeared predominantly in the basal, rather than the apical medium (Edwards and Leese, 1993). A study performed in human fallopian tube epithelial cells also grown as a monolayer in Transwells (Dickens *et al.*, 1996) has confirmed the above findings, suggesting the net movement of glucose into the apical compartment and net movement of lactate to the basal compartment. Interestingly, in rabbit oviduct epithelial cells, there was a decrease in the proportion of glucose and lactate appearing in the apical side of the cells, three days after mating compared to when the animals were at oestrous (Edwards and Leese, 1993), potentially indicating the passage of the embryo from the oviduct into the uterus and the observed decrease in oestrogen and increase in progesterone that is known to occur after ovulation (Leese *et al.*, 2001).

Oestrogen and progesterone may affect the transepithelial flow of Cl^- ions, which are associated with oviduct fluid secretion (Leese *et al.*, 2001). This has been explored in a study published in our laboratory (Simintiras *et al.*, 2016), where varying levels of oestrogen, androgens and testosterone have been shown to modify bovine oviduct epithelial cell secretions and to alter the gene expression profile of bovine oviduct epithelial cells (Simintiras *et al.*, 2016).

Different rates of fluid transport are observed in different areas of the oviduct and at different stages of the cycle. The direction of the fluid flow is not constant, but, as a general pattern, the fluid moves from the ampulla towards the abdominal cavity (Hunter, 2012), following the path of least resistance. It is worth noting that glucose, lactate and pyruvate enter the lumen of the ampulla at higher rates compared to the isthmus in the rabbit, most likely due to the larger surface of mucosa bordering the lumen of the ampulla (Leese, 1983).

As mentioned above, the oviduct provides the environment where male and female gametes combine to create an embryo. The importance of the oviduct in gamete activation (Coy *et al.*, 2012) is demonstrated by its important role in the formation of a sperm reservoir, which is associated with the maintenance of sperm viability and the induction of sperm capacitation.

1.4. Sperm

Spermatozoa in the epididymis are inactive and become activated when they are mixed with seminal plasma (Coy *et al.*, 2012); this state is described as a state of "activated motility" (Yanagimachi, 1994). After ejaculation, the sperm begins its journey into the female reproductive tract. However, only a very small percentage of the sperm produced during ejaculation reaches the site of fertilization (Coy *et al.*, 2012). Movement of the sperm towards the site of fertilization is achieved through the combined action of both the spermatozoa and the muscular contractions of the female reproductive tract. The spermatozoa that manage to reach the isthmus are often bound to the ciliated epithelial cells of the oviduct (Coy *et al.*, 2012), although electron microscopy scans have also shown binding to secretory cells (Suarez, 2002).

Sperm cannot fertilise oocytes until they complete a process called capacitation. Sperm capacitation was defined by Suarez and Ho as "the process during which the sperm attains full fertilizing capacity" (Suarez and Ho, 2003). Sperm capacitation, which occurs within the female reproductive tract, is a reversible process, (Johnson, 2007), which is associated with removal of a coating of substances from the epididymis and the seminal plasma (de Lamirande, Leclerc and Gagnon, 1997). The removal of these substances is associated with sperm binding to the oviduct epithelium (de Lamirande, Leclerc and Gagnon, 1997). The interaction between the epithelial cells of the oviduct and the spermatozoa is possibly performed through interactions between carbohydrate residues, mainly glycoproteins localized on the epithelial cells, and proteins localized on the sperm head, as loss of proteins of the sperm head has been observed and associated with sperm capacitation (Suarez, 2002;). Furthermore, net transfer of sperm cholesterol to HDL detected in bovine oviduct fluid has been observed, with a potential to affect sperm capacitation (Ehrenwald, Foote and Parks, 1990). Overall, sperm capacitation and hyperactivation, as well as release from the oviduct are controlled by the environment of the oviduct (Coy *et al.*, 2012), and, according to Fazeli (2011), they are a result of communication between sperm and the oviduct in the female reproductive tract (Fazeli, 2011).

The interaction between sperm and oviduct epithelial cells results in the formation of a sperm reservoir (Coy *et al.*, 2012), observed across mammalian species (Suarez, 2002). The sperm reservoir has multiple functions. On one hand, it enables the prevention of polyspermy by releasing only few spermatozoa at a time (Suarez, 2002). Release of the sperm from the sperm reservoir can be performed after sperm capacitation and hyperactivation (Suarez, 2002), since capacitated spermatozoa lose their binding affinity to the epithelial cells (Smith and Yanagimachi, 1991) and hyperactivation assists their detachment from the epithelial cell surface (Suarez, 2002). In hamsters, capacitated spermatozoa have been found to be released into the lumen of the oviduct, whereas non-capacitated spermatozoa remain firmly attached to the ampullary and isthmic mucosa (Smith and Yanagimachi, 1991). The activation of the oocyte, after penetration of its zona pellucida, results in increasing numbers of activated spermatozoa, which approach the oocyte, although polyspermic penetration will not be possible (Hunter, 2012). After establishment of the block of polyspermy, which prevents the zygote from becoming triploid or polyploid (Johnson, 2007) binding of the spermatozoa relaxes and they are released from the oviduct epithelium (Hunter, 2012).

On the other hand, the sperm reservoir maintains the fertilization capacity of the sperm, until the moment of entry of the oocyte in the oviduct (Suarez, 2002); sperm incubation with bovine oviduct epithelial cells has shown increased maintenance of sperm motility (Chian, Lapointe and Sirard, 1995). Activation of the spermatozoa hours before ovulation would result in their metabolic exhaustion before ovulation (Hunter, 2012). After the sperm becomes released from the sperm reservoir, it reaches a state of "hyperactivated motility". This motility pattern can be observed in sperm located in the ampulla at fertilization (Suarez and Ho, 2003) and it is characterized by flagellar beating showing asymmetry and high amplitude (Coy *et al.*, 2012).

The presence of sperm is associated with alterations in the oviduct fluid viscosity, since the oviduct can sense the presence of sperm and increase the levels of antioxidants and heat-shock protein-70, to reduce the levels of stress in sperm (Georgiou *et al.*, 2005). This increase in protein concentration is associated with an increase in viscosity (Li and Winuthayanon, 2017), with the involvement of TRPV4 receptors (Andrade *et al.*, 2005) and this, in turn, results in modified patterns of ciliary beating frequency, aiming to

enhance sperm movement (Li and Winuthayanon, 2017). In addition, the oviduct responds to the presence of sperm via regulation of muscular contractions. These contractions are regulated by prostaglandins (PG), as PGE_2 and PGF_{2a} increased muscular contractions, whereas PGE_1 was associated with reduced contractions (Wånggren *et al.*, 2008). In addition, interstitial Cajal-like cells of the muscle layer of the human oviduct express oestrogen and progesterone receptors, suggesting potential regulation of contractions via steroid hormones (Cretoiu *et al.*, 2009).

1.5. Fertilisation

Fertilisation is the process in which the sperm, after entering the oviduct through the utero-tubal junction and becoming hyperactivated, meets the oocyte, which has also entered the oviduct through the infundibulum and is waiting at the ampullary – isthmic junction. The spermatozoa that reach the oocyte bind to the zona pellucida (Johnson, 2007) and they undergo acrosome reaction, which is a process associated with the release of proteolytic enzymes from the acrosome of the sperm head, which enables sperm to penetrate the glycoprotein coat of the egg (Eisenbach and Giojalas, 2006). In particular, the acrosome in the sperm head swells and its plasma membrane fuses with the plasma membrane of the oocyte and the contents of the acrosome are released through exocytosis (Allen and Green, 1997). The sperm bind to specific proteins on the oocyte surface called ZP proteins, ZP2 and ZP3 being more directly involved in binding the sperm head (Hinsch and Hinsch, 1999).

After penetration of the zona pellucida, the spermatozoon lies tangentially in the perivitelline space, which is located between the oocyte membrane and the zona pellucida and the membranes of the sperm and the oocyte fuse (Johnson, 2007). The union between sperm and oocyte is accompanied by intracellular calcium (Ca^{2+}) oscillations, which are triggered by sperm-specific phospholipase C (PLCζ), localised on sperm and contributing to the initiation of embryo development (Saunders *et al.*, 2002). The Ca^{2+} oscillations are linked to the release of the content of cortical granules (reviewed by (Coy and Avil, 2010)), which are adjacent to the oocyte membrane, to the perivitelline space, a process called the cortical reaction (Johnson, 2007). Release of ovastacin, a protein detected in cortical granules before fertilisation, results in cleavage of ZP2 (Burkart *et al.*, 2012), which in turn is associated with hardening of the zona and establishment of the block of polyspermy (Li and Winuthayanon, 2017). At this stage, the male nucleus enters the oocyte to become the male pronucleus , thus successfully creating a euploid zygote (Li and Winuthayanon, 2017).

1.6. The pre-implantation embryo

1.6.1. Pre-implantation embryo development

After successful fertilisation, early embryo development involves a series of cellular divisions, which are called cleavage. These are time-sensitive and species-specific (Johnson, 2007). Embryos are characterised as cleavage-stage from the stage of 2-cell embryo up to the morula stage. Embryo cell divisions can be synchronous or asynchronous (Prados *et al.*, 2012; Brison, Sturmey and Leese, 2014). Each division occurs normally at approximately 18-20 hours (Prados *et al.*, 2012). After each division, the number of embryo cells, called blastomeres, increases, however the size of each blastomere is reduced; consequently, even though cell number increases, the net size of the embryo does not (Johnson, 2007). The homogeneity between the blastomeres is believed to be driven by the even distribution of the cell components after each division (Brison, Sturmey and Leese, 2014).

Around the 8- to 16-cell stage (Johnson, 2007), which is the stage when the embryo will enter the uterus in the bovine (Lonergan *et al.*, 2016), the individual blastomeres undergo a process called compaction (Brison, Sturmey and Leese, 2014) (Figure 1.10). This process results in a change in the blastomere cell shape from spherical to a flat, epithelial-like cells (Coticchio *et al.*, 2019) and the formation of a morula stage-embryo (Lonergan *et al.*, 2016). During compaction, the blastomeres become tightly connected, as demonstrated by movement of the cell-cell junction protein E-cadherin from the cell cytoplasm to the cell membranes at that stage (Alikani, 2005) and the detection of tight junctions in embryo cells at the morula stage (Gualtieri, Santella and Dale, 1992). As reviewed by Coticchio *et al* (2019), the process of compaction enables the segmentation of embryo cell populations into inner and outer cells and the preservation of a spherical shape of the embryo as well as cell-to-cell contact (Coticchio *et al.*, 2019).

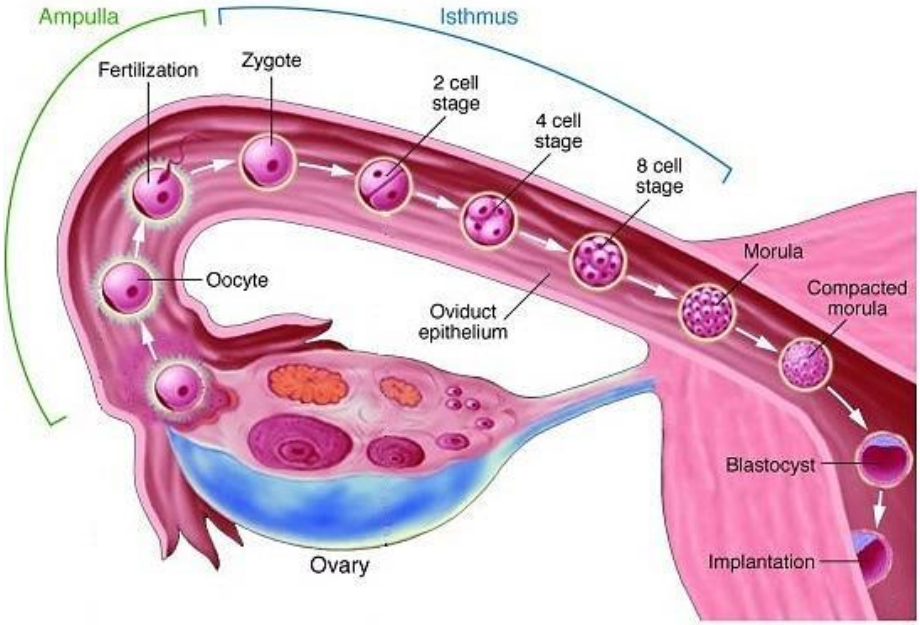


Figure 1.10.: Preimplantation embryo development. After fertilisation in the ampllary-isthmic junction, the zygote undergoes cleavage division, resulting in an increase in the number of blastomeres. Around the 8-to-16 cell stage, the embryo undergoes compaction, forming the morula, which will then become a blastocyst. Figure adapted from (Schuel, 2006).

Depending on this cell polarisation, the cells of the morula will then become either the inner cell mass (ICM) cells or the trophoblast cells, also called cells of the trophoectoderm (TE) (Johnson and McConnell, 2004). It is debated whether the allocation of cells which will form one cell population or the other is established at the 4- to 8-cell stage, or even earlier (Brison, Sturmey and Leese, 2014; White *et al.*, 2018); however it is believed that during the transition of 8- to 16-cell embryo, cell positioning and cell fate have already been made, but the allocation to a specific lineage is still reversible (Coticchio *et al.*, 2019).

In general, there is a level of uncertainty over the mechanisms behind the commitment of the inner cells of the morula into cells of the ICM of the blastocyst and the outer cells of the morula into the trophoectoderm cells of the blastocyst (Coticchio *et al.*, 2019), although an important role in the allocation to the ICM of the TE seems to be played by the Hippo signalling pathway, which is a tumour suppressing pathway, originally detected in the Drosophila (Xenopoulos, Kang and Hadjantonakis, 2012; Coticchio *et al.*, 2019). The trophoblast cells of the blastocyst surround the blastocoelic cavity, containing blastocoelic fluid as well as the inner cell mass (ICM) cells, which are embedded into the wall of the trophoblast (White *et al.*, 2018). Before implantation, the

inner cell mass population is subdivided into the cells of the epiblast, which is located in the interior of the ICM and the primitive endoderm, which is located in the periphery of the ICM and in contact with the blastocyst cavity. The epiblast cells will be the one which will provide most of the embryonic tissues, whereas the primitive endoderm and the cells of the trophoectoderm will form the extraembryonic tissues (Xenopoulos, Kang and Hadjantonakis, 2012; White *et al.*, 2018).

During early cleavage, the embryo relies principally on mRNA that was synthesised in the oocytes, but as development progresses, there is a transition from the use of maternally-derived mRNA, that is, mRNA stored in the oocyte before fertilisation, to embryo-produced mRNA, a process previously described by Jansz and Torres-Padilla as "embryonic genome activation" (EGA) (Jansz and Torres-Padilla, 2019). This process occurs in the oviduct, at different time points during development in different species. For cattle, this is around the 8-to-16 cell stage (reviewed by (Sirard, 2012))Embryonic genome activation is a highly regulated process, occurring in a stepwise manner, with an initial genome activation occurring at earlier stages, before a major genome activation event (Latham, 2001). For humans, Braude *et al* reported that the 4-to-8 cell stage was the stage after which genomic activity was detected in the embryo (Braude P., Bolton V and Moore S., 1988).

Epigenetic modifications

Epigenetic modifications have been defined by Handy, Castro and Loscalzo (2011) as "heritable alterations that are not due to changes in the DNA sequence"; they are changes which modify the accessibility of DNA and the structure of chromatin, through processes such as histone modifications and DNA methylation or demethylation, thus regulating gene expression (Handy, Castro and Loscalzo, 2011). Chromatin is believed to be remodelled during EGA; in particular, CpG-rich promoters and chromatin regions overlapping with DNA hypomethylated domains are described as "accessible chromatin loci" before EGA, and the majority of these become inaccessible after EGA, in a manner that is transcription-dependent (Wu *et al.*, 2018). A process of de-methylation and re-methylation of specific parts of the genome occurs during EGA, which enables the removal of epigenetic modifications acquired during gamete maturation and the establishment of new methylation patterns in the embryo (Reik, Dean and Walter,

2001). Histone modifications are also associated with this process (reviewed by (Wu *et al.*, 2018). Conceptus-derived genes are now responsible for RNA and protein synthesis, nucleotide and amino acid transport, as well as maturation processes occurring in a variety of cell systems, such as the Golgi apparatus and the mitochondria (Johnson, 2007).

1.6.2. Pre-implantation embryo metabolism

Pyruvate is the primary energy substrate consumed by early stage embryos, with increasing levels of glucose consumption, as the blastocyst stage approaches (Sturmey, Brison and Leese, 2008; Leese, 2012). Indeed, oxygen consumption is relatively constant up to the morula stage, with a sharp increase being observed from the blastocyst stage onwards (Houghton *et al.*, 1996; Sturmey and Leese, 2003; Leese, 2012). Glucose consumption follows a similar pattern, being undetectable up to the blastocyst stage, when it becomes the main substrate consumed by the embryo (Houghton *et al.*, 1996; Sturmey and Leese, 2003; Leese, 2012; Guerif *et al.*, 2013).

Bovine and porcine embryos depend on oxidative phosphorylation throughout development (Thompson *et al.*, 1996; Sturmey and Leese, 2003). The relationship between oxidative phosphorylation and glucose and pyruvate consumption is linear, suggesting an increase in consumption of all substrates to accommodate the increasing needs for ATP (Thompson *et al.*, 1996). Other substrates, such as amino acids (Sturmey, Brison and Leese, 2008; Leese, 2012), fatty acids (R. G. Sturmey *et al.*, 2009) and lactate (Leese, 2012) are also used by embryos in all stages to provide the embryo with a variety of energy sources to use. In a comprehensive review focusing on pre-implantation embryo metabolism, Leese highlights the relatively small body of evidence investigating the metabolism of the preimplantation embryo *in situ* and suggesting a need for further research in this field (Leese, 2012).

The importance of the concentrations of energy substrates in the oviduct is demonstrated by their impact on embryo development *in vitro*. In particular, as suggested by the Quiet Embryo hypothesis by Leese (2002), the maintenance of low levels of embryo metabolism is associated with the achievement of highest embryo viability (Leese, 2002). More recently, this hypothesis has been adjusted to reflect the

fact that an ideal environment for optimal embryo development is a balanced environment, where the energy substrates are available to the embryo within an optimal range of concentrations, a concept called the “Goldilocks hypothesis” (Leese *et al.*, 2016). In support of this hypothesis, highest embryo viability in the bovine is associated with a limited range of pyruvate consumption (Guerif *et al.*, 2013).

Maternal pathophysiology has been previously associated with altered or compromised embryo metabolism. Blastocysts produced by overweight and obese women had lower glucose consumption rates compared to women of healthy body mass index, as well as increased amino acid consumption/production and reduced triglyceride concentration in the embryos (Leary, Leese and Sturmey, 2015). In addition, embryo development under high NEFA conditions, which is a common symptoms of metabolic disturbances, resulted in altered patterns of gene expression related to REDOX maintenance in the embryo (Van Hoeck *et al.*, 2013). In both these studies, the authors suggest that the observed modified patterns of embryo metabolism are traced back to the ovarian environment and its potential effect on oocyte health. Analysis of the follicular fluid of obese women is believed to be altered compared to women of healthy BMI (Robker *et al.*, 2009; Valckx *et al.*, 2012).

1.7. Developmental Origins of Health and Disease hypothesis

There is growing evidence that supports the notion that metabolic diseases arise from a combination of genetic and environmental factors, as well as epigenetic modifications (Rando and Simmons, 2015). The link between developmental events and health in later life was most effectively described by David Barker. His original work reported on association between adverse foetal environment and the development of cardiovascular disease (Barker, 1990, 1997; Barker and Martyn, 1992), however these now include a wider range of metabolic conditions. According to the Developmental Origins of Health and Disease Hypothesis (DOHaD), a number of diseases and syndromes appearing later in life, including type II diabetes and obesity, as well as the risk of developing coronary heart disease, are associated with deficiencies in nutrient support or with the physiology of the maternal environment during pregnancy (Barker *et al.*, 2002; Barker, 2004; Fazeli, 2011). This was further explored and it is now known that exposure to an adverse or stressful environment during prenatal and early post-natal development, could contribute to the development of chronic conditions later in life (Martin-Gronert and Ozanne, 2006; Feuer and Rinaudo, 2016). The environment in which development occurs appears to be influenced by a variety of factors, including the maternal body size and composition, nutritional status and metabolism; these, in turn, are affected by the environment at which the maternal organism was exposed as a foetus, a child and an adolescent (Martin-Gronert and Ozanne, 2006), as well as the lifestyle factors that confer health. Chronic conditions are believed to develop as a result of a mismatch between the energy provision during early development and the energy provision during the adult life (Feuer and Rinaudo, 2016).

Given the molecular, epigenetic and developmental plasticity of the developing preimplantation embryo, this stage represents a particularly sensitive window of development. Exposure of the early embryo to a suboptimal environment during critical stages of development can increase the susceptibility of resultant offspring to chronic metabolic conditions, including obesity, diabetes and cardiovascular diseases, during the adult life (Feuer and Rinaudo, 2016) (Figure 1.11). This is because embryo development in an adverse environment can impact on a wide range of processes, including cell proliferation and physiology as well as gene expression patterns (Watkins,

Papenbrock and Fleming, 2008). It is presumed that the maternal reproductive tract can usually buffer the adverse effects of the external environment to some extent (Rando and Simmons, 2015); however, a suboptimal uterine environment, such as that which might be seen in a diabetic or obese woman, is related to the development of disease in the adult life of the offspring (Barker, 2004; Rando and Simmons, 2015), such as type II diabetes, coronary heart disease and hypertension (Barker 2004).

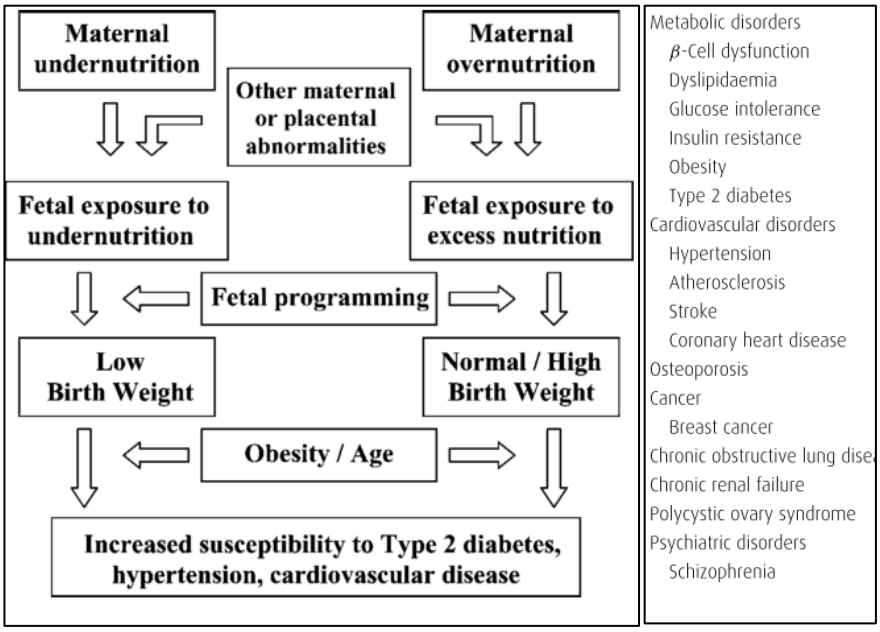


Figure 1.11: Impact of under- and over-nutrition on offspring health and diseases associated with malnuntrition during foetal life. A suboptimal environment in the female reproductive tract, originating from maternal overnutrition or undernutrition, which the embryo is exposed to, is associated with higher chance of susceptibility to metabolic conditions such as Type 2 diabetes, hypertension and cardiovascular disease (Martin-Gronert and Ozanne, 2006)

Maternal undernutrition during pregnancy has been linked to the development of metabolic diseases in the adult life of the offspring, despite the buffer of the maternal environment (Rando and Simmons, 2015). The adaptations that a developing conceptus makes in response to the environment that it encounters during development, are intended to prepare the offspring for post-natal conditions that it "predicts" that it will encounter. However, disease susceptibility is presumed to be raised when the *in utero* conditions mismatch the post-natal conditions (Tarry-adkins and Ozanne, 2016). A well-known example in support of this hypothesis is the Dutch Hunger Winter, observed during the Nazi-occupied Netherlands in the winter of 1944-1945. During that time, previously well-nourished individuals suffered a reduced calorie daily intake of between

400 and 800 calories. This was later linked to the appearance of a variety of metabolic diseases in the offspring conceived during this period (Ravelli *et al.*, 1998).

1.8. Obesity

According to the World Health Organisation, “overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health” (WHO, http://www.who.int/topics/obesity/en/). The most widely used measure of obesity is Body Mass Index (BMI), which is determined as the ratio of body weight in kg to the height in metres squared (WHO, http://www.who.int/topics/obesity/en/). Obesity can be distinguished as mild, moderate and severe and it can be influenced by demographic characteristics and the sex of the individual (Azziz, 1989). According to the World Health Organisation, healthy body mass index (BMI) ranges from 18.5 – 24.99, whereas people with a BMI higher than 30.0 are characterised as obese, and people between with a BMI between 25.0 and 30.0 are overweight (WHO, 1995). However, BMI does not make the distinction between muscle-associated and fat-associated weight, and is not consistent across population. Therefore, the waist-to-hip ratio has also been identified as a useful tool for the measurement of abdominal fat accumulation, even though population restrictions also apply to it. More recently, the World Health Organisation has reported that the measurement of waist circumference as a method of identifying overweight patients who are at risk of developing diseases associated with obesity, attributed to abdominal fat distribution (World Health Organization, 2000).

Obesity is characterised as a metabolic disorder and has been associated with cardiovascular disorders, type II diabetes and cancer (Van Der Steeg *et al.*, 2008). The increasing incidence of obesity has led to the definition of the metabolic syndrome. The metabolic syndrome is associated with glucose intolerance, insulin resistance, abdominal obesity, hypertension and dyslipidaemia (Eckel, Grundy and Zimmet, 2005). As reviewed by Stumvoll *et al*, insulin resistance is defined as the state when insulin exerts biological effects inferior to the expected ones, in terms of glucose disposal and suppression of glucose production (Stumvoll, Goldstein and Haeften, 2005). In abdominal obesity, insulin resistance results in compensatory development of hyperinsulinaemia (Brewer and Balen, 2010). Both insulin resistance and hyperinsulinaemia have been described to be major driving forces for the development of obesity, together with hyperandrogenaemia and disrupted steroidogenesis (Silvestris *et al.*, 2018).

1.8.1. Obesity and fertility

The relationship between obesity and fertility has been described in multiple studies (Metwally, Li and Ledger, 2007; Van Der Steeg *et al.*, 2008; Robker *et al.*, 2009; Lane *et al.*, 2015), however it remains unclear which of the symptoms of obesity (ie. high adiposity, hyperglycaemia, hyperinsulinaemia) are associated with the poor quality of oocytes and consequently with poor embryo development (Lane *et al.*, 2015). Reduced fertility in obese women has been attributed to dysregulation of steroidogenesis or production of poor quality embryos (Robker *et al.*, 2009). Indeed, the latter has been supported by the detection of alterations in the follicular microenvironment of women with abnormally high BMI, which shows signs of dyslipidaemia, hyperinsulinaemia and inflammatory response (Robker *et al.*, 2009). Obesity is associated with dysregulation of the hypothalamic-pituitary-ovarian axis (Silvestris *et al.*, 2018), associated with a healthy endocrinological profile (Figure 1.13).

Obesity has risen dramatically in recent decades, which has led to increasing evidence that maternal overnutrition during pregnancy, or even before the establishment of pregnancy, plays an important role in the development of metabolic diseases, even in early childhood (Rando and Simmons, 2015; Tarry-adkins and Ozanne, 2016). An obesogenic environment during gestation has been associated with the development of many metabolic diseases (Tarry-adkins and Ozanne, 2016), (Figure 1.12). A strong predictor of foetal growth is placental weight, which mediates the impact of maternal obesity, gestational diabetes and gestational weight gain on foetal growth and birth BMI (Ouyang *et al.*, 2013). Balanced foetal and placental growth are associated with low rates of perinatal mortality (Matsuda *et al.*, 2018). It is therefore believed that the placenta may play an important role in future development of metabolic conditions (Ouyang *et al.*, 2013). It has also been reported that, the higher the offspring weight at birth and the lower the parental BMI before puberty, the lower the risk for the offspring to develop the above conditions (Barker *et al.*, 2002).

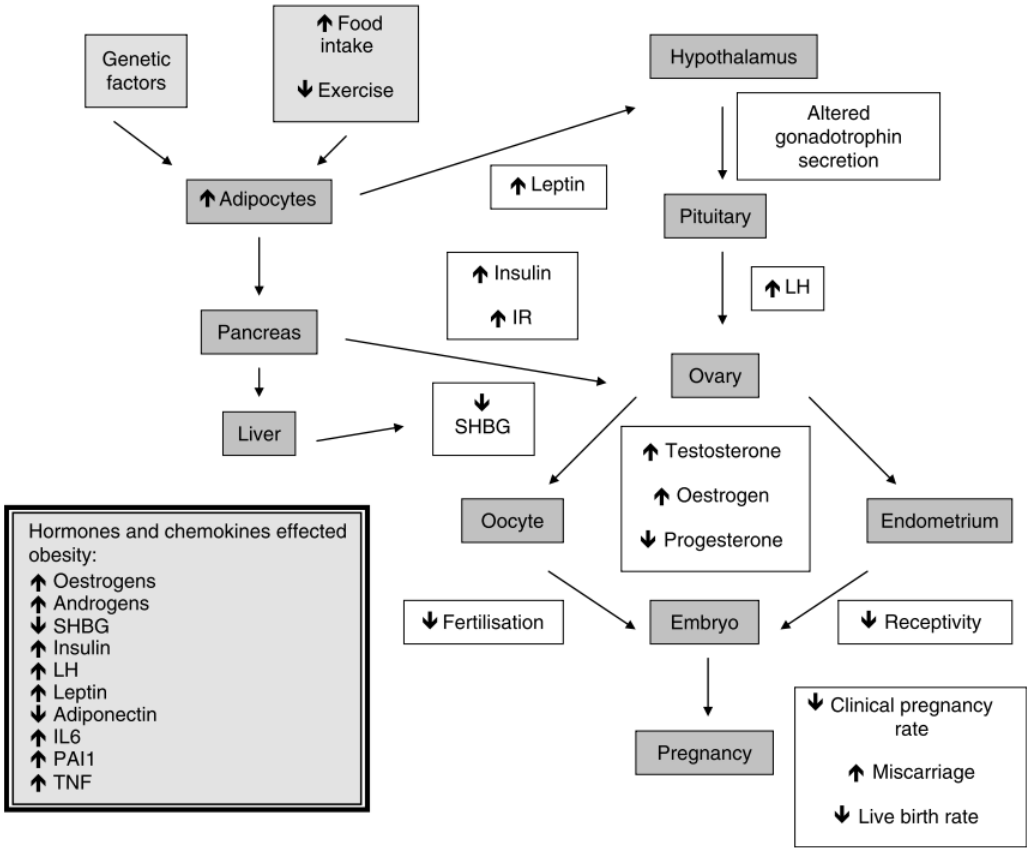


Figure 1.12: Summary of the impact of obesity on the regulation of the hypothalamic-pituitary-ovarian axis (Brewer and Balen, 2010). Obese women present a modified gonadotrophin secretion pattern from the hypothalamus, and increase LH secretion from the cells of the pituitary (Brewer and Balen, 2010). High levels of oestrogen are observed as a result of increased peripheral androgen aromatisation, as well as hyperandrogenaemia, attributed to insulin resistance and resulting hyperinsulinaemia (Silvestris *et al.*, 2018). Leptin, a protein produced by the cells of adipose tissue (Broughton and Moley, 2017), is associated with this dysregulation (Brewer and Balen, 2010). Obese women have higher circulating levels of leptin than healthy-weight control subjects (Broughton and Moley, 2017) (Silvestris et al., 2018). High levels of leptin induce a reduction in oestrogen (Greisen et al., 2000) and progesterone (Brannian, Zhao and McElroy, 1999) production by luteinised granulosa cells. In addition, as reviewed by Silvestris *et al* (2018), obese women exhibit a reduction in the levels of sex hormone binding globulin (SHBG), insulin-like-growth factor binding proteins (IGFBP) and growth hormone (GH) (Silvestris et al., 2018). Furthermore, insulin resistance and the consequent hyperinsulinaemia results in reduced levels of SHBG, hyperandrogenaemia and deranged insulin-growth factor system, all of which contribute to the development of menstrual and ovulatory disturbances (Brewer and Balen, 2010).

It is now widely acknowledged that embryo development is largely affected by the maternal diet just before and at the time of conception, and this is to some extent associated with gamete quality (Lane *et al.*, 2015). The exact mechanisms that underlie the links between maternal diet and gamete/embryo quality remain unclear, despite considerable research. For example, there are increased levels of expression of markers of oxidative stress and inflammation in the multiple tissues of obese women, which, in turn, can be associated with development of obesity in their offspring (Rando and

Simmons, 2015). Pre-gestational maternal obesity is associated higher expression of proinflammatory cytokines in the placenta and higher levels of circulating inteleucin-6 during pregnancy (Roberts *et al.*, 2011). Additionally, experiments in rats have shown that exposure of the females to a Western diet, containing high concentrations of carbohydrate and fat, before or during pregnancy is associated with the development of inflammation and oxidative stress, which in turn result in increased adiposity in the offspring, with altered patterns of adipogenic and lipogenic genes. Importantly, addition of an antioxidant supplement was associated with reduced adipogenity and normal glucose tolerance (Sen and Simmons, 2010).

Obese women need longer to conceive (Jensen *et al.*, 1999; Van Der Steeg *et al.*, 2008; Robker *et al.*, 2009) compared to women of normal weight. Obesity in young age or early adulthood has also been associated with menstrual problems, such as earlier onset of puberty, obese girls having been reported to experience the onset of menses earlier (Lash and Armstrong, 2009), as well as high risk of developing hypertension during pregnancy and subfertility in adulthood (Lake, Power and Cole, 1997; Lash and Armstrong, 2009). Additionally, obese women encounter low contraceptive efficacy, potentially associated with the variations observed in the distribution and metabolism of steroid hormones (Lash and Armstrong, 2009). Moreover, high BMI is associated with impaired success of assisted reproductive technologies (Broughton and Moley, 2017)(Lash and Armstrong, 2009), obese women requiring higher levels of gonadotrophin for ovarian stimulation (Brewer and Balen, 2010).

Obese women also produce a significantly smaller number of oocytes compared to overweight women (Robker *et al.*, 2009) and both overweight and obese women produce significantly fewer oocytes than women of normal BMI (Leary, Leese and Sturmey, 2015). Oocytes from overweight and obese women are smaller than oocytes from women with weight within the healthy range and appear less likely to cleave and form blastocysts after fertilisation (Leary, Leese and Sturmey, 2015).

There is no clear conclusion on the impact of obesity on successful establishment of pregnancy and embryo health and viability. As reviewed by Schulte *et al,* metabolic alterations, such as the ones observed in obesity, are associated with increased rates of

miscarriage and overall reduced fertility, by affecting endometrial receptivity (Schulte, Tsai and Moley, 2015). A study by Leary *et al* (2015) suggested that fertilised oocytes of obese women reached the morula stage earlier and the blastocysts were smaller and contained fewer cells, compared to the control group. Furthermore, the embryos were metabolically distinct from the embryos derived from women with normal BMI; they consumed lower amounts of glucose, their amino acid profile was altered and their triglyceride levels were increased compared to embryos from oocytes of women with normal BMI (Leary, Leese and Sturmey, 2015).

On the other hand, a study by Styne-Gross *et al* (2005) showed that the implantation rates, the pregnancy rates and the rates of spontaneous miscarriage of embryos produced by fertilisation of the oocytes of women with healthy BMI are not affected by the BMI of the recipient. The results of this study highlight the importance of a healthy oocyte in achieving successful pregnancy outcomes, regardless of the presence of a suboptimal uterine environment (Styne-Gross, Elkind-Hirsch and Scott, 2005). Robker *et al* showed that obese women produced significantly fewer embryos than overweight and moderate women; however, the fertilisation rate of the oocytes was similar in all BMI groups (Robker *et al.*, 2009). A study by Valckx *et al* (2012), indicated no association between BMI and success rates of IVF / ICSI. However, the number of oocytes collected and the total number of embryos produced was significantly lower in obese women compared to women with normal BMI (Valckx *et al.*, 2012). Finally, a strong association has been reported between maternal hyperglycaemia and the development of chromosomal degradation and cell apoptosis at the blastocyst stage (Pampfer, 2000), which could be attributed to downregulation of glucose transporter gene expression by the blastocyst cells (Moley, Chi and Mueckler, 1998).

These studies indicate a crucial link between maternal metabolic health and early development. A key component of metabolic ill health is disrupted regulation of glucose.

1.9. **Glucose homeostasis and metabolism**

1.9.1. Glucose homeostasis

Glucose serves as the main energy source for most cell types, especially the brain. Glucose is normally derived in the body as a product of digestion and absorption of dietary polysaccharides, mainly starch, or disaccharides (mainly sucrose and lactose) or it is synthesized in the liver (Ostrowska, Jarczak and Zwierzchowski, 2015). The liver is crucial for glucose homeostasis, through glucose release via glycogenolysis or storage via glycogenesis (Röder *et al.*, 2016). Glycogen is important for glucose homeostasis in mammals (Han *et al.*, 2016). The importance of glucose is associated with its role as an energy fuel for biological processes, through its participation in the citric cycle, for the generation of ATP, as well as a metabolic intermediate, as it can be converted into glycogen or triacyloglycerols and stored within tissues (Ostrowska, Jarczak and Zwierzchowski, 2015).

Blood glucose levels are tightly regulated in the body, for the maintenance of normal body function. The maintenance of energy homeostasis in the body, performed by regulation of digestion and metabolism of macromolecules, is achieved by the release of hormones and digestive enzymes by the pancreas (Röder *et al.*, 2016). Digestive enzymes are secreted by the exocrine or acinar cells (Röder *et al.*, 2016), through the pancreatic ducts and into the duodenum (Chandra and Liddle, 2009). The acinar cells comprise approximately 75% to 90% of the pancreas. The cells of the pancreatic ducts secrete ions and fluid which deacidify the gastric fluid entering the duodenum (Chandra and Liddle, 2009). Finally, pancreatic hormones are released directly into the circulation. The endocrine cells form clusters called the islets of Langerhans, localised among the exocrine cells and comprise only 1% to 2% of the pancreas (Röder *et al.*, 2016). The islets consist mainly of: alpha cells, which produce the hormone glucagon, beta cells, secreting insulin, amylin and C-peptide, delta cells, releasing somatostatin and PP cells or gamma cells, producing pancreatic polypeptide ((Röder *et al.*, 2016), also reviewed by (Chandra and Liddle, 2009)).

It has been long established that normoglycaemia is established through the balance between glucagon production by the alpha cells and insulin production by the beta cells

of the pancreas. In cases of high blood levels of glucose, the beta cells secrete insulin and the alpha cells reduce glucagon production, while in low glucose, glucagon is released from the alpha cells (Göke, 2008). Additionally, under the action of glucagon or insulin, glycogenolysis or glycogenesis, respectively, is performed in the liver (Röder *et al.*, 2016). This regulatory mechanism ensures that glucose levels are maintained between 4 and 6mM in humans (Röder *et al.*, 2016).

It is well known that diabetes occurs from an imbalance created between glucagon and insulin secretion by the pancreatic cells (Unger and Orci, 1975). Type I diabetes is an autoimmune condition, which is characterised by destruction of the beta-cells of the pancreas. Its pathogenesis is mainly genetic, although an environmental component contributing to the disease cannot be excluded, even though it is still unknown (Gillespie, 2006). Patients with Type 1 diabetes have an obligatory requirement for insulin. On the other hand, type II diabetes is mainly associated with lifestyle, especially overweight and obesity, whereas genetic influence is secondary. Similar to type I diabetes, type II diabetes occurs as a result of beta cell dysfunction (Stumvoll, Goldstein and Haeften, 2005; Göke, 2008), combined with insulin resistance (Göke, 2008). In diabetic patients, beta cells have reduced mass, a fact which triggers the remaining beta cells to increase insulin secretion as a compensatory mechanism (Stumvoll, Goldstein and Haeften, 2005; Göke, 2008). Increasing levels of glucose observed in the circulation will result in beta-cell dysfunction due to glucose toxicity (Stumvoll, Goldstein and Haeften, 2005). Insulin levels will eventually decrease, therefore resulting in a surge in glucose levels (Göke, 2008). An association between type II diabetes and obesity has been established (Stumvoll, Goldstein and Haeften, 2005).

1.9.2. Glucose metabolism

Cell membranes are impermeable to glucose (Nishimoto *et al.*, 2006; Zhang *et al.*, 2012), therefore, its entry into cells is performed with two different mechanisms (Ostrowska, Jarczak and Zwierzchowski, 2015): facilitated transport, performed by glucose transporters (GLUT), which carry glucose molecules without consuming energy, using facilitated diffusion across the cell membrane (Gould and Holman, 1993; Ostrowska, Jarczak and Zwierzchowski, 2015; Dupont and Scaramuzzi, 2016), and SGLT transporters, which actively transport glucose molecules, a process requiring energy consumption

(Ostrowska, Jarczak and Zwierzchowski, 2015). GLUT proteins are coded by *SLC2A* genes (Ostrowska, Jarczak and Zwierzchowski, 2015). Once inside the cell, glucose can be metabolised through several different pathways, namely glycolysis, glycogen synthesis, the pentose phosphate pathway and the hexosamine pathway, the first step of all being the conversion of glucose to glucose 6-phosphate, mediated by hexokinase (Dupont and Scaramuzzi, 2016). These are summarised on Figure 1.13.

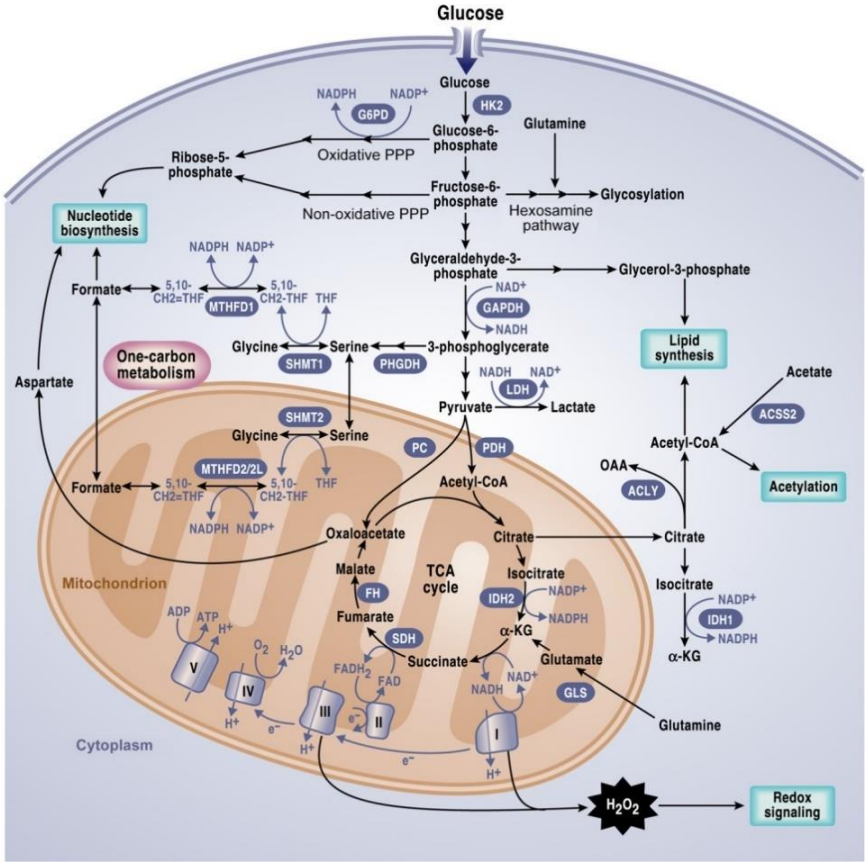


Figure 1.13.: Different pathways of glucose metabolism (figure by (DeBerardinis and Chandel, 2016)). Glycolysis can be divided in three stages: (1) Conversion of glucose to fructose 1,6-biphosphate (2) Conversion of fructose 1,6-biphosphate to two three-carbon interconvertible molecules, dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP), and (3) Oxidation of these molecules to pyruvate, with the formation of 2 ATP molecules (Berg, Tymoczko and Stryer, 2002c), (Stryer, 1995). Pyruvate may be converted to ethanol, which has been observed in the yeast, it may be reduced to lactate by NADH, which is catalysed by lactate dehydrogenase, or it may progress to the citric acid cycle, also called the TCA cycle. This latter process generates the highest amounts of energy compared with the other two. Conversion of pyruvate to acetyl CoA initiates the TCA cycle, which will generate electron carriers which will be fed to the electron transport chain, where oxidative phosphorylation occurs (Berg, Tymoczko and Stryer, 2002c). The NADH and $FADH_2$ created through fatty acid oxidation, which is not described here, glycolysis, and the citric acid cycle contain electrons which are used to create ATP through the process of oxidative phosphorylation, a process occurring in the mitochondria, and containing a series of transformations collectively called cellular respiration. The electrons from NADH or $FADH_2$ flow to the electron transport chain, in the inner mitochondrial membrane, by electron carriers, and the resulting pH gradient created by the uneven proton distribution leads to the creation of a proton-moving force. Proton flow back to the

mitochondrial matrix results in ATP synthesis (Berg, Tymoczko and Stryer, 2002d). The pentose phosphate pathway results in the synthesis of ribose 5-phosphate from glucose 6-phoshate and the production of ATP. This pathway is associated with the synthesis of fatty acids, cholesterol, neurotransmitters and nucleotides (Berg, Tymoczko and Stryer, 2002f). Lipid synthesis is largely dependent on intermediate products of the glycolytic pathway. The conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate (Berg, Tymoczko and Stryer, 2002c) and the subsequent reduction of this to glycerol 3-phosphate are the initial step for this conversion (Berg, Tymoczko and Stryer, 2002e). Citrate, a component of the TCA cycle is also an important regulator of fatty acid synthesis, by regulation of the acetyl-CoA carboxylase (Berg, Tymoczko and Stryer, 2002a). Finally, a variety of amino acids, such as serine, glutamine, glycine and cysteine are synthesised by intermediates of the glycolysis pathway and the citric acid cycle (Berg, Tymoczko and Stryer, 2002b)

Glycolysis is a sequence of reactions which involves the conversion of glucose into pyruvate and the generation of small amounts of ATP. In the presence of oxygen, the resulting pyruvate is subsequently oxidised to CO_2 and H_2O in the mitochondria, whereas when oxygen is limited, glucose conversion to lactate is the only pathway (Stryer, 1995; Berg, Tymoczko and Stryer, 2002c).

Another pathway of glucose metabolism, commonly encountered in diabetes, is the polyol pathway (Figure 1.14), which includes the conversion of glucose to sorbitol which in turn may be converted into fructose. Increased sorbitol concentrations are associated with increased levels of osmotic stress, which may eventually be associated with cell damage (Singh Grewal *et al.*, 2015).

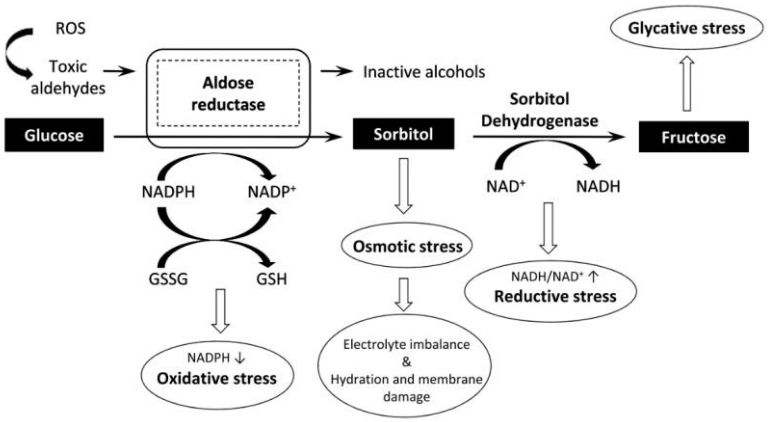


Figure 1.14: Polyol pathway, consisting of glucose conversion to sorbitol and then fructose, observed usually in diabetic complications. Sorbitol accumulation is associated with osmotic stress, electrolyte imbalance, membrane damage and hydration, all of which could result in cell damage (Singh Grewal *et al.*, 2015).

1.9.3. Glucose in reproductive tissues

High levels of glucose have detrimental effects later in foetal development (Greene *et al.*, 1989), (Tarry-adkins and Ozanne, 2016); for example, glucose is known to cross the placental barrier, whereas insulin does not, and the combination of these two events leads to macrosomia in the embryo (Tarry-adkins and Ozanne, 2016), which is a high birth weight, defined by the American College of Obstetricians and Gynaecologists as "over 4000 grams or of 4500 grams irrespective of gestational age (Ng *et al.*, 2010). High levels of glucose also delay embryonic stem cell (ESC) differentiation to neuronal cell types, thus impairing normal development of the nervous system in the embryo (Yang *et al.*, 2016). However, the impact of high levels of glucose on early embryo development, from the zygote to the blastocyst stage, remains largely unknown (Yang *et al.*, 2016).

Glucose has been implicated in ovarian follicular development, dominant follicle maturation and ovulation and formation of corpus luteum (Zhang *et al.*, 2012; Dupont and Scaramuzzi, 2016). Glucose transporters are present in ovarian tissues, their expression varying in different species, dependent on the hormonal levels in the follicle. So far, the evidence on the impact of intrafollicular glucose on follicle size and status is inconclusive (Dupont and Scaramuzzi, 2016). Although glucose transporters, as well as insulin receptor, are expressed in ovarian tissues, they are not believed to be associated with fertility; their ablation resulted in healthy, fertile females (Dupont and Scaramuzzi, 2016). In the bovine, both GLUT1 and GLUT3 transport glucose in the follicle, ovary and corpus luteum, among others. GLUT3 has a high affinity for glucose, whereas GLUT1 is a common glucose transporter across blood barriers (Ostrowska, Jarczak and Zwierzchowski, 2015). Hormonal changes are known to affect glucose uptake in the ovary, by modifying GLUT1 and GLUT3 mRNA expression (Dupont and Scaramuzzi, 2016).

As reviewed by Dupont and Scaramuzzi (2016), GLUT4 is associated with insulin-stimulated glucose uptake (Ostrowska, Jarczak and Zwierzchowski, 2015; Dupont and Scaramuzzi, 2016), whereas GLUT1 is a transporter which is insulin-independent (Ostrowska, Jarczak and Zwierzchowski, 2015). GLUT1, 3 and 4 are expressed in different stages of ovarian tissue, but GLUT4 is preferentially expressed in fat, heart and muscle.

Gene expression of glucose transporters in the follicles appears to vary according to the cell type; although GLUT1 has similar levels of expression between granulosa and theca cells, the former express higher levels of GLUT3 and lower levels of GLUT4 than the latter. Additionally, the expression of GLUT1 and 3 in follicles is comparable to their expression in other tissues (heart, brain, muscle), indicating their importance in this tissue, whereas GLUT4 has a very low expression compared to its expression in the muscle. During the luteal phase of the cycle, there is a decrease in the expression of GLUT1 and an increase in the expression of GLUT3, whereas the pattern of GLUT4 expression was similar to that of progesterone. Finally, the concentration of glucose in the follicular fluid had a significant negative correlation with the expression of GLUT1 and GLUT3 mRNA (Nishimoto *et al.*, 2006).

Glucose transporters have also been identified in the uterus (Frolova and Moley, 2011a). The uterine endometrium expresses glucose transporters 1,3,4 and 8. GLUT1 was the first transporter to be identified in the endometrium (Frolova and Moley, 2011a), and is the most abundant (Frolova and Moley, 2011b) with a role in progression of early pregnancy (Frolova and Moley, 2011a), as well as the process of endometrial decidualisation (Frolova and Moley, 2011b). Decidualisation is the process of morphological and functional adaptation of the endometrial stroma, with a crucial role in the successful establishment of pregnancy (Okada, Tsuzuki and Murata, 2018). GLUT3 has also been identified in the uterus, although it has been studied much less than GLUT1. Similar to GLUT1, GLUT3 is expressed in the stromal cells and the decidua of the uterus and has an important role in stromal cell decidualisation, as well as during pregnancy (Frolova and Moley, 2011a). GLUT1, 3 and 4 have been detected in the uterine tissue of rats; in particular, GLUT1 staining increased in decidual cells with progression of decidualisation, whereas GLUT3 and 4 were detected throughout rat pregnancy in the uterus, with the exception of the endometrial glands and the smooth muscle cells of the uterus (Türkay Korgun *et al.*, 2001). GLUT4 expression in the uterus is believed to be quite low, resulting in contradictory results in different studies (Frolova and Moley, 2011a). Finally, GLUT8 is believed to be involved in endometrial decidualisation (Frolova and Moley, 2011a).

Tadokoro *et al* (1995) have reported the expression of glucose transporter 1 in the ampulla and the isthmus of the rat oviduct (Tadokoro *et al.*, 1995). Additionally, a study by Kuchiiwa *et al* demonstrated the expression of Glut1 and Glut3 in rat oviduct secretory epithelial cells (Kuchiiwa *et al.*, 2011) and glucose transporters have been identified in the uterotubal junction in the bat (Roy and Krishna, 2013). In humans, high levels of GLUT1 expression have been detected in adenocarcinomas in the fallopian tube epithelium, compared to benign tissues (Kalir *et al.*, 2005). However, research in glucose transporters in the oviduct remains limited.

A study by Moley *et al* (1998) in mouse embryos showed that gene and protein expression of glucose transporters was affected by the glucose content of the environment in embryos at different stages of development. Specifically, *SLC2A1* expression was downregulated in diabetic mouse embryos compared to control mouse embryos at 48 and at 96 hours post-hCG administration, whereas *SLC2A2* and *SLC2A3* expression was significantly higher in control mouse embryos compared to diabetic mouse embryos 96 hours after hCG administration. This reduction in glucose transporter gene and protein expression could have been associated with the reduced viability observed at the blastocyst stage in diabetic rats. However, it is worth noting that glucose transporter regulation is cell type – specific and has to be determined for every system studied (Moley, Chi and Mueckler, 1998).

Evidence focusing on the impact of insulin on the oviduct epithelium is limited. A study by Takeuchi *et al* showed no impact of insulin addition on the growth of human fallopian tube epithelial cells in culture (Takeuchi *et al.*, 1991). Additionally, insulin receptors were not identified in the oviduct epithelial cells of the water buffalo, even though they were expressed in the early embryo and the blastocyst stage embryo (Daliri *et al.*, 1999). The impact of moderate concentrations of basal insulin supplementation on BOECs grown in air-liquid interface has been previously found to be beneficial for the morphology of the oviduct epithelium (Palma-Vera, Einspanier and Schoen, 2014).

1.10. Hypothesis and Aims

Based on the evidence that currently exists, it is reasonable to assume a link between hyperglycaemia and obesity. Barker's hypothesis has highlighted an important link between an adverse environment during pregnancy and the development of metabolic abnormalities during puberty and adulthood (Barker, 2004) and research has demonstrated these may be traced back to the ovarian stage follicle. However, very little is known with regards to whether these adverse conditions affect the environment where the pre-implantation embryo spends the first few days of development, as well as embryo growth and metabolism.

Therefore, this thesis sets out to test the hypothesis that abnormally high levels of glucose are associated with modified oviduct epithelial cell secretions, which may affect preimplantation embryo development. In order to test this hypothesis, the thesis had the following aims:

1) To confirm and explore further the *in vitro* model of the bovine oviduct, previously established in the laboratory.
2) To determine the impact of high glucose levels in the presence or absence of insulin on oviduct epithelial cells grown according to the above model, in terms of the composition of cell secretions and patterns of gene expression.
3) To determine the impact of the modified embryo environment characterised in 1) and 2) on bovine embryo development and metabolism.

Chapter 2

Materials and Methods

2.1. Bovine Oviduct Epithelial Cell collection and maintenance

2.1.1. Tissue harvest and Bovine Oviduct Epithelial cell seeding

Bovine reproductive tracts at mixed stages of the oestrous cycle were harvested from a local abattoir, and transported to the laboratory approximately an hour after slaughter. Tracts were immersed in "Transport Buffer", which comprised of HBSS (+phenol red, -$CaCl_2$, –$MgCl_2$), 10mM HEPES and 1µM aprotinin from bovine lung, which inhibits the action of proteases by blocking their binding sites (Sigma-Aldrich). The animals from which tissue was dissected were 18-24 months old, usually after their first calving.

Isolation of Bovine Oviduct Epithelial Cell (BOEC) population was performed as described by (Simintiras *et al.*, 2016). The connective tissue surrounding the oviducts was dissected and the oviducts were immersed in HBSS. Oviduct cells were mechanically removed into HBSS, by gentle palpation of the isolated oviduct to free the epithelial cells, and centrifuged twice at 630x*g* for 5 minutes, in order to wash the cells. The cells were next re-suspended in 10mL BOEC cell culture medium (Table 2.1) and a cell count was performed with the use of a haemocytometer. Cell viability was assessed using a Trypan Blue exclusion test; cells with an intact cell membrane do not take up Trypan Blue. $2x10^6$cells are seeded in T_{75} flasks in a final volume of 10ml of BOEC cell culture medium. $2x10^6$cells are seeded in T_{75} flasks and they were incubated at 39^oC, 5% CO_2.

Table 2.1.: Composition of the medium used to feed Bovine Oviduct Epithelial Cells.

	Volume	**Concentration**
DMEM	500mL	N/A
Nutrient Mix F12	500mL	N/A
Pen/Strep	26.5mL	239.2u/mL Pen/ 239.2 µg/mL Strep
Amphotericin B	20mL	4.51µg/mL
L-Glutamine	10mL	1.8mM
Newborn Calf Serum (NCS)	25.5mL	2.3% v/v
Fetal Bovine Serum (FBS)	25.5mL	2.3% v/v
Bovine Serum Albumin (BSA)	7.5g	0.68% w/v

After 18 hours of incubation, BOECs were isolated based on their different adhesion times, as described by (Cronin *et al.*, 2012). This is based on the observation that

fibroblasts adhere to plastic cell culture vessels faster than epithelial cells; thus, after 18h the majority of fibroblasts in the cell suspension are adhered, whereas the epithelia remain in suspension. The purity of the epithelial cells was confirmed using fluorescent immunohistochemistry and haematoxylin and eosin staining, as described in sections 2.3 and 2.4.

Isolated BOECs were seeded in Transwells™, at cell density of $1x10^6$ cells per Transwell (Figure 2.1). The cells were maintained by basal and apical supply of medium, to a final volume of 2mL in each chamber.

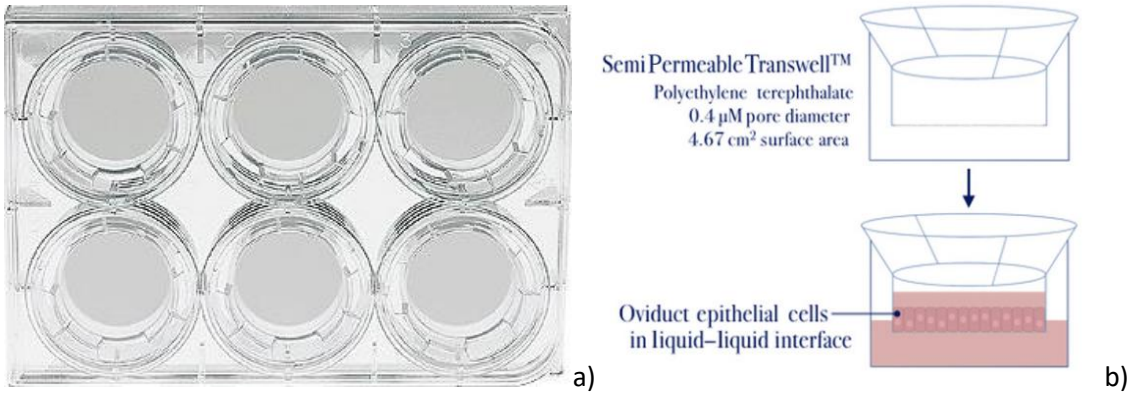


Figure 2.15: a) Transwell plate used for growing Bovine Oviduct Epithelial Cells in a polarised epithelium. Picture from the Cole-Palmer website. b) Schematic diagram of a single Transwell. The bovine oviduct epithelial cell monolayer, growing on a semi-permeable transport, is indicated. Media is applied to the apical and basal side of the cells during cell growth (pink colour). Figure modified from (Simintiras *et al.*, 2016).

2.1.2. Cell maintenance

BOECs had their media replenished 24h after the first seeding and every 48 hours after that. Only primary BOEC cultures were used for the experiments described in this thesis, as passaging bovine oviduct epithelial cells in culture has been reported to impact on the expression of selected genes, specifically coding for mucins (*MUC1, MUC4, MUC16*), proteins associated with cell metabolism (*GAPDH, SDHA, HK1*), as well as *OVGP1* gene expression and release, and IL8 release (Danesh Mesgaran *et al.*, 2016).

Attainment of an electrically integral confluent epithelial monolayer was determined by measuring the TransEpithelial Electrical Resistance (TEER), which is a useful measure for determining the creation of a barrier of cells growing on filter support (Chen, Einspanier and Schoen, 2015). Cell electrical resistance was measured using an EVOM

Voltohmmetre, connected to an STX2 electrode set (EVOM). Resistance measurements were performed by immersing the electrodes into the apical and basal chambers of the transwell inserts. The resistance of the medium was between 12Ω and 15Ω and the resistance of a blank transwell, containing only medium, was on average 130Ω. The surface areas of a Transwell and a Snapwell were 4.67cm^2 and 1.12cm^2 respectively. The TransEpithelial Electrical Resistance was therefore calculated using the following formula:

TEER = (resistance of cells in the transwell-(resistance of blank transwell–medium resistance)) *surface area

Attainment of a TEER above a threshold value of >700 Ωcm^2 indicated the formation of an electrochemically confluent cell monolayer (Simintiras and Sturmey, 2017).

2.2. Confirmation of cell identity

The presence of the epithelial cell-specific surface protein Cytokeratin-18 (CK18) was used to confirm the identity of the cells isolated. BOECs were seeded on glass coverslips, that had been pre-coated with 0.1mg/mL poly-L-lysine in double-distilled water (ddH_2O) and left to air dry for 10 minutes, in order to enhance cell attachment to the coverslips (Sigma-Aldrich, 2008). BOECs were then cultured on the glass coverslips for 6 days in culture medium. Then, the cells were fixed stained with the antibody for against Cytokeratin-18 (Applied Biosystems), using appropriate controls.

Cytokeratin-18 staining was performed follows. Unless stated otherwise, the cells were washed with PBS before each step. BOECs were fixed in 4% (w/v) paraformaldehyde PFA in PBS for 30 minutes, and then permeabilised with 0.25% v/v Triton X-100 in PBS for 10 minutes. This was followed by incubation with 1%BSA/ 10% normal goat serum/ 0.3M glycine in 0.1% PBS-Tween for 1 hour, to block non-specific binding, as suggested by the manufacturer. Following this, the cells were incubated with the anti-Cytokeratin-18 mouse primary antibody (Applied Biosystems, ab82254, diluted 1:100, or 1% BSA in 0.1% PBS-Tween (primary antibody control) at 4^{o}C overnight. The following day, the cells were washed in PBS and incubated with goat anti-mouse, FITC-conjugated secondary antibody (Applied Biosystems, ab6785, dilution 1:3000 in blocking buffer) or with blocking buffer (secondary antibody control) for 1 hour in the dark. The coverslips were

then incubated with anti-fade DAPI stain for 15 minutes and mounted on coverslides before observation under a Zeiss LSM 710 confocal microscope, using the ZEN2010 B software.

2.3. Haematoxylin and Eosin (H&E) staining

Haematoxylin and Eosin staining was performed using the following protocol to confirm successful isolation of epithelial cells of the oviduct. BOECs were seeded on coverslips at a density of $2x10^5$cells/ml that had been pre-coated with poly-L-Lysine, as described in section 2.2. The cells were washed with PBS and fixed with 4% (w/v) PFA in PBS for 15 minutes. After being washed for a second time with PBS, they were incubated with Hematoxylin Solution which had been filtered before use, for 3 minutes and then rinsed with distilled water. The slides were next incubated in 1% eosin for 3 minutes and washed 3 times in distilled water, for 1 minute each time. The slides were mounted using Hydromount and observed on a bright-field microscope (x20 magnification). Haematoxylin stained the BOEC nuclei dark purple, whereas eosin stained the BOEC cytoplasm pink. Post-acquisition image was modified to increase contrast for presentation purposes.

2.4. Transmission Electron Microscopy

In order to show the formation of an epithelial cell monolayer by BOECs in culture, Transmission Electron Microscopy (TEM) was performed on BOECs grown to confluence. The experiment was performed with microscopy facility technical assistance. Briefly, the day before the cells were ready for the experiment, a resin block was prepared and allowed to harden for 48 hours at 60^{o}C. On the day of the experiment, the cells were fixed in glutaraldehyde in cacodylate buffer for 1 hour at room temperature. The cells were then washed twice for 5 minutes in cacodylate buffer and this was followed by fixation at 4 degrees for 1 hour in 1% Osmium tetra-oxide in cacodylate buffer. After washing the cells again twice in cacodylate buffer, they were incubated in 1% uranyl acetate for an hour, and then in increasing ethanol concentrations (30%, 50%, 70%, 90%, 100%, 100%) for 10 minutes each. The cells were incubated in 1:1 ethanol:resin for 60 minutes and then overnight in resin. The following day, the cells were incubated in fresh resin for 3 hours and then new resin was made and further incubated of 3 hours. Then, the cells were embedded onto a hardened resin block using fresh epon/araldite, for 72

hours at 60°C. After three days, they were removed from the oven and allowed to stand for 48 hours at room temperature. Once the resin was cured, a hacksaw was used to cut the block into smaller pieces and 70nm sections were cut with the use of glass knives using a Leica UC6 Ultramicrotome. Sections were collected onto carbon-coated copper grids and allowed to dry. Finally, images were obtained using a Gatan Ultrascan 4000 camera attached to a Jeol 2010 Transmission Electron Microscope running at 120kV.

2.5. Fluorescein rejection assay of epithelial monolayer integrity

A fluorescein rejection assay was used to confirm the establishment of a physical intact epithelial monolayer of BOECs and validate the use of TEER as a non-invasive measurement of the epithelial barrier integrity. Fluorescein is a non-permeable dye of a molecular weight of 332.31g/mol and so is unable to cross an intact epithelial monolayer. Fluorescein was used at a concentration of 200 µM (Simintiras, 2016). BOECs were grown to confluence, determined by attainment of a TEER>700Ω.cm^2 and then they were incubated in Krebs-Ringer buffer (Table 2.2), for 30 minutes, at 39°C, 5%CO_2.

Table 2.2: Composition of Krebs - Ringer buffer

	Component	**Amount**	
Stock 1:	NaCl	6.896 g	Dissolved in 100mL ddH_2O
	KCl	0.353 g	
	KH_2PO_4	0.161 g	
	$MgSO_4.7H_20$	0.291 g	
Stock 2:	$NaHCO_3$	2.092 g dissolved in 100mL ddH_2O	
Stock 3:	$CaCl_2.H_2O$	0.187 g dissolved in 100mL ddH_2O	
Final medium:	**Stock 1**	**10mL**	
	Stock 2	**10mL**	
	Glucose	**0.1g**	
	ddH_2O	**70mL**	
	Stock 3	**10mL**	

Fluorescein transport from the basal to apical compartment and vice versa was determined. Fluorescein was added to the receiving chamber and 50µl samples, collected at 30-minute time intervals for a total of 240 minutes, from both the apical

and basal compartments. Samples were plated in a 96-well PCR plate. Every time a sample was collected, the TEER was also measured. The collected samples were then used to determine fluorescence in the basal and apical compartments, using a BMG plate reader, at excitation/emission of 485nm/520nm respectively.

At 210 minutes of incubation, EDTA was added. EDTA is known to chelate the Ca^{++} in the tight junctions between the cells in the monolayer (Noach *et al.*, 1993) and disrupt the barrier properties. This served as a positive control for fluorescein transport.

2.6. *In vitro* model of the oviduct

When BOECs cultured on Transwells had reached a TEER >700 ohms, the medium from the apical compartment was aspirated, but not replaced, and the cells were incubated in moist air at 39^{o}C, 5% CO_2. After 24 hours, a thin film of fluid, which had formed at the apical compartment, was collected. The fluid volume was measured using a Gilson's pipette and the sample was stored at -20^{o}C for short-term storage or at -80^{o}C for long term storage. This fluid was termed *in vitro* Derived Oviduct Fluid (*iv*DOF) (Simintiras *et al.*, 2016).

2.7. Metabolic assays

The quantification of energy substrates in ivDOF and spent culture media was determined using microfluorometric assays based on those developed by (Leese and Barton, 1984) and the principles of Lowry (Lowry *et al.*, 1951). These assays work by coupling the enzymatic conversion of NADH to NAD to substrate-level reactions and measuring the resulting change in fluorescence.

The glucose assay is based on the conversion of glucose to glucose-6-phosphate, a reaction facilitated by hexokinase. Glucose-6-phosphate is then converted to 6-phosphogluconate through the activity of G6PD, and during this reaction, $NADP^{+}$ is reduced to NADPH. Consequently, the higher the levels of glucose which are present in the solution, the higher the levels of fluorescence produced (Figure 2.2). The fluorescence was detected using a plate reader fitted with excitation and emission filters of 355nm and 460nm, respectively.

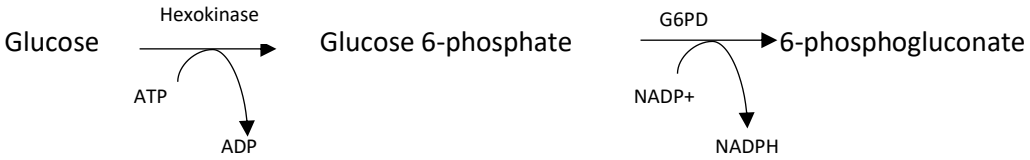


Figure 2.2: Glucose conversion to glucose-6-phosphate and then to 6-phosphogluconate. This reaction results in the production of NADPH, which produces fluorescence that can be detected by a plate reader.

The principle behind the lactate assay relies on the conversion of lactate to pyruvate, a reaction catalysed by LDH, during which NAD^+ is reduced to NADH. Consequently, the higher the levels of lactate which are present in the solution, the higher the levels of fluorescence produced (Figure 2.3). The fluorescence was detected at excitation/emission of 355nm /460nm, using the BMG plate reader.

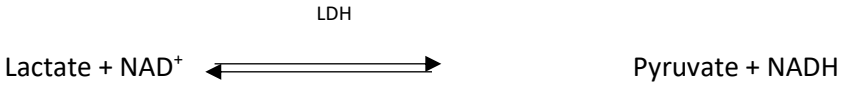


Figure 2.3: Conversion of lactate conversion to pyruvate, during which NAD^+ is converted to NADH, which produces fluorescence that can be detected by the BMG plate reader.

The pyruvate assay works on the on the conversion of pyruvate to lactate, a reaction catalysed by LDH, during which NADH is oxidised to NAD^+. Consequently, the higher the levels of pyruvate which are present in the solution, the lower the levels of fluorescence produced (Figure 2.4). The fluorescence was detected at excitation/emission of 355nm /460nm, using the BMG plate reader.

Figure 2.4: Conversion of pyruvate to lactate, during which NADH is oxidised to NAD+. The more the NADH that gets oxidised to NAD, the lower the fluorescence.

2.7.1. Glucose assay

The quantification of glucose present in ivDOF and basal culture media, as well as in spent media from embryo culture, was carried out using the method described by (Guerif *et al.*, 2013). Briefly, 1 µL of sample was added to 9 µL of glucose cocktail, consisting of 0.42mM Dithiothreitol, 3.08mM magnesium sulphate, 1.3mM NADP, 0.45mM ATP and 20 IU/ml Hexokinase/G-6-P, all dissolved in EPPS buffer at pH=8.0. The solution was aliquoted and stored in -20^{o}C for a maximum of three months. The concentrations of the samples were determined against a standard curve 0-1mM (Figure 2.5). Any standard curve with an R^2<0.98 was rejected and the respective samples run with it were excluded. All samples were analysed in triplicates.

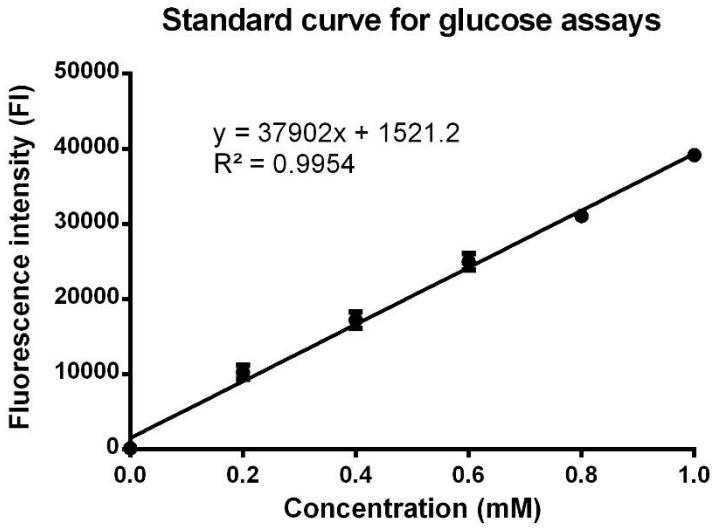


Figure 2.5: Example of standard curve used for glucose assays

2.7.2. Lactate assay

The measurement of the amount of lactate was performed according to (Guerif *et al.*, 2013). Briefly, 1 µL of sample was added to 9 µL of lactate cocktail; this consisted of glycine buffer (which in turn included 4mM glycine, 1.6mM hydrazine sulphate, 0.02mM EDTA, all made up to a pH of 9.4), 4.8mM NAD and 40 IU/ml Lactate dehydrogenase (LDH). The solution was aliquoted and stored in -20^{o}C for up to three months. The concentrations of the samples were determined against a standard curve 0-2.50mM (Figure 2.6). Any standard curve that yielded an R^2<0.98 was rejected and the respective samples run with it were excluded and re-analysed. All samples were determined in triplicates.

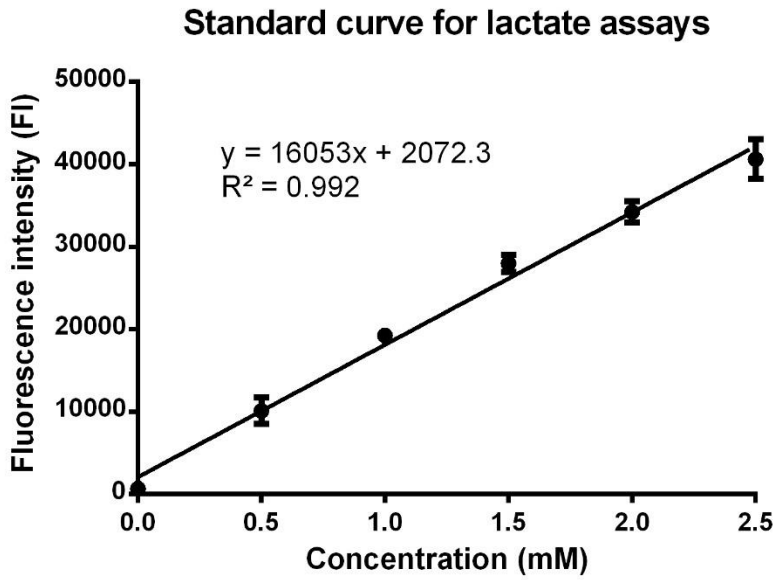


Figure 2.6: Example of standard curve used for lactate assays.

2.7.3. Pyruvate assay

Pyruvate determination was performed according to Guerif et al 2013 (Guerif *et al.*, 2013). Briefly, 1 µL of sample was added to 9 µL of pyruvate cocktail; this included EPPS, NADH Disodium salt, LDH and sodium hydroxide. The solution was aliquoted and stored in -20^{o}C for up to three months. The concentrations of the samples were determined against a standard curve 0-0.45mM (Figure 2.7). Any standard curve with an R^2<0.98 was rejected and the respective samples run with it were excluded. All samples were analysed in triplicate.

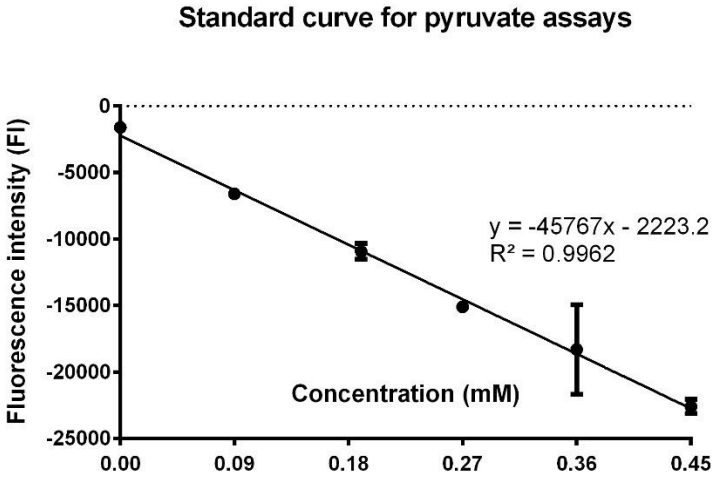


Figure 2.7.: Example of standard curve used for pyruvate assays.

2.8. High-Performance Liquid Chromatography

Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) was used to determine the amino acid composition of the fluid collected and the basal media. Using RP-HPLC, 17 amino acids could be detected: aspartic acid, asparagine, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine and lysine (Figure 2.8).

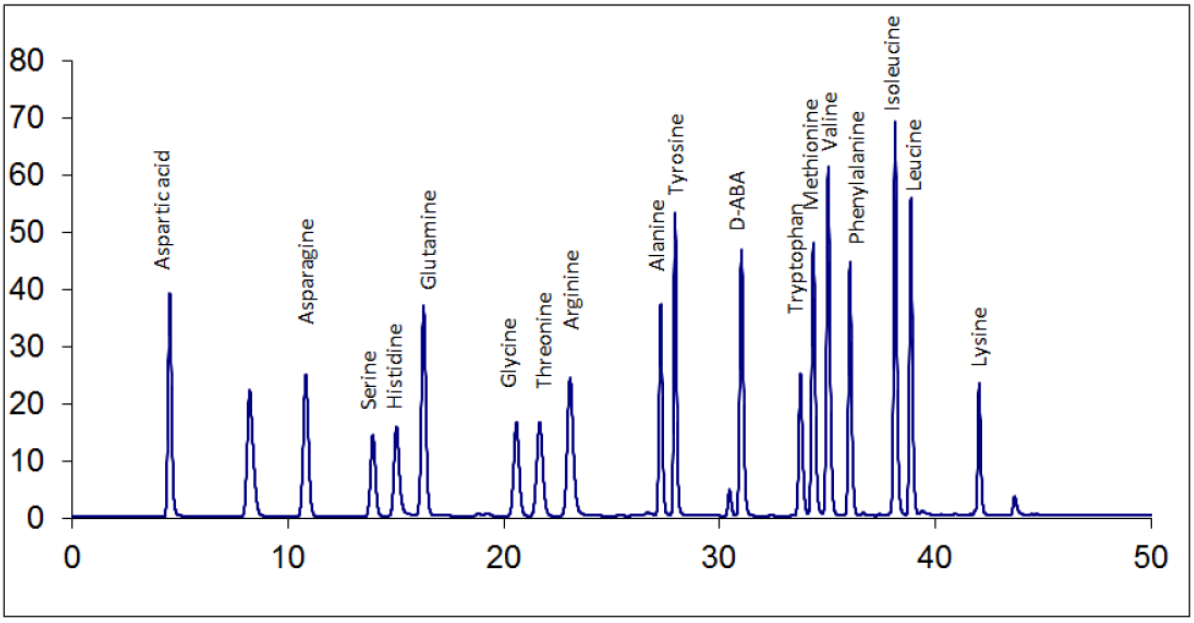
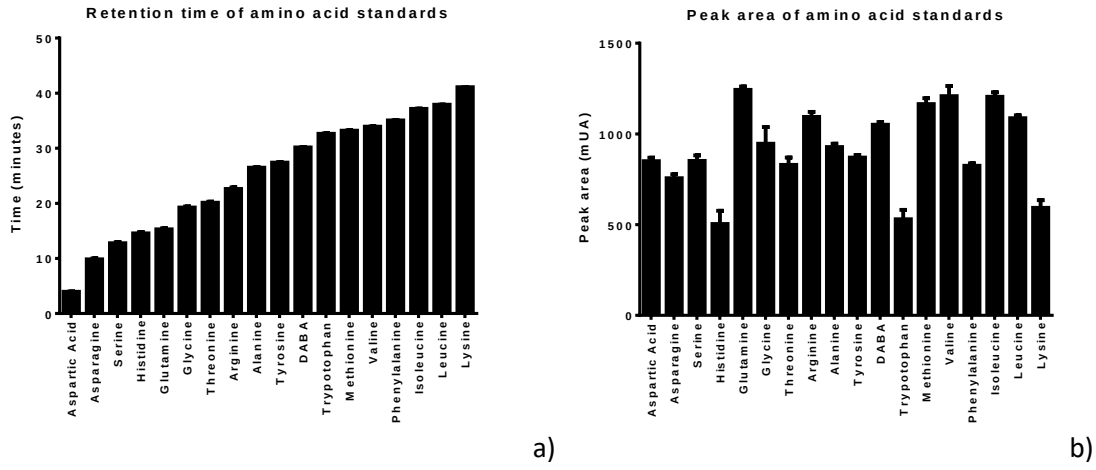


Figure 2.8: Amino acid detection with the use of HPLC. The peaks correspond to different amino acids, which are indicated above the respective peaks.

The method of amino acid detection was performed as previously described (Houghton *et al.*, 2002)(Partridge and Leese, 1996) (Humpherson, Leese and Sturmey, 2005), which separates amino acids on the basis of size and hydrophobicity. Briefly, mixtures containing the amino acids underwent pre-column derivatisation with O-phthaldialdehyde (OPA), supplemented with 1mg/ml 2-mercaptoethanol, which resulted in the formation of amino acid conjugates with OPA. This step labels the amino acids to generate a fluorescent signal, detectable at 450nm, which may be detected by a fluorescence detector attached to the HPLC system. Derivatisation and sample loading was performed using an Autosampling Injecting Unit connected to the HPLC system. Following derivatisation, samples were injected onto a HyperClone™ 5 µm ODS (C18) 250 x 4.6 mm column (Phenomenex, UK). Individual amino acids were eluted from the column using a gradient method comprising two buffers: Buffer A (80% 83mM sodium

acetate : 19.5% methanol : 0.5% tetrahydrofuran) and Buffer B (80% methanol : 20% 83mM sodium acetate), both at pH 5.9.

Chromatography was performed at a flow rate of 1.3ml/min A gradient of Buffer A to Buffer B was applied throughout the sample run: The system ran at 1.3mL/min buffer A for the first 9.5 minutes of the run. Then, the proportion of Buffer B gradually increased to 14%, where it remained stable for 5 minutes. Over the course of the next 7 minutes, the proportion of buffer B gradually increased to reach 50%, and remained stable at this for 3 minutes. Following this, the proportion of buffer B increased for the next 13 minutes, until it reached 100%, where it remained stable for 6 minutes. Finally, the proportion of Buffer B was brought back to 0%. Due to the different proportions of Buffers A and B, the organic phase of the solvent increased and the aqueous phase decreased, thus resulting in the different retention times of the amino acids. The buffer gradient ensured amino acid separation at different retention times, based on the interaction with the solid phase of the column. The concentration of each amino acid was quantified according to the area under the curve (AUC) and compared to those given by validated standard samples (Sigma, AAS18) of known concentrations (12.5uM) (Figure 2.9). Prior to loading samples, the efficiency of the method was tested, by running standard samples and measuring the coefficient of variation (standard deviation of peak area /mean peak area); any coefficient of variation below 5% was considered acceptable.



a) b)

Figure 2.9: Example of amino acid separation of amino acid standard samples as detected using the HPLC. The different retention times (a) and different areas under the curve (AUC) (b) are presented.

2.9. RNA extraction

RNA was extracted from cells grown in Transwells immediately after *iv*DOF collection. BOEC cells were detached from the Transwell membrane using Trypsin, applied on the cells for 15 minutes. The trypsin was inactivated by adding of cell culture medium, which contains FBS and can de-activate trypsin. The detached cells were centrifuged at 630x*g* for 5 minutes and then washed twice in Hank's Balanced Salt Solution at 13,000x*g*, 4°C for 6 minutes. Following this, 1ml of Trizol and 200µl chloroform were added and the samples mixed and incubated at room temperature for 20 minutes. These were then centrifuged at 12,000x*g* for 10 minutes, at 4°C, to achieve phase separation: the top, aqueous layer contained the RNA, the interphase and the bottom, organic layer contain the DNA and proteins (Rio *et al.*, 2010). The aqueous phase was then removed and precipitated with isopropanol prior to storage in -20°C (minimum time 20 minutes, maximum 6 months).

Following isopropanol precipitation, the samples were centrifuged at 12,000x*g*, 4°C for 40 minutes, and the pellet was resuspended in 70% (v/v) EtOH in ddH_2O and centrifuged at 12,000x*g*, at 4°C for 10 minutes. Then, any residual organic solution was removed by pulse centrifugation and air-drying of the samples for 10 minutes. Finally, the RNA was re-suspended in 50µl of ddH_2O. The concentration and the purity of the RNA isolated was assessed using the ratio of A_{260}/A_{280}, which is indicative of protein contamination of the sample. The absorbance was measured using a Nanodrop spectrophotometer and samples of known concentration and purity were immediately stored at -80°C.

2.10. Complementary DNA (cDNA) synthesis

cDNA libraries were generated from the extracted RNA using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368814). The RNA was diluted with water, so that all samples contained 11.1 ng/µl of RNA, and to a final volume of 10µl. A master mix (Table 2.3) containing necessary components for cDNA synthesis was prepared fresh, prior to each cDNA synthesis experiment.

Table 2.3: Master mix prepared for cDNA synthesis

Component	Volume (ul)	Final concentration in master mix
10xRT buffer	2	1x
25xdNTP mix (100mM)	0.8	8mM
10xRT random primers	2	1x
MultiScribe™ Reverse Transcriptase	1	5 U/µl
ddH_2O H_2O	4.2	n/a
Total volume	**10**	

The master mix was then mixed with the diluted RNA, the samples were gently mixed loaded onto an Applied Biosystems 2720 Thermal Cycler for cDNA synthesis. The conditions are described in Table 2.4. cDNA samples were stored at -20°C for short term use or at -80°C for long term storage.

Table 2.4.: Thermocycler conditions for cDNA synthesis.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2.11. Polymerase Chain Reaction – agarose gel electrophoresis

2.11.1. Primer design

Forward and reverse primer design was carried out using the PrimerBlast tool by Pubmed (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), for genes encoding for β-actin, OVGP1, ERα, ERβ, PR, GLUT1, GLUT3 and INS-R. Primers for the RPL19 reference gene had been previously designed in the laboratory and were therefore not re-designed. The primer sequences were designed to match the exon sequences of the target genes only, and for this purpose, the cds sequence was used, where introns are excluded. The PCR target product sizes were set to a maximum of 250bp, although 100bp was considered as the optimal. The ideal CG content was 50% and each primer was aimed to contain 20 nucleotides. Where possible, the primers were designed to span exon-exon junctions and the melting temperature (T_m) was set between 58°C and 62°C, with optimal T_m of 60°C. The resulting primers were tested using PrimerBlast for product specificity.

2.11.2. Optimal annealing temperature

Polymerase Chain Reaction was performed using the Platinum Taq DNA polymerase kit (Invitrogen, 10966018), in order to amplify target sequences using the specificity of the primers designed according to section 2.11.1 and used for qRT-PCR. The cDNA synthesized previously was used and a master mix was prepared as described in Table 2.5:

Table 2.5: PCR master mix for determining optimal annealing temperature.

Component	Volume per PCR reaction	Final concentrations
10x reaction buffer – $MgCl_2$	5	1x
50mM $MgCl_2$	1.5	1.5 mM
10mM dNTPs	1	200µM
10µM Forward Primer	1	200 nM
10µM Reverse Primer	1	200 nM
Taq polymerase	0.25	2U/reaction
cDNA (25ng/µl)	1	0.5 ng/µl
ddH_2O	39.25	-
Total volume	**50**	

In order to delineate the optimal primer-cDNA annealing conditions, a temperature gradient was applied to determine the optimal annealing temperature, which was set to annealing temperatures of 70°C, 68.9°C, 67.1°C, 64.4°C, 60.9°C, 59.5°C, 56.4°C and 55°C, by adjusting the thermocycler to apply all the above temperatures in different wells of the thermocycler within the same run. The thermocycler conditions were set as described in Table 2.6.

Table 2.6: Thermocycler conditions for PCR reactions.

STEP	TEMP (°C)	Time
Initial denaturation	94	3 minutes
Annealing (x50 cycles)	94	45 seconds
	gradient of T_{ann}	30 seconds
	72	90 seconds
Final extension	72	10 minutes
Hold	4	forever

A 50x stock solution of Tris-Acetic Acid- EDTA (TAE) buffer was made by mixing 24.2g of Tris, 5.71ml of glacial acetic acid and 10mL of 0.5M EDTA (pH=8.0), to a final volume of 100mL and the solution was stored at room temperature. The PCR products were then

loaded on a 2% agarose gel in TAE buffer. with the addition of 10000x SYBRSafe DNA staining dye. TAE buffer was made up at a 50x concentration, by mixing 24.2g Tris, 10ml of 0.5M EDTA, pH=8.0 and 5.71mL glacial acetic acid. Loading dye, which makes the cDNA solutions more visible during loading, was used to load the buffers to the agarose gel and a 50bp ladder was used to determine the size of the products. Samples were run at 100V for approximately 1 hour. The DNA bands were then observed using a VersaDoc™ (BioRad) Molecular Imager, using the VersaDoc software.

For all genes, the optimal annealing temperature was determined to be 60°C. Additionally, all gene expression experiments were performed according to the MIQE guidelines (Bustin *et al.*, 2009).

2.11.3. Optimal primer concentration

The optimal primer concentration was determined with the use of qRT-PCR. Serial dilutions of primer concentrations were assessed, ranging from 200nM to 12.5nM. Cycle threshold (Ct) values were assessed, which describe the number of cycles which are required for the fluorescence produced by the PCR reaction to cross the background level. The expected outcome of this experiment was higher C_t values for lower primer concentrations. The optimal primer concentration was the one where the C_t values produced during qRT-PCR were the same as those of the immediately higher primer concentration; this suggested that the minimum amount of primer that would give highest PCR efficiency, as well as complete absence of primer dimers (Figure 2.10).

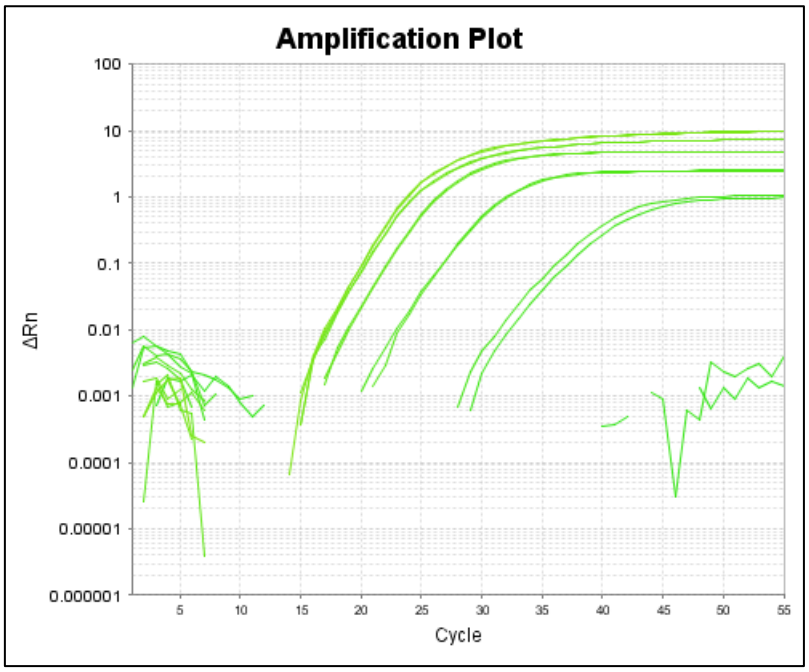


Figure 2.10: Representative amplification plot for the determination of the optimal primer concentration. 200µM and 100µM primer concentrations appear to produce the same Ct values; therefore, 100µM primer concentration is considered to be optimal.

The master mix used for this experiment is described in Table 2.7:

Table 2.7: Master mix for qRT-PCR reactions for house-validated genes.

Component	Volume (µl)
2xSYBR Green master mix	12.5
Forward and Reverse primer mix	4
ddH_2O	7.5
cDNA (25ng per reaction)	1
Final volume	**25**

For this experiment, the thermocycler conditions used were as described in Table 2.8, as previously validated in the lab to be optimal for BOEC gene expression experiments:

Table 2.8: Thermocycler conditions for qRT-PCR for house-validated genes.

STEP	TEMP (°C)	Time
Initial denaturation	94	5 minutes
Annealing (x55 cycles)	94	30 seconds
	60	45 seconds
	72	45 seconds
Melting curve	95	15 seconds
	60	1 minute
	95	15 seconds

2.11.4. qRT-PCR efficiency

When primers bind to the target sequence efficiently and exclusively, they produce two copies of the target sequence for every cycle of PCR. Such an instance would be described as 100% efficiency. Any efficiency less than 90% suggests that the primers lack specificity to the target and so do not amplify the product as efficiently as they are expected to, i.e. they produce less than 2 copies of the DNA template per cycle. Extremely high efficiency, on the other hand, suggests that the primers bind and amplify efficiently multiple target sequences, beyond the target sequence, thus making it impossible to determine the amplification of the target template.

The efficiency of the qRT-PCR reaction was determined by assessing the ability of the primers to produce two copies of the target gene in each cycle, with the use of serial dilutions of the cDNA template (Figure 13). The annealing temperature and the primer concentrations were used as determined from the previous experiments (Sections 2.11.2, 2.11.3). The master mix used for this experiment was the same as for primer concentration validation (Table 2.7, section 2.11.3), modified accordingly during optimisation to fit higher cDNA concentrations. The amounts of cDNA tested were 25ng, 12.5ng, 6.25ng, 3.125ng and 1.5625ng per PCR reaction, corresponding to 1ng/µl, 0.5 ng/µl, 0.25 ng/µl, 0.125 ng/µl, 0.0625 ng/µl per reaction. The thermocycler conditions are described in Table 2.8. and a representation of the expected amplification plots is shown in Figure 2.11.

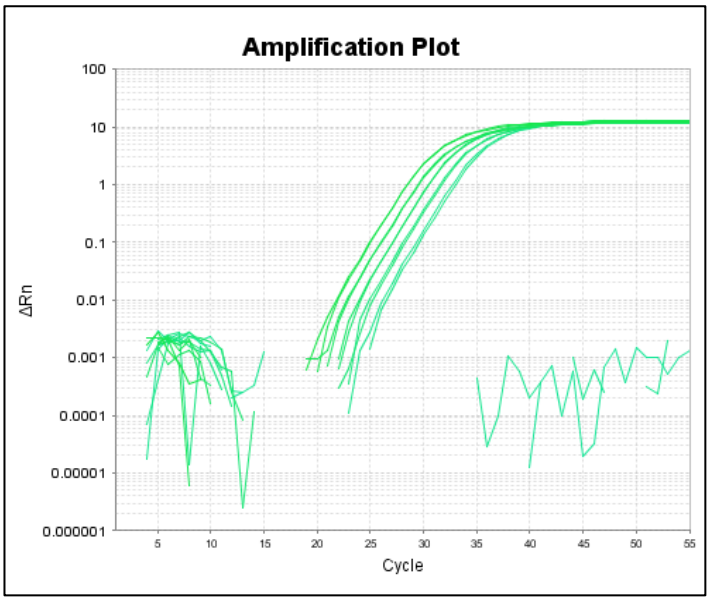


Figure 2.11: Representative amplification plot of serial cDNA dilutions. Decreasing amount of cDNA added to the qRT-PCR reaction resulted in an increase in the Ct value.

The C_t values were plotted against the log of the cDNA concentration, in order to determine the efficiency of the PCR reaction. The efficiency of the PCR reaction is calculated using the following formula:

$$\textbf{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Based on the above calculation, a slope of -3.315 suggests optimal efficiency, which is equal to 1 (100% efficiency), but any efficiency values between 90% and 110% were considered acceptable. Example of an efficiency curve can be found on Figure 2.12.

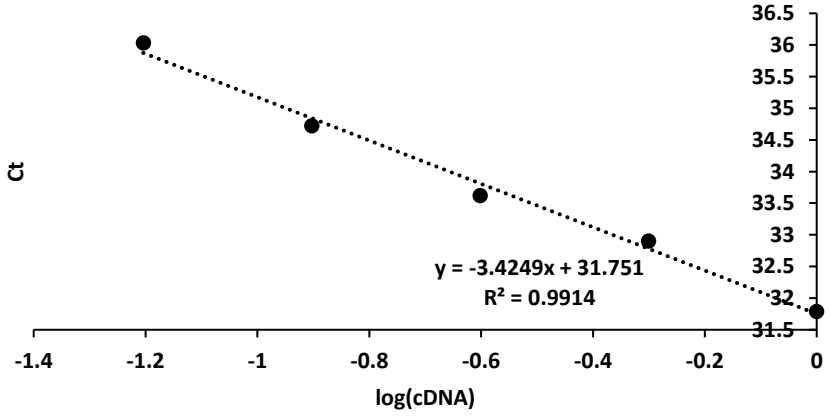


Figure 2.12.: Example of efficiency curve, plotted as Ct values against log [cDNA]. The slope is -3.4249, suggesting an efficiency of 95.88%. Therefore, this primer pair was considered acceptable for use in gene expression experiments.

Accordingly, primers encoding for β-Actin, GLUT1 and ER-α and were used on the basis that they met inclusion criteria on the basis of efficiency (Table 2.10) and primers for RPL19 had, as mentioned, been previously designed in the laboratory. Primers for the remaining genes of interest had either extremely high or extremely low efficiency curves and therefore pre-validated primers were purchased (PrimerDesign Ltd), designed against cDNA libraries extracted from BOECs. The primer concentrations used for internally validated and externally designed primers are shown in Tables 2.9 and 2.10 respectively.

Table 2.9: Primer sequences for house-validated primers.

Primer name	Primer Sequence
RPL19 Forward	GAAAGGCAGGCATATGGGTA
RPL19 Reverse	TCATCCTCCTCATCCAGGTT
b-actin Forward	AGATCAAGATCATCGCGCCC
b-actin Reverse	TAACGCAGCTAACAGTCCGC
SLC2A1 Forward	TAACCGCAACGAGGAGAACC
SLC2A1 Reverse	AGAAAACAGCGTTGATGCCG
ESR1 Forward	TGGCCATTCATTCGCTCACT
ESR1 Reverse	CCTGGAATCCCTTTGGCTGT

Table 2.10: Primer sequences for externally validated primers.

Primer name	Primer Sequence
INSR Forward	CAACGAGGCTTCGGTCATGA
INSR Reverse	GGAACGGAGGTAACTCTTCAGG
OVGP1 Forward	GGACCAGCATCTCCAGGGAA
OVGP1 Reverse	CGCTCTCCGGACAAAGCAG
SLC2A3 Forward	CTCGGTTGCTACCATAGGCTC
SLC2A3 Reverse	GCTCTTCCAAAGTGTAATTGAGAAAG

For the genes which were externally validated, the master mix prepared and the thermocycler conditions are shown in Tables 2.11 and 2.12:

Table 2.11: Master mix for externally validated primers.

Component	Volume (µl)
Precision®PLUS qPCR Master Mix	10
Primer/Probe mix	1
Template (25ng)	5
RNase/DNase free water	4
Final volume	**20**

Table 2.12: Thermocycler conditions for qRT-PCR for externally-validated primers.

STEP	TEMP (°C)	Time
Initial denaturation	95	2 minutes
Annealing (x40 cycles)	95	10 seconds
	60	60 seconds
Melting curve	95	15 seconds
	60	1 minute
	95	15 seconds

The results of the qRT-PCR were exported and analysed. The threshold was adjusted to fit the linear part of the amplification curve and the ROX internal standard was confirmed to be stable throughout the assay for all the genes involved.

The Ct values of the two reference genes (RPL19 and beta-actin) were averaged. For each sample and each gene, this average was subtracted from the Ct value of that gene, producing the ΔCt value. The ΔCt value from a sample which was determined as the control was then subtracted from the different ΔCt values of the test samples, producing a ΔΔCt value. The $2^{-\Delta\Delta Ct}$ value was then calculated, which represents the fold change. This was set to 1 for the control samples and the respective fold changes were calculated for the target samples. These values were then assessed statistically.

2.12. Measuring ion flow across an epithelial monolayer

In order to determine the integrity of the epithelial monolayer in a dynamic manner, an Epithelial Voltage/Current Clamp (World Precision Instruments) system was used. This system can measure the potential difference between the apical and basal compartment of the epithelial cells and present the values in millivolts. The membrane potential is then modified and temporarily set to a specific value, which in this case was zero, so that the ion flux from the basal to the apical compartment can be measured. These values can then be divided to calculate resistance.

The voltage clamp was used to compare the ionic flux from apical and basal compartments through the epithelial cell monolayer, in response to different treatments. Cells were grown on Snapwells^{TM} permeable membranes which have been shown previously to have the same selective transportation properties as Transwells. BOECs were grown on Snapwells at a density of $0.5x10^6$ cells/ml. Since EVOM voltohmmetre is not suitable for measuring cell resistance in Snapwells, the cells were grown for 7 days, same as the cells grown on Transwells, and then they were used for the voltage clamp experiments. For the acute hyperglycaemia experiments, the cells were cultured under normal glucose conditions, with differing treatments applied during the voltage clamp. For the chronic hyperglycaemia experiment, the cells were grown under treatment conditions and then the respective treatments were added on the basal side.

The voltage clamp experiment briefly consisted of detaching the Snapwells and placing into an Ussing chamber. The chamber was sealed and Krebs Ringer buffer, prepared as described in section 2.5 and pre-warmed at 39^oC and at 5% CO_2, was added in both chambers. The chambers were also gassed with 95% O_2 /5% CO_2 and maintained at 37^oC. The voltage was measured using of electrodes enclosed within glass rods in both the apical and basal compartment. The voltage was then clamped and the current was measured. Treatments were then added and the voltage and current were measured again. Finally, at the end of the experiment, ATP was added as a positive control, which initiates a sharp increase in voltage and a sharp decrease in current (Figure 2.13).

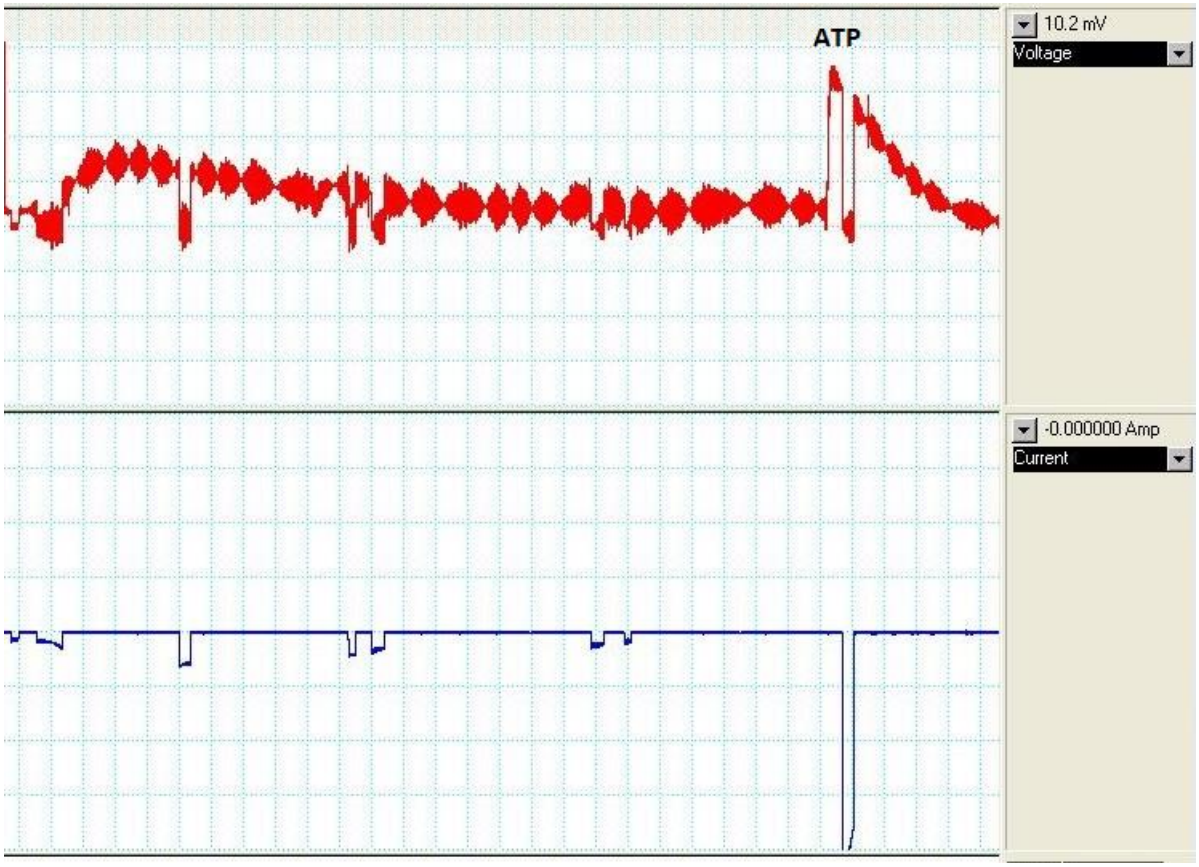


Figure 2.13: Example of ATP control positive reaction using the voltage clamp. The voltage is increased and the current is decreased.

2.13. *In vitro* embryo production

2.13.1. Stocks for oocyte maturation, fertilisation and embryo production

A series of stock solutions were necessary to enable the production of culture media for oocyte and embryo culture. Epidermal Growth Factor (EGF) stock was prepared by dissolution of the contents of the EGF stock vial in 20ml of maturation medium (see below for maturation medium components). Fibroblast Growth Factor (FGF) stock was made by addition of 0.4ml maturation medium to each of two vials. Once prepared, the EGF and FGF stocks were mixed in the ratio 4ml stock EGF : 185µl stock FGF and stored at –20^{o}C, for final concentrations of 0.4µg/ml EGF and 2.2ng/ml FGF. FSH: LH stock was made up by addition of 7.5ml M199 (1x) to 1 Menopur/Merional vial, to give a final concentration of 0.01 IU FSH, 0.01 IU LH. GlutaMAX was aliquoted into 1ml aliquoted and was stored at 5^{o}C. M199 (10x) was stored at 5^{o}C and gentamycin was added to it at

the opening of a new bottle. Mineral oil (M1840) was stored at 5°C. Finally, maturation additives were prepared as described in Table 2.13 and stored at -20°C.

Table 2.13.: Maturation additives preparation.

Chemical	Content
Apotransferin	0.025g
PVA	0.025g
Pyruvate	0.11g
B-mercaptoethanol	7µl
Embryo-tested water	24ml
Glutamax II	1ml

Stock B was prepared as per Table 2.14. The osmolarity was measured to be 430 and stock B was stored for 2 weeks at +5°C.

Table 2.14: Stock B

Chemical	MW	Concentration	g/**20ml**
$NaHCO_3$	84.01	230mM	0.42

Stock BSA fatty-acid free was prepared as per Table 2.15. It was stored for 6 weeks at +5°C.

Table 2.15.: Stock BSA-fatty-acid free.

Chemical	Concentration	Content/**10ml**
BSA fatty acid free	20% w/v	2g

Stock H was prepared as per Table 2.16. The osmolarity was measured to be 375 and stock H was stored for 6 weeks at +5°C.

Table 2.16.: Stock H

Chemical	MW	Concentration	Content/**50ml**
Hepes free acid	238.30	126mM	1.5000g
Hepes sodium salt	260.30	125mM	1.6250g

Heparin stock was prepared as per Table 2.17. The stock was stored for up to 3 months at -20°C.

Table 2.17.: Heparin stock

Chemical	Content/**20ml**
Heparin	0.2g

Stock K was prepared as per Table 2.18. The osmolarity was measured to be 105 and stock K was stored for 6 weeks at +5°C.

Table 2.18.: Stock K

Chemical	MW	Concentration	Content/**10ml**
Kanamycin Sulphate	382.60	130mM	0.5g

Stock C was prepared as per Table 2.19. The osmolarity was measured to be 060 and stock Cwas stored for 2 weeks at +5°C.

Table 2.19.: Stock C

Chemical	MW	Concentration	g/10ml
Sodium Pyruvate	110.04	327mM	0.036

Stock D was prepared as per Table 2.20. The osmolarity was measured to be 405 and stock D was stored for 6 weeks at +5°C.

Table 2.20.: Stock D

Chemical	MW	Concentration	g/10ml
$CaCl_2.2H_2O$	147.01	171mM	0.252

Stock L was prepared as per Table 2.21. It was stored for 6 weeks at +5°C.

Table 2.21.: Stock L

Chemical	Concentration	Components
Na Lactate Syrup	4.7% v/v	0.47ml
Sterile Water	-	9.53ml

Stock M was prepared as per Table 2.22. The osmolarity was measured to be 120 and stock M was stored for 6 weeks at +5^{o}C.

Table 2.22.: Stock M

Chemical	MW	Concentration	g/10ml
$MgCl_2.6H_2O$	203.30	49mM	0.1

Percoll additives were prepared as per Table 2.23, they were aliquoted and stored for up to three months at -20^{o}C.

Table 2.23.: Percoll additives

Chemical	g/20ml
$CaCl_2.2H_2O$	0.616
$MgCl_2.2H_2O$	0.162
NaCl	0.935
KCl	0.046
NaH_2PO_4	0.009
Gentamycin	1ml
Hepes Free Acid	0.2384
Hepes Na^+ Salt	0.2602
Na Lactate Syrup	0.74

SPTL solution was prepared as per Table 2.24, it was aliquoted and stored for up to three months at -20^{o}C.

Table 2.24.: SPTL solution

Component	Conc (mM)	Amount
H_2O	-	50.15ml
NaCl	100	0.292g
KCl	3.78	0.014g
NaH_2PO_4	0.38	0.003g
Gentamycin	0.6%	0.313ml
Hepes-Free acid	5.28	0.0745g
Hepes-Na Salt	6.24	0.0814g
Na-Lactate	0.42%	0.2313ml
$CaCl_2.2H_2O$	1.67	0.0123g
$MgCl_2.2H_2O$	0.32	0.0032g
Phenol Red	0.5%	0.25ml

TL solution was prepared as per Table 2.25 and it was stored for 6 weeks at 5^oC.

Table 2.25.: TL solution (10x)

Chemical	MW	Concentration	g/50ml
NaCl	58.44	1.141mM	3.333
KCl	74.55	32mM	0.119
$NaH_2PO_4.2H_2O$	119.98	5mM	0.031
Gentamycin	-	5% v/v	2.5ml

Stock G was prepared as per Table 2.26. The osmolarity was measured to be 060mOsm and stock G was stored for 6 weeks at +5^oC.

Table 2.26.: Stock G

Chemical	MW	Concentration	g/10ml
Glucose	180.16	60mM	0.108

Stock GLN was prepared as per Table 2.27. The osmolarity was measured to be 003 and stock GLN was stored for 2 weeks at +5°C.

Table 2.27.: Stock GLN

Chemical	MW	Concentration	Content/**10ml**
L-Glutamine	146.14	20mM	0.0146g

Stock S2 was prepared as per Table 2.28 and it was stored for 6 weeks at +5°C.

Table 2.28.: Stock S2

Chemical	MW	Concentration	g/50ml
NaCl	58.44	1.077M	3.147
KCl	74.55	143mM	0.534
KH_2PO_4	136.09	12mM	0.081

2.13.2. *In vitro* oocyte maturation

2.13.2.1. Solution preparation before tissue arrival

Ovaries were transported in warmed PBS supplemented with 1:2000 Antibiotic-Antimycotic solution. During ovary transit to the laboratory, oocyte holding media was prepared as outlined in Table 2.29.

Table 2.29.: Holding media.

Sterile water	80ml
199 medium 10x	10ml
Stock B	2ml
Stock H	6ml
Stock K	0.1ml
BSA Fr V	0.2ml

Bovine maturation media (BMM) was also prepared as per Table 2.30. The solution was pH-ed to 7.2.

Table 2.30.: Bovine maturation media (BMM)

Sterile water	8ml
199 medium 10x	1ml
Stock B	1ml
EGF:FGF	0.1ml
FSH:LH	0.05ml
Glutamax	0.1ml
Maturation additives	0.222ml
FCS (optional)	1ml

The osmolarity of these media was measured to be between 270 and 300 mOsm and they were filter-sterilised. Following that, 50ml of holding media were removed into a separate 50ml tube and 0.2ml of heparin were added. Both holding media tubes were pre-warmed at 37^{o}C for at least 2 hours.

Wash dishes and *in vitro* maturation dishes were prepared by adding 400µl BMM into each of the four wells of a dish. Water (3ml) with antibiotic/antimycotic (30µl) was added to the intermediate space between the wells in order to minimise evaporation. The dishes were incubated at 5% CO_2 at 39^{o}C for at least 2 hours before it was required to allow equilibration.

2.13.2.2. Tissue collection and *in vitro* maturation

Ovaries were harvested from 18- to 24-month old animals, and were transported to the lab approximately an hour after slaughter in phosphate buffer saline (PBS). The ovaries were rinsed first with water at 35^{o}C, and then twice in pre-warmed PBS+antibiotic/antimycotic, before being immersed into the same solution. Ovarian follicles between 2-8mm in diameter were punctured with a 18G needle fitted to a syringe and the contents aspirated into holding medium supplemented with heparin. The follicular aspirate was added to a 50ml falcon tube and allowed to sediment at 37^{o}C for a minimum of 20 minutes. The supernatant was removed and discarded and the sedimented oocytes were resuspended in holding medium without heparin. These were then placed onto a 12cm petri dish with a grid scored on the underside and oocytes were selected under a stereomicroscope fitted with a heated stage set at 37^{o}C. When the number of follicles allowed it, only oocytes with at least two layers of cumulus cells were selected. These were then washed twice more in holding medium and then washed

three times in maturation medium at which they allowed to mature for 24 hours (39°C, 5% CO_2).

2.13.3. Fertilisation media preparation

HEPES TALP and FERT TALP solutions were prepared as per Tables 2.31 and 2.32:

Table 2.31.: HEPES TALP solution

	For 20ml
Sterile water	15.0ml
10xTL	2.0ml
Stock B	0.16ml
Stock H	1.2ml
Stock C	0.16ml
Stock D	0.24ml
Stock L	0.6ml
Stock M	0.2ml
OSMOLARITY CHECK	
Stock BSA Fr V	0.4ml

Table 2.32.: FERT TALP solution

	For 20mls
Sterile water	14.4ml
10xTL	2.0ml
Stock B	2.0ml
Stock C	0.16ml
Stock D	0.24ml
Stock L	0.6ml
Stock M	0.2ml
OSMOLARITY CHECK	
Stock BSA FAF	0.4ml
Pencillamine/Hypotaurine	0.2ml
Heparin	20µl

The pH of FERT TALP was adjusted to 7.2 and the osmolarity of both solutions was measured to be between 270 and 290 mOsm, with care taken to ensure the osmolarity of the two differed by less than 10mOsm. Similar to BMM preparation, wash dishes and fertilisation dishes were prepared by placing 400µl of FERT TALP into each of the wells and also water in the space between them to prevent evaporation. These were placed into the incubator at 39°C, 5% CO_2 at least 2 hours before fertilisation.

2.13.4. *In vitro* fertilisation

On the day following oocyte isolation, sperm was prepared for *in vitro* fertilisation. The sperm was thawed from liquid nitrogen in warm distilled water before being layered on top of discontinuous Percoll gradient for the separation of motile from immotile sperm and the retention of motile spermatozoa. The Percoll gradient consisting of a 45% Percoll solution was layered on top of a 90% Percoll solution (Byeong-Seon Jeong, 2001). Percoll gradient was prepared as per Table 2.33.

Table 2.33.: Percoll gradient preparation

90% Percoll	**45% Percoll**
4.5ml Percoll	2.0ml 90% Percoll
600µl Percoll additives	2.0ml 1x SPTL

After centrifugation, the supernatant was removed and the pelleted sperm was resuspended in 4ml of HEPES TALP solution, followed by another centrifugation at 1200rpm, for 5 minutes. The pelleted sperm was resuspended in 200µl of FERT TALP and a sperm count was performed using an Improved Neubauer haemocytometer.

Whilst sperm was being prepared, matured oocytes were washed three times through FERT TALP medium before being placed into IVF droplets of the same medium. Sperm was added at a final concentration of $0.5x10^6$ sperm/ml and the gametes co-incubated 20-22h at 39^oC, 5% CO_2.

2.13.5. *In vitro* embryo culture solutions preparation

HEPES synthetic oviduct fluid (SOF) was prepared as per Table 2.34

Table 2.34.: HEPES SOF

	For 10ml
Sterile water	7.1ml
Stock S2	1.0ml
Stock B	0.2ml
Stock H	0.8 ml
Stock C	0.1ml
Stock D	0.1ml
Stock L	0.1ml
Stock M	0.1ml
Stock G	0.25ml
Pen/Strep	60µl
Stock BSA Fr V	0.2ml

Based on the glucose, lactate, pyruvate and amino acid concentrations detected in *iv*DOF (Chapter 4), the modified SOFaaBSA was prepared as per Table 2.35.

Table 2.35.: Synthetic oviduct fluid for group embryo culture. All units are in µl.

	7.3mM	**8.5mM**	**11mM+I**	**11mM-I**	**control**
Water (µl)	3779.53	4930.37	4281.32	2920.70	5590.00
Stock S2	1000.00	1000.00	1000.00	1000.00	1000.00
Stock B	1000.00	1000.00	1000.00	1000.00	1000.00
Stock C	169.02	90.06	90.37	146.16	100.00
Stock D	100.00	100.00	100.00	100.00	100.00
Stock L	137.41	143.53	160.04	150.83	100.00
Stock M	100.00	100.00	100.00	100.00	100.00
Stock G	601.65	167.89	604.24	1486.37	250.00
Stock GLN	1452.39	808.15	1004.03	1435.93	1000.00
Tyrosine stock (10x)	1000.00	1000.00	1000.00	1000.00	
Amino acids non ess	200.00	200.00	200.00	200.00	100.00
Amino acids ess					200.00
Pen Strep	60.00	60.00	60.00	60.00	60.00
Stock BSA	400.00	400.00	400.00	400.00	400.00

The pH of all media was adjusted to 7.2 and media were aliquoted and stored at -20^{o}C for a maximum period of 3 months. HEPES TALP was made up fresh and SOFaaBSA were defrosted on the day that the fertilised oocytes would be placed into culture.

Finally, the individual culture stock, used for metabolic analyses, was produced as follows (Table 2.36):

Table 2.36.: Synthetic oviduct fluid for individual embryo culture

	7.3mM	**8.5mM**	**11mM**	**11mM-I**	**control**
Water	to 100ml	to 100ml	to 100ml	to 100ml	to 100ml
NaCl (g)	0.629	0.629	0.629	0.629	0.629
KCl (g)	0.053	0.053	0.053	0.053	0.053
KH2PO4 (g)	0.016	0.016	0.016	0.016	0.016
NaHCO3 (g)	0.21	0.21	0.21	0.21	0.21
Glucose (g)	0.065	0.018	0.065	0.16	0.009
Glutamine (g)	0.021219	0.011807	0.014669	0.020979	0.0029
Pyruvate (ml)	6.141145	3.272044	3.283314	5.310658	0.0035g
CaCl2.2H2O (g)	0.025	0.025	0.025	0.025	0.025
MgCl12.H2O (g)	0.01	0.01	0.01	0.01	0.01
Pen/Strep (mL)	0.5995	0.5995	0.5995	0.5995	0.5995
amino acids (mL)	2	2	2	2	2
BSA (g)	0.799	0.799	0.799	0.799	0.799
Stock L (µl)	1374.089	1435.261	1600.424	1508.31	-
Tyrosine stock (10x) (mL)	10	10	10	10	-

For the four different treatments, a 9mM stock of pyruvate was made up. Stock L was also added, even though it was not present in the control group.

For both the group embryo culture and the individual embryo culture experiments, the solutions were placed in embryo tested petri-dishes and were incubated at 39^{o}C, 5% CO_2 for at least 2 hours before time for embryo culture.

2.13.6. *In vitro* embryo culture

After approximately 20-22h post fertilisation, presumptive embryos were selected and vortexed in HEPES SOF to remove cumulus cells and dead spermatozoa. The zygotes were then transferred into SOF medium and cultured in groups between 15 and 23 embryos for 6 days at 39^{o}C in 5% CO2/5%O2/90%N2. The rate of cleavage was assessed visually on day 2 of individual culture and the embryos were cultured. Blastocyst rates were also monitored between days 6 and 8 of group culture.

In order to determine the impact of the modified culture media on embryo metabolism at the cleavage stage, the embryos were vortexed and transferred immediately into individual culture in 4µl droplets of the individual culture medium for 20-25 hours. The time that the embryos were moved into and out of individual culture was monitored. Additionally, embryos on day 7 of group culture were transferred into individual culture, for 20-25 hours. The time that the embryos were moved into and out of individual culture was monitored. After embryo removal from the individual culture droplets, the dishes containing the droplets were sealed and stored immediately in -80^{o}C for further metabolic analyses (Sections 2.7,2.8).

2.14. Statistical analysis

Statistical tests performed for each experiment are analysed in the respective chapter's Materials and Methods section.

Chapter 3

The *in vitro* model of the oviduct

3.1. Introduction

3.1.1. Use of cattle as a model for studying the oviduct

The importance of studying the reproductive system in the cow arises mainly from its commercial roles in the production of milk and meat for consumption. The past 40 years have seen a dramatic improvement in dairy cow performance in terms of milk yield (Oltenacu and Broom, 2010). However, this has corresponded with a decline in the reproductive efficiency of the dairy cows, attributed mainly to high levels of stress, a drop in appetite and poor metabolic health (Leroy *et al.*, 2014).

Beyond the importance of herd fertility for commercial agriculture, the cow provides a useful model for studying reproductive processes in general (Harper *et al.*, 2012). Cows, similar to humans, ovulate a single oocyte which, following fertilisation, produces an embryo of comparable size between the two species. Embryonic Genome Activation occurs in similar developmental stages and the cow and human and a similar proportion of embryos progress to form blastocysts. Finally, their pattern of energy metabolism is also similar (Baumann *et al.*, 2007).

As a consequence, there are many studies that have exploited the use of bovine tissue in reproductive biology (Ellington *et al.*, 1990; Hugentobler, Morris, *et al.*, 2007; Hugentobler *et al.*, 2008; Palma-Vera, Einspanier and Schoen, 2014; Marey *et al.*, 2016). The bovine has similarities in genome structure (Elsik, Tellam and Worley, 2009) and embryonic development (Ménézo and Hérubel, 2002) to the human, and bovine tissue is relatively easy to obtain from local abattoirs, where meat goes to the food chain and the reproductive tracts would otherwise be disposed of. Additionally, co-culture of human sperm with bovine oviduct epithelium has been found to induce similar improvement in sperm function to the one described for the human oviduct epithelium (Ellington *et al.*, 1998).

3.1.2. Bovine Oviduct Epithelial Cells

The oviduct is a tubular structure (Leese, 1988), lined with epithelial cells (Winuthayanon *et al.*, 2015), which form a single cell monolayer (Clyman, 1966; Winuthayanon *et al.*, 2015). The oviduct epithelial cells can be either secretory or

ciliated (Joshi, 1995), the former being involved in the secretion of oviduct fluid (Leese, 1988), and the latter in gamete and embryo transport (Comer, Leese and Southgate, 1998). Epithelial cells are joined together by tight junctions giving rise to a confluent layer (Furuse *et al.*, 1993), the formation of which can be followed by measuring TransEpithelial Electrical Resistance (TEER) (Chen, Einspanier and Schoen, 2015).

3.1.3. Use of Transwells as a system mimicking the *in vivo* environment

Traditionally, cells tend to be cultured in flasks, as a cell culture flask is inexpensive, disposable, requires no cleaning or sterilization, and allows approximately 10^7 cells to be cultured in an adherent manner (Sigma-Aldrich, no date b). However, such an arrangement does not mimic the in vivo architecture of epithelial tissues such as the oviduct, which, as described in section 3.1.2, consists of a monolayer of cells connected to each other with tight junctions (Furuse *et al.*, 1993) forming a polarised layer (Walter, 1995). In the whole animal, the basal side is in contact with a layer of stroma and capillaries and the apical side is exposed to the oviduct lumen (Clyman, 1966). To mimic this arrangement in culture, the Transwell system is increasingly being used (Walter, 1995; Gualtieri *et al.*, 2013; Simintiras *et al.*, 2016), as has been previously established for a variety of cell types (Pascoe *et al.*, 1996; Watanabe *et al.*, 2013). In the case of the oviduct, Simintiras et al used a transwell arrangement to study hormone transportation from the basolateral to the apical compartment (Simintiras *et al.*, 2016), (Figure 3.1).

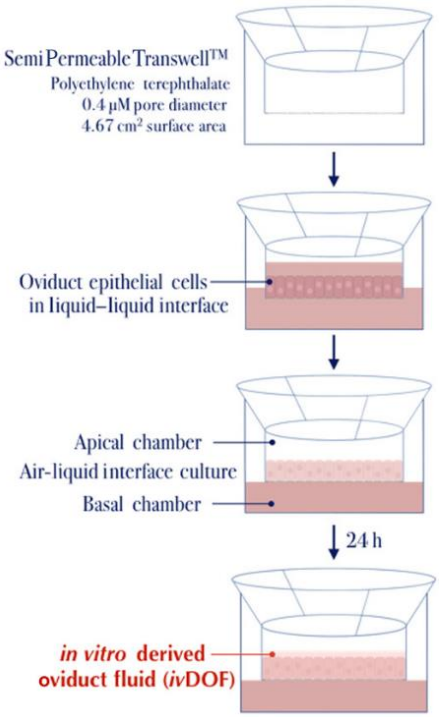


Figure 3.1.: *In vitro* model of the oviduct. (Simintiras *et al.*, 2016)

For the purposes of the present experiments, as described in Materials and methods (Section 2.1), the cells were physically removed from the bovine oviduct (Simintiras *et al.*, 2016). This method was preferred to enzymatic epithelial cell removal, which is a commonly used technique for primary cell isolation, but there is the risk of alterations in fragile structures, such as cilia, from the enzyme activity, due to their larger surface area (Ulbrich *et al.*, 2010).

Potential markers of epithelial cell function

In previous work (Simintiras *et al.*, 2016), Simintiras *et al* used, among others, the following markers to characterise their model of bovine oviduct epithelial cells *in vitro*:

(i) Amino acids: The importance of studying the impact of long-term culture in the amino acid composition of spent media derives from the important role of the latter in various processes associated with homeostasis and nutrition. This includes both their role as structural components of proteins, and their regulatory role in various processes such as stress response, metabolism, nucleic acid synthesis, osmoregulation and cell signalling (Wu, 2010).

(ii) Gene expression: Gene expression profiling is important for the determination of cell identity in a variety of cellular processes, including differentiation of embryonic cells in the preimplantation-stage embryo and epithelial-to-mesenchymal transition (Toyoda *et al.*, 2010). Bovine oviduct epithelial cells (BOECs) produce oviduct specific glycoproteins (OVGPs), which are suggested to be localized in secretory granules of the secretory cells (Oliphant and Ross, 1982). It has also been reported that BOECs express *OVGP1* (Simintiras *et al.*, 2016).

The *in vitro* model had been established in the laboratory (Simintiras *et al.*, 2016); therefore, some confirmatory experiments were performed in order to determine the successful isolation and culture of the cells as per the previous model. Additional experiments were also performed in order to further describe and explore the *in vitro* model of the oviduct.

3.2. Hypothesis and aims

The work described in this chapter will test the hypothesis that the cell culture vessels used for BOEC culture and the long-term culture of BOECs in an air: liquid interface, may influence the gene expression profile of the cells and the composition of the *in vitro* Derived Oviduct Fluid (*iv*DOF) secreted by the cells.

Consequently, the aims of this chapter were:

(a) To determine the impact of the vessel used to culture the cells on the composition of spent media and on BOEC gene expression

(b) To determine the impact of a long-term BOEC culture on oviduct epithelial cell secretion volumes and composition

3.3. Experimental design

All techniques described below were performed as detailed in Materials and Methods.

In order to replicate and further validate the *in vitro* model of the oviduct described by (Simintiras *et al.*, 2016), the cells were seeded and grown to confluence as described in section 2.1. The cells were visualised using confocal microscopy for the epithelial cell marker cytokeratin-18, and for haematoxylin and eosin staining. Transmission electron microscopy was used, as described in section 2.4 of Materials and Methods. The barrier properties of the epithelial cell monolayer were confirmed using TransEpithelial Electrical Resistance (TEER) measurements and fluorescein transport assay, which indicated that the epithelial monolayer enables the selective transport of molecules from the basal to the apical side of the cells. Since these experiments had previously been performed in the laboratory (Simintiras *et al.*, 2016), not all were performed in triplicate, as had been the case in the original study. In particular, the staining experiments, described in more detail in (Simintiras *et al.*, 2016) were only performed in one biological replicate. Also, for the purposes of all transport experiments performed in this thesis, the TransEpithelial Electrical resistance was measured at 48-hour intervals, to confirm formation of epithelial monolayer; however, in order to indicate the maintenance of a confluent monolayer, previously established in the laboratory (Simintiras and Sturmey, 2017), TEER measurements on a daily basis were only performed once.

3.3.1. Impact of culture vessel on cell secretions and molecular mechanisms

In order to determine the impact of the type of cell culture vessel on the gene expression patterns of the cells, BOECs were seeded and grown on Transwells and T_{25} flasks for 7 days. On day 7, the medium was replaced and the cells incubated for 24 hours under standard culture conditions (39^{o}C, 5% CO_2). The spent medium was then collected and maintained at -20^{o}C for short-term storage (up to 4 weeks). Long term storage was not performed. This experiment was performed in three biological replicates with two technical replicates in each. Glucose, lactate, pyruvate and amino acid concentrations of the spent media, as well as gassed media (39^{o}C, 5% CO_2) that had not been in contact with BOECs, were measured using ultrafluorometric assays and high-performance liquid

chromatography as described in sections 2.7, 2.8 of Materials and Methods. The cells were removed and gene expression experiments were performed as described on sections 2.9-2.11 of Materials and Methods. Gene expression experiments were performed for *OVGP1* and oestrogen receptor 1 (*ESR1*), as these were previously reported to be expressed in the bovine oviduct epithelium (Simintiras *et al.*, 2016). *SLC2A1* and *SLC2A3,* which code for glucose transporters, and *INSR,* which codes for insulin receptor, were also assessed, as they were going to be used in the experiments described in subsequent chapters of this thesis.

3.3.2. Impact of long-term culture of BOECs on *iv*DOF composition

In order to determine the impact of extended culture on the volume of the *in vitro* Derived Oviduct fluid secreted by BOECs, the amino acid composition of the apical fluid and the resistance of the monolayer, BOECs were isolated and grown to confluence on Snapwells, and they were left in air: liquid interphase for 43 days. The amino acid composition of the fluid was determined using high performance liquid chromatography and the resistance of the monolayer was evaluated using TransEpithelial Electrical Resistance measurements respectively, as described on sections 2.8 and 2.1 of Materials and Methods. For reasons of time limitations, this experiment was only performed in one biological replicate, of between 3 and 10 technical replicates, according to the number of available Snapwells with an intact membrane; therefore, it was considered to be an observational experiment.

3.3.3. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6. All data were analysed using the Shapiro-Wilk test in order to determine whether a parametric or non-parametric analysis as required. When the data was normally distributed, One-Way ANOVA was used for analysis, with Tukey's *post-hoc*, and the mean ± standard deviation plotted. Wherever the data were not normally distributed, or the n-number was too small to assess normality, a Kruskal-Wallis test was performed with Dunn's *post-hoc*, and the median ± interquartile range plotted. For the statistical analysis of the amino acid data, Two-Way ANOVA with Tukey's *post-hoc* was used and the mean ± standard deviation plotted. For the gene expression analysis, all datasets were too small to

determine whether the data were normally distributed and therefore a Mann-Whitney test was performed between gene expression patterns in flasks and Transwells.

3.4. Results

3.4.1. Successful isolation of bovine oviduct epithelial cell population

The identity of harvested cells was confirmed using confocal microscopy using the epithelial cell marker cytokeratin-18. (Figure 3.2).

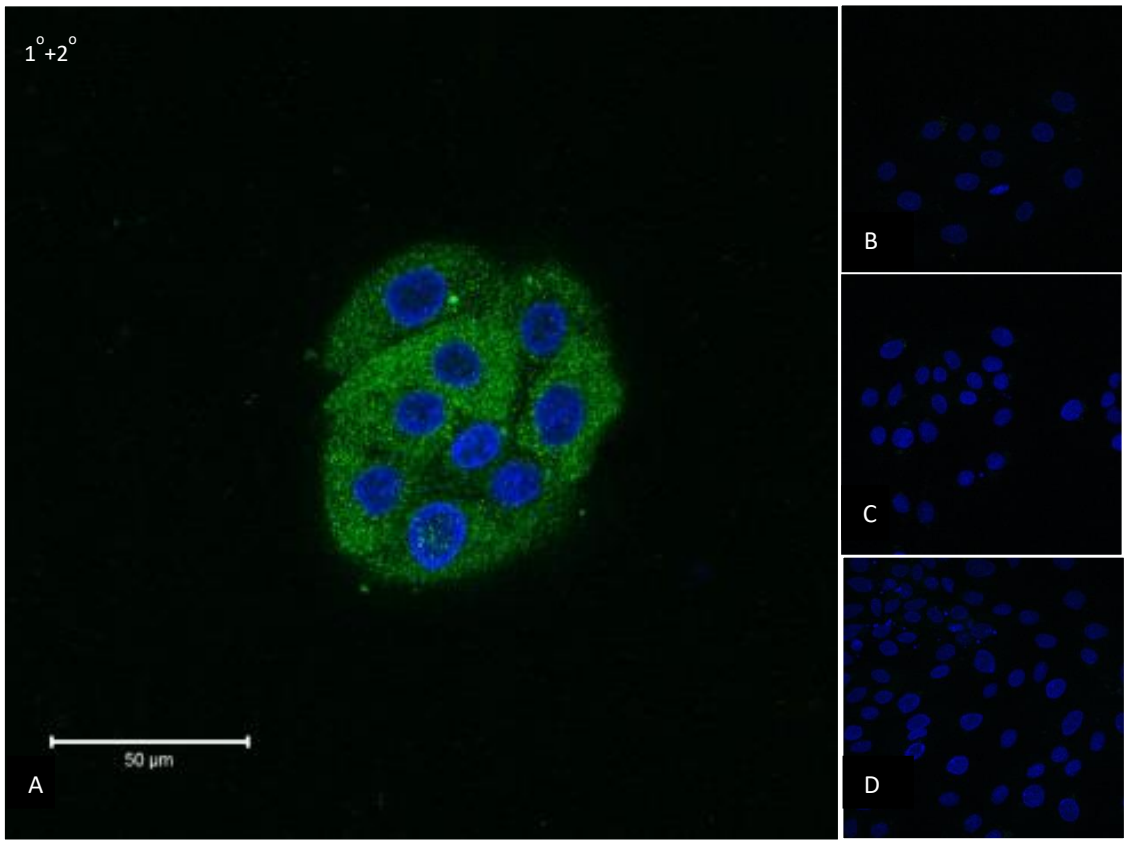


Figure 3.2.: Epithelial cell staining with cytokeratin-18. Bovine Oviduct Epithelial Cells are positively stained for cytokeratin (3.1.a), whereas controls only show nuclear (DAPI) stain (3.1.b: primary antibody control, 3.1.c: secondary antibody control, 3.1.d: DAPI stain only). Representative of n=3, scaling bar represents 50µM.

The epithelial cells were also visualised under an inverted microscope (data not shown), and they were also visualised in a bright-field microscope when they were also stained with haematoxylin and eosin. Positive haematoxylin and eosin staining was indicated by a purple colour-stained nucleus and a pink-colour stained cytoplasm, which confirmed the epithelial identity of the cells, as they were observed to be similar to previously published images of the epithelium (Simintiras *et al.*, 2016) (Figure 3.3).

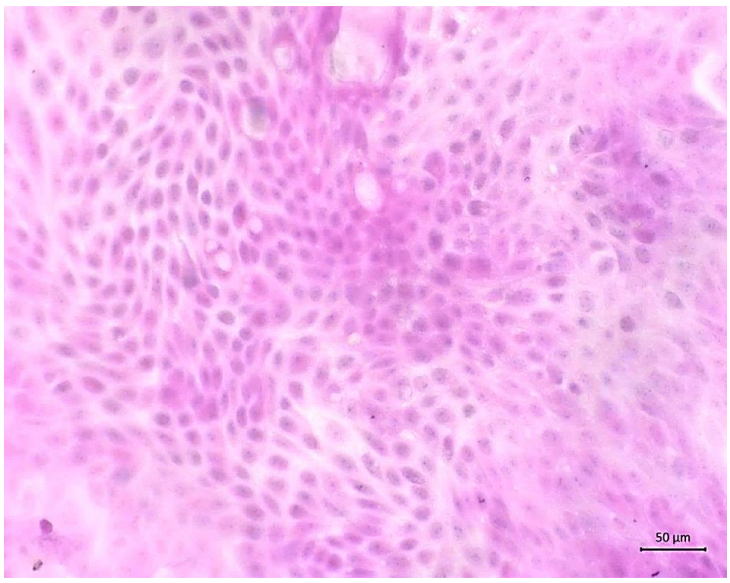


Figure 3.3.: Haematoxylin and eosin staining of BOECs. Scaling bar represents 50µM.

3.4.2. Successful formation of a single cell, semi-permeable BOEC monolayer

Transmission Electron Microscopy of BOECs grown on a Transwell membrane confirmed that the cells grew as a single-cell monolayer, attaching to the membrane. Membrane protrusions were observed on the apical side of the cells (Figure 3.4).

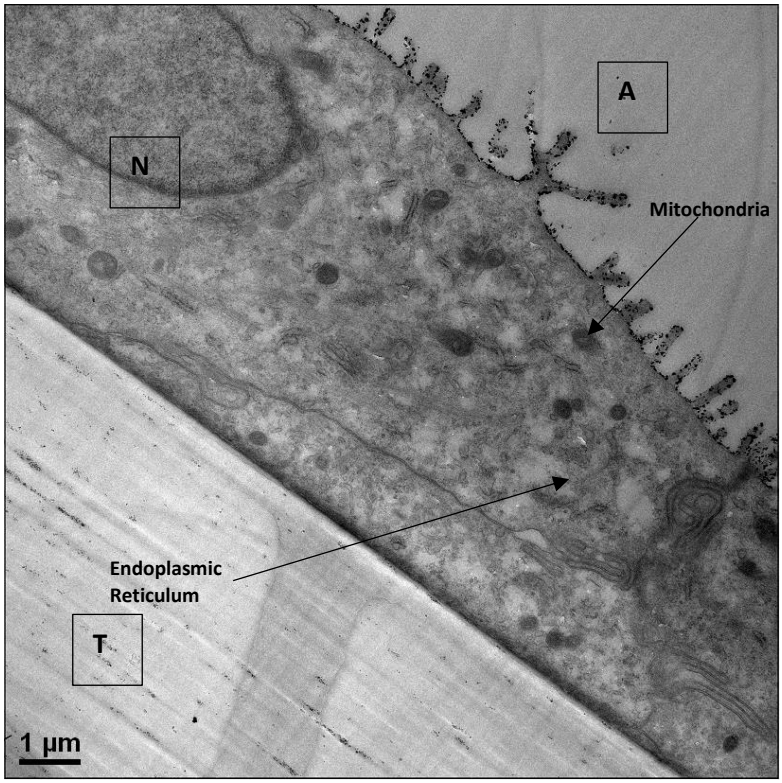


Figure 3.4.: Cross-section of a Bovine Oviduct Epithelial Cell grow on a transwell membrane (indicated by T), with the apical compartment indicated (A). The cell nucleus (N) is visible, as well as membrane protrusions at the apical surface. Mitochondria and endoplasmic reticulum are also visible. Scale: 1µm

After confirmation of the cells' epithelial nature after isolation, the formation of a confluent monolayer was monitored with TransEpithelial Electrical Resistance (TEER) measurements over time. The data indicate that TEER increased slowly over days 1-3 of culture, then exponentially between days 3 and 6 after which it plateaued with no further increase (Figure 3.5).

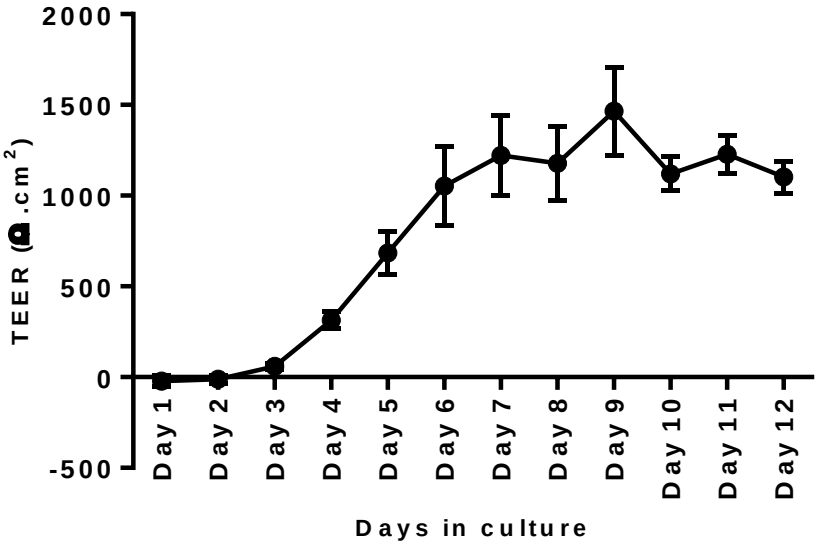


Figure 3.5.: TransEpithelial Electrical Resistance with time of culture. Data are presented as mean from 10 technical replicates.

Finally, after establishing the formation of a monolayer, its selective transport properties were examined using the inert molecule, fluorescein. Figure 3.6 shows that fluorescein flux across the monolayer was minimal indicating that the monolayer was intact. During this period, the TEER was stable. However, after addition of EDTA at 180min, to disrupt the tight junctions, a flux of fluorescein from the basal to the apical compartment was observed, reflected as a reduction of fluorescence in the basal compartment and an increase of fluorescence in the apical compartment. EDTA addition also resulted in a sharp reduction of the Trans Epithelial Electrical Resistance, suggesting the disruption of the monolayer. Taken together, these findings confirm the presence of a confluent, transporting epithelium of BOECs.

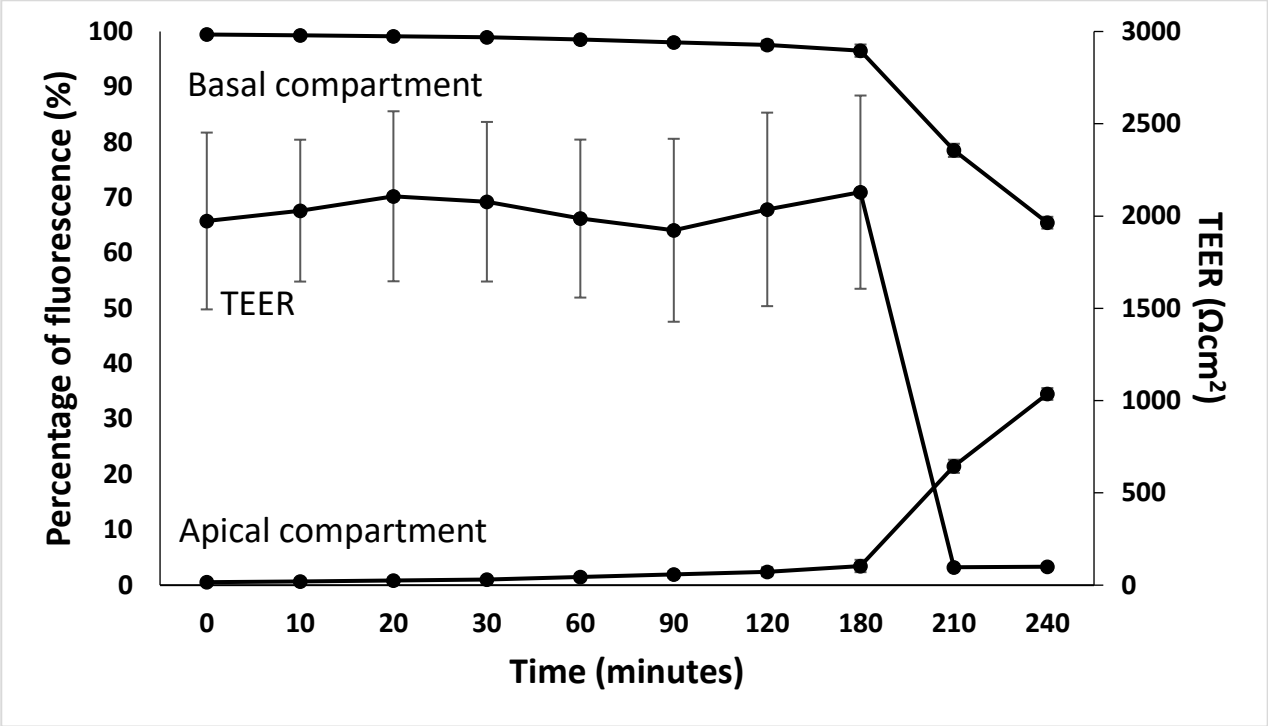


Figure 3.6.: Fluorescein flux from the apical to the basal compartment of BOECs over time. EDTA was added on time point 180 (180 minutes after the beginning of incubation). n=3 biological replicates

3.4.3. Long-term BOEC culture modified the amino acid composition of oviduct fluid

An observational experiment was performed, seeking to investigate the impact of long-term culture of BOECs in an air: liquid interphase on the secretion volumes, the TEER and the amino acid content of the fluid secreted by the cells. Figure 3.7 shows that the volume of *iv*DOF secreted by the cells during this extended-culture experiment was not significantly modified throughout the experiment. TEER over time for this experiment is shown in Figure 3.12_Appendix.

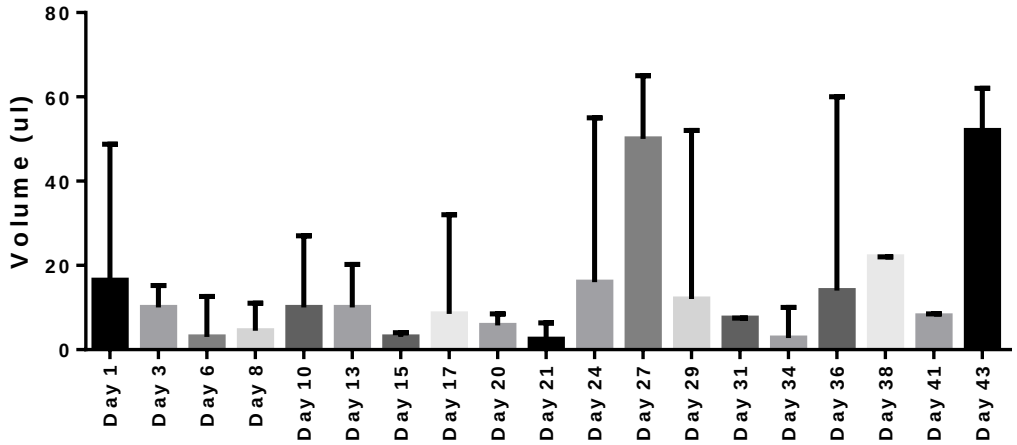
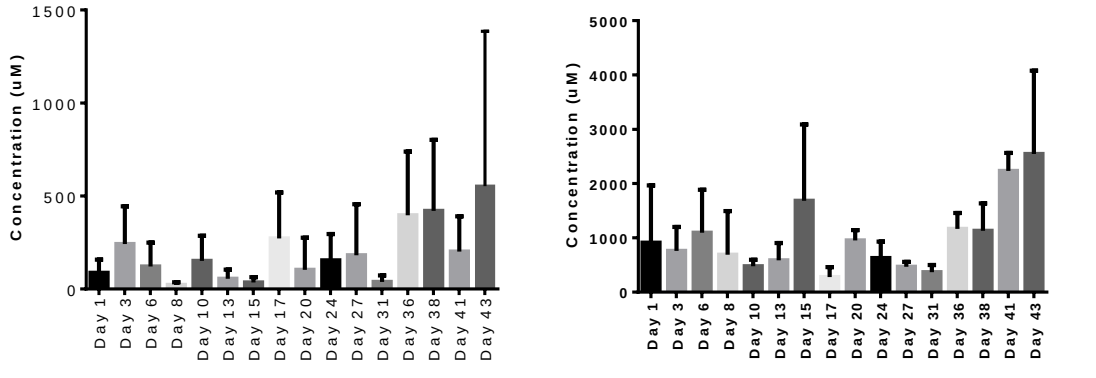


Figure 3.7.: The impact of long-term *in vitro* culture on the secretion volumes of *iv*DOF. n=3-10 technical replicates, depending on Snapwell availability

The amino acid composition of *iv*DOF over the duration of the observation are given in figure 3.8. These data revealed that the concentrations of nine amino acids in ivDOF; glutamine, glycine, threonine, arginine, alanine, valine, isoleucine, leucine and lysine were significantly altered over time. In particular, in all cases there was a trend for the amino acid concentration to increase towards the end of the incubation period (appendix). Only the amino acids whose concentrations were found to be significantly modified throughout the cultured period are presented.



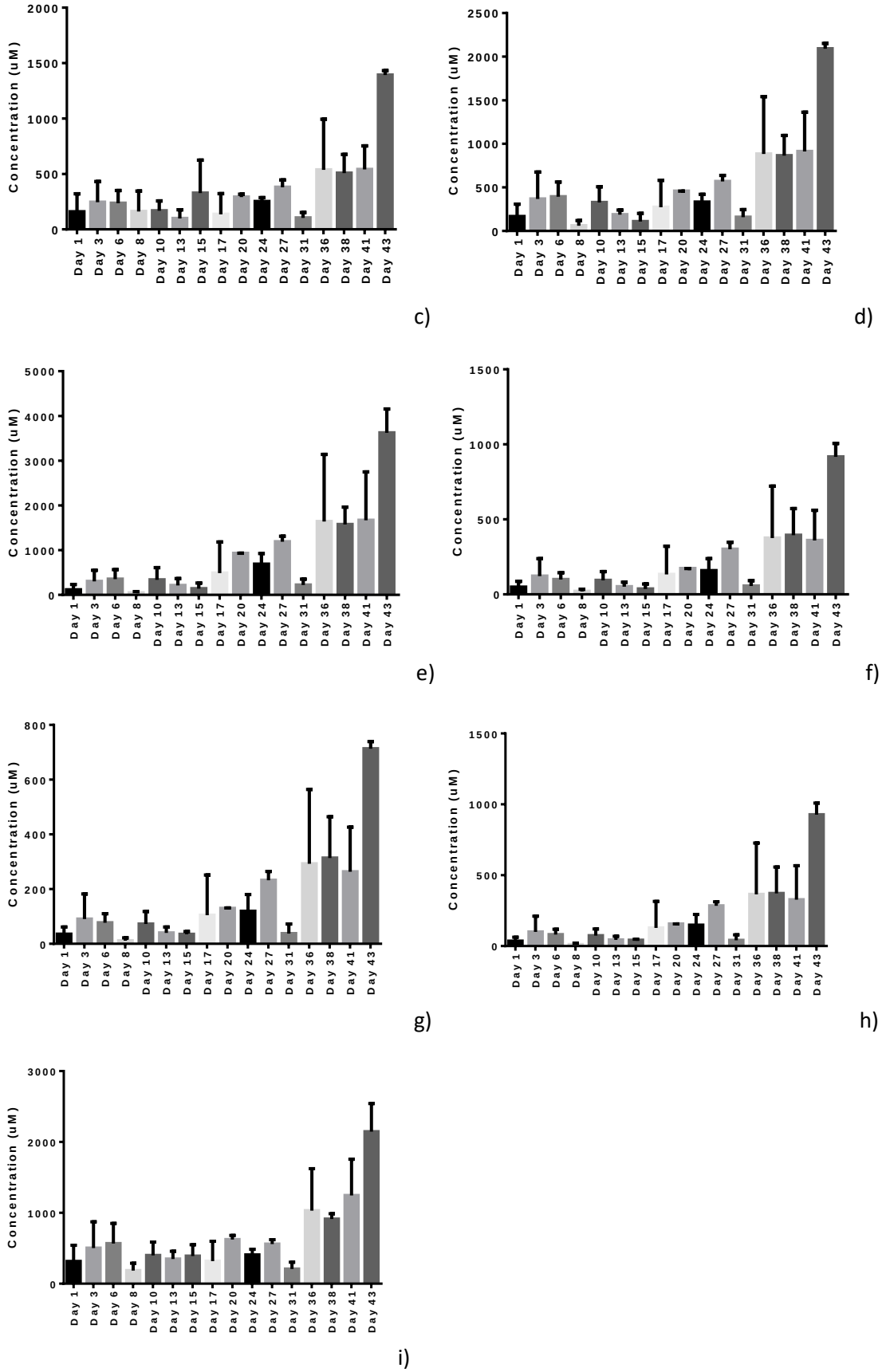


Figure 3.8.: Bar charts representing the amino acid profile of ivDOF collected every 48 hours during long term BOEC culture, for the amino acids where the amino acid concentrations were modified in a statistically significant way.

n=3-10 technical replicates depending on *iv*DOF availability ±SD. a) glutamine, b) glycine, c) threonine, d) arginine, e) alanine, f) valine, g) isoleucine h) leucine, i) lysine

The appendix contains tables with the amino acids that were significantly increased towards the end of the culture period (Table 3.1_Appendix, Figure 3.13_Appendix).

3.4.4. Different culture systems for BOECs modify the amino acid composition of spent media but do not alter energy substrate concentrations or gene expression

Next, the impact of different culture systems was assessed. BOECs were grown on T25 flasks and on Transwells and the glucose, lactate and pyruvate concentrations, the amino acid concentrations of spent media and the gene expression levels in the cells were measured at day 7 of culture.

Figure 3.9a shows that the concentration of glucose was not significantly altered by the culture vessel used, or compared to gassed medium. Similar to glucose, pyruvate concentration (Figure 3.9c) was not significantly modified in any of the culture vessels. Lactate concentration measured in spent media collected from the basal compartment of the Transwell was significantly higher than that in medium that had not been in contact with any culture vessel (Figure 3.9b). There was also a trend for a higher concentration of lactate in the apical compartment of the transwell and the T25 flask; however, these values did not reach significance. The above results show that the concentrations of glucose, lactate and pyruvate were essentially unchanged under the different culture systems used and that with the exception of lactate concentration, there were no other differences in the concentrations detected when the cells were grown in T25 flasks or Transwells (Figure 3.9).

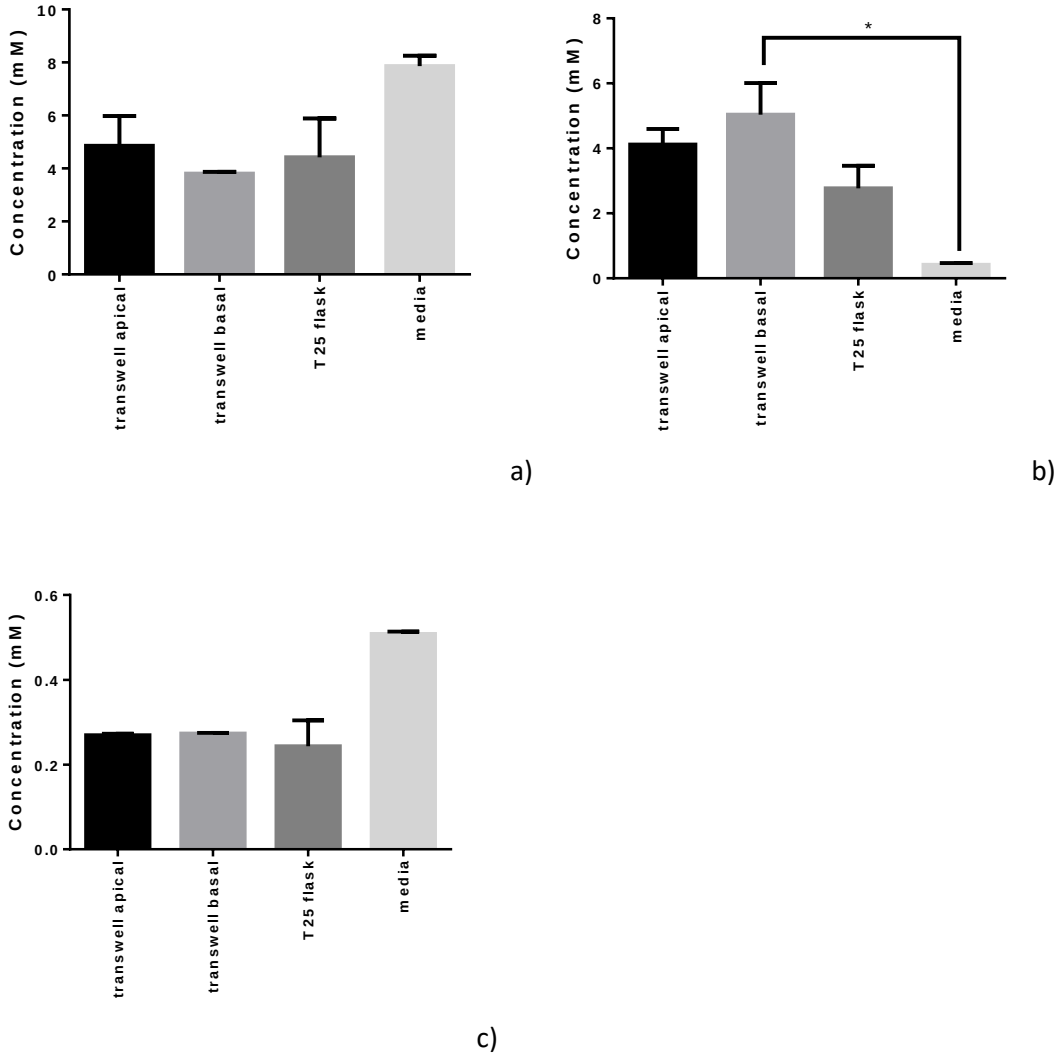
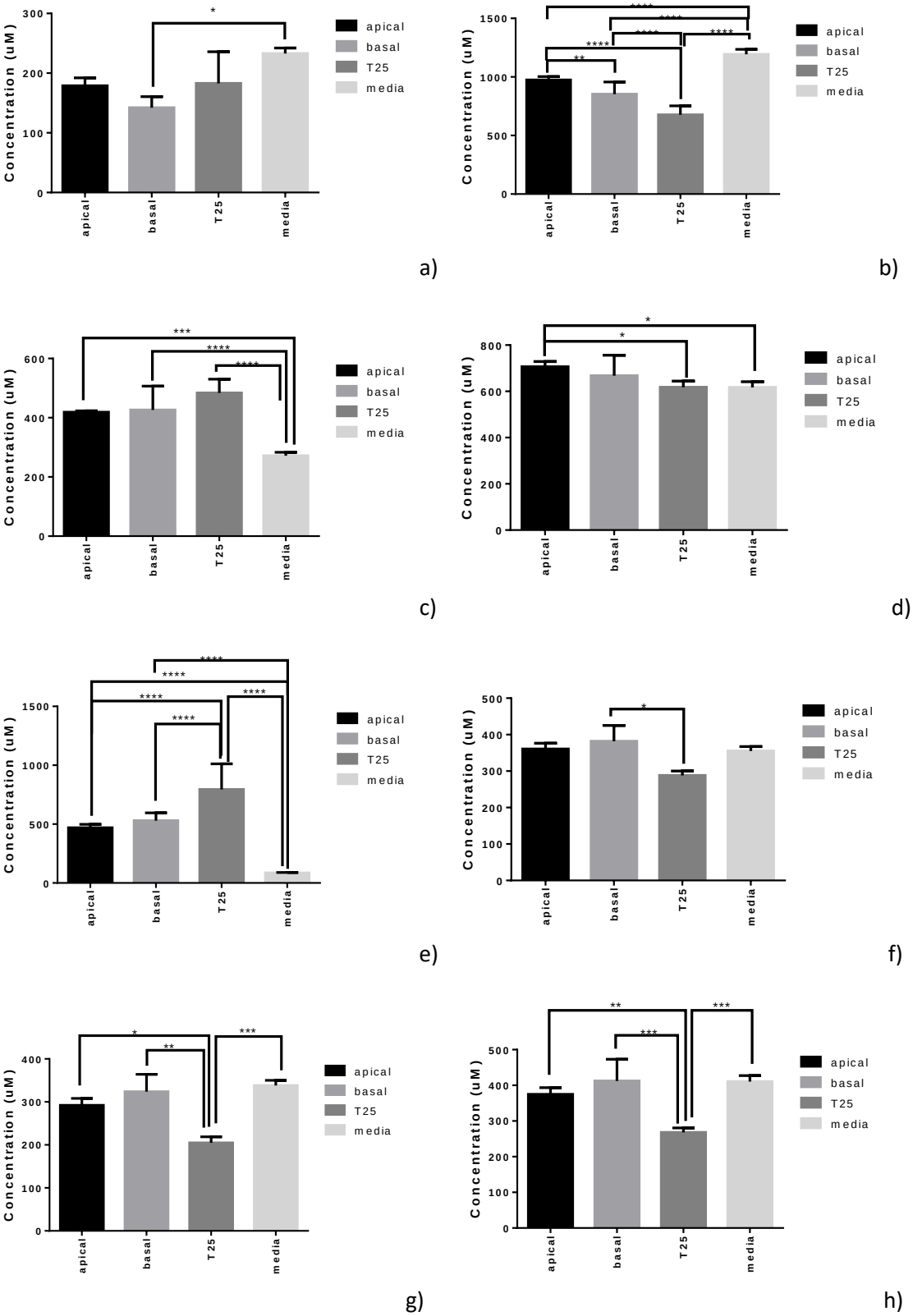
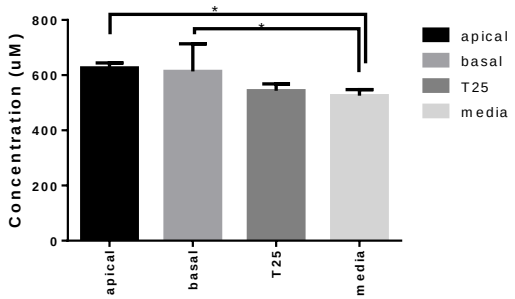


Figure 3.9.: Concentrations of a) glucose, b) lactate, c) pyruvate detected in media from cells under different culture conditions. n-=3 biological replicates

The amino acid composition of spent medium from cells grown in flasks compared to those in Transwells showed a number of interesting differences. For example, there was significantly less serine in the basal compartment medium of cells grown in Transwells compared to the concentration in gassed medium (Figure 3.10 a). Glutamine, isoleucine and leucine (Figure 3.10 b, g and h respectively) concentrations were significantly lower in the spent media from the T25 flask compared to that from the apical and basal compartments of the transwell ($p<0.001$ and $p<0.01$ respectively for glutamine, $p<0.05$ and $p<0.01$ for isoleucine, $p<0.01$ and $p<0.001$ for leucine). Glycine concentrations were significantly lower in gassed media compared to spent media in all vessels ($p<0.001$ for spent media from the apical compartment and $p<0.0001$ for spent media from the basal compartment and from the T25 flask). Additionally, marginally significant differences

were observed for arginine, valine and lysine concentrations (Figure 3.11 d, f and i) between the different culture vessels.





i)

Figure 3.10.: Amino acid concentrations detected in media collected from cells grown under different culture conditions. a) serine, b) glutamine, c) glycine, d) arginine, e) alanine, f) valine, g) isoleucine, h) leucine, i) lysine, n=3 biological replicates

Finally, analysis of the gene expression patterns of key genes associated with glucose transport and BOEC function, has shown that mode of culture does not significantly impact the gene expression profile of key genes expressed by BOECs ($p>0.05$) (Figure 3.11). However, there was a trend towards significant differences, in that, with the exception of *INSR*, the gene expression levels were higher in cells grown on Transwells compared to the gene expression patterns of cells grown on T25 flasks; conversely, *INSR* expression was significantly higher in cells grown on flasks compared to cells grown on Transwells.

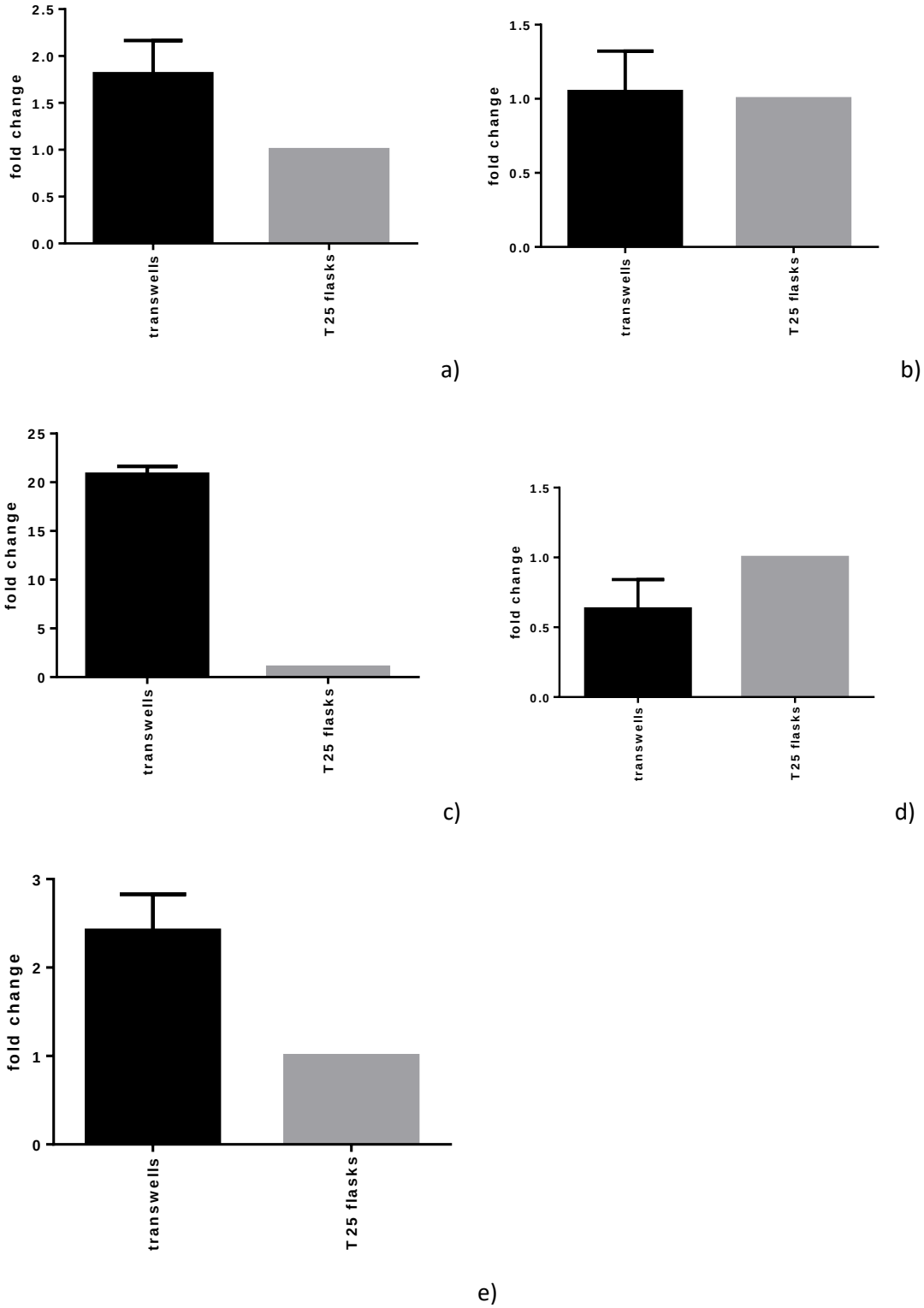


Figure 3.11.: Gene expression levels of key genes a) *SLC2A1*, b) *ESR1*, c) *SLC2A3*, d) *INSR* and e) *OVGP1,* n=3 biological replicates

3.5. Discussion

The data presented in this chapter have validated and expanded the *in vitro* model of the oviduct, which had previously been shown to mimic closely the *in vivo* environment of the oviduct (Simintiras *et al.*, 2016). A Bovine Oviduct Epithelial Cell population may now be readily isolated from bovine reproductive tracts, and used to grow a confluent epithelial cell monolayer, that enables the study of selective transport of molecules from the basal to the apical compartment. Repetition of some of the experiments previously performed, has confirmed the method to be robust and reproducible.

In addition to the successful replication of previously performed experiments, Transmission Electron Microscopy was used to has confirm visually the formation of a single cell monolayer. It was not possible to confirm that the cells in this preparation adopted a columnar phenotype, possibly as a result of the cell positioning on the Transwell membrane at the time of fixation. However, the TEM data were in agreement with previous reports regarding ciliation, since cilia had been reported to disappear from BOEC surface under the *in vitro* conditions as epithelial cells become de-differentiated (Walter, 1995; Reischl *et al.*, 1999; Ulbrich *et al.*, 2010). This was reported to be restored in the porcine, after prolonged culture in air-liquid interface (Miessen *et al.*, 2011), and in humans, following the addition of oestradiol to the basal medium (Comer, Leese and Southgate, 1998). Whether maintenance of an air-liquid interface also restores ciliation in the bovine, without the addition of oestradiol, remains to be confirmed, although Simintiras *et al* hinted at the restoration of a ciliated phenotype (Simintiras *et al.*, 2016).

An interesting finding highlighted in this chapter was that long-term culture of BOECs in an air:liquid interface did not change the volume and the TransEpithelial Electrical Resistance of the monolayer, suggesting there was no impact of the air:liquid interface model on cell survival or on the ability of the cells to maintain the monolayer integrity and produce *iv*DOF. However, this prolonged exposure to the air-liquid interface ultimately resulted in a modified *iv*DOF amino acid profile. In particular, culture over a 43-day long period, in an air-liquid interface, led to a significant increase in 9 out of 17 amino acids measured in the oviduct cell-derived apical fluid. Previous studies in the porcine discussed the importance of a 3-week culture period in an air-liquid interface,

as a minimum for the establishment of the characteristics of oviduct epithelial cells, as well as the ability of this cell type to be cultured in an air:liquid interface for up to 6 weeks (Miessen *et al.*, 2011). The work presented here, however, suggests that, even though this might be the case, long-term culture leads to significant alterations in the amino acid content of the fluid produced by the cells at the apical compartment. Indeed, when compared to the amino acid concentrations detected in bovine oviduct fluid collected with the use of catheter, which represents the *in vivo* Derived Oviduct Fluid (Hugentobler, Morris, *et al.*, 2007), the amino acid concentrations detected during the first days of culture were comparable to the concentrations detected *in vivo*. However, after approximately 30 days of long-term culture of the cells in an air: liquid interface, the concentrations of some key amino acids increased significantly and reached levels that higher by 2-fold (for glutamine and glycine), 5-fold (for valine and leucine) 6-fold (for alanine), 10-fold (for threonine, isoleucine and lysine) and 20-fold (for arginine) compared to the levels reported *in vivo*. Although substrate differences might be predicted between the *in vitro* and the *in vivo* conditions (Simintiras *et al.*, 2016), the amino acid profile over the long-term culture was significantly modified, since more than half of the amino acids examined were significantly higher than the conditions reported *in vivo* (Hugentobler *et al*., 2007), which suggests a potential discrepancy between the *in vivo* and *in vitro* extended culture conditions, which in turn needs to be taken into account when this type of experiments are performed.

The impact of extended culture of oviduct epithelial cells has been reported previously in the porcine and was found to impact on gene expression, and in particular with genes related to amino acid degradation (Jankowski *et al.*, 2018). The same study reported that long-term culture of pig oviduct epithelial cells led to decreased levels of gene expression related to the cells' pluripotency (Jankowski *et al.*, 2018). The above suggest that, although the *in vitro* model of the oviduct can mimic the *in vivo* environment, it cannot sustain this for long periods of time, in agreement with the findings presented in this chapter, which suggest a period of 36 days as a maximum time of maintenance of the air-liquid interface. This air-liquid interface, *in vitro* model of the bovine oviduct therefore provides a suitable model to investigate the function of the bovine oviduct as well as to investigate health and disease, as long as it is used within the first days of the air-liquid interface.

Finally, the impact of different culture systems, namely Transwells and culture flasks, in which to grow BOECs was investigated. The results show that BOECs expressed *ESR1* and *OVGP1* in both culture conditions, and consequently still retained their bovine oviduct epithelial cell characteristics; therefore, both culture conditions can be used for experiments related to BOEC work. Additionally, there was no impact of the culture conditions on the concentrations of glucose, lactate and pyruvate, which were found to be similar in spent media collected from either the apical or the basal compartment of a Transwell and in spent media collected from a T25 flask. Interestingly however, the culture conditions affected the amino acid composition of the above collected media, with nine amino acids being modified. Specifically, the concentrations of glutamine, isoleucine and leucine were significantly lower and alanine was significantly higher in media collected from T25 flasks compared to Transwells and gassed media. Even though the gene expression data suggest that there are differences at the transcriptomic level associated with the two different culture conditions, these differences were not found to be statistically significant.

The differences observed under the two culture systems could potentially be attributed to the difference in the materials of which the two types of vessels are made; Transwell inserts are made of polyester (Sigma), whereas T25 flasks are made of polystyrene (Sarstedt, no date). In addition, the cell distribution is different in the two vessels; as mentioned previously, epithelial cells growing in Transwells are exposed to media at both their apical and their basal compartments, thus resembling the *in vivo* conditions (Furuse *et al.*, 1993), whereas cells grown in T25 flasks are exposed to media only through their apical compartment, since they adhere to the flask surface (Sarstedt). Finally, cell growth in flasks does not necessarily follow the single cell monolayer formation pattern, which has been observed with the use of Transwells (Simintiras *et al.*, 2016), as visual assessment reveals the formation of epithelial cell clusters in the flasks, which are not uniformly distributed (Simintiras 2016). The results described in this chapter, together with the above described differences in cell culture in the two different vessels, indicate that the same culture conditions should be applied for each experiment, so that any comparisons performed between different treatments represent biological significance rather than the method of culture. Moreover, the fact

that some genes were differentially expressed between the two culture systems, but this difference did not reach significance, especially for the case of *SLC2A3,* suggests that a repetition of this experiment with higher n-numbers could further clarify the gene expression patterns of BOECs.

Overall, the results of this chapter suggest that the *in vitro* model of the oviduct is a valid representation of the oviduct *in vivo*. There are, of course, limitations to the use of this model, and in particular to the cell culture time during which this remains valid. However, this remains a robust model for the isolation and culture of Bovine Oviduct Epithelial Cells and it could be very useful in the characterisation of various pathophysiological conditions, such as the investigation of the impact of hyperglycaemia in the composition of the BOEC secretions and the molecular mechanisms underlying BOEC function, described in Chapter 4 of this thesis.

Chapter 4

The hyperglycaemic oviduct

4.1. Introduction

Obesity is a condition known to be associated with the development of metabolic syndrome, characterised by impaired glucose tolerance or impaired fasting glycaemia, as well as insulin resistance, dyslipidaemia, hypertension and central obesity (Eckel, Grundy, & Zimmet, 2005). According to the NHS, normal blood sugar levels for humans should be 4-7mM before a meal and below 8.5-9mM two hours after a meal (NHS, no date). Hugentobler et al. has reported that normal glucose concentrations in bovine plasma vary between 5.82mM and 7.70 mM, depending on the day of measurement (Hugentobler *et al.*, 2008).

Maternal provision of nutrients to the developing embryo is dependent on the nutritional status of the mother, as well as maternal metabolism, body size and body composition. These, in turn, are in part established during the mother's development from foetal life to adolescence (Martin-Gronert and Ozanne, 2006). It has been hypothesized that the existence of a mismatch between energy provision during foetal life, and during adolescence and adult life, can potentially result in the development of metabolic perturbations later in life (Martin-Gronert and Ozanne, 2006; Feuer and Rinaudo, 2016). Depending on the type, the extent, and the timing of the environmental challenge, prenatal development can be severely impaired (Feuer and Rinaudo, 2016). The changes that it may undergo could affect epigenetic modifications, which are processes associated with DNA accessibility and chromatin structure (Handy, Castro and Loscalzo, 2011) occurring during early development (reviewed by (Martin-Gronert and Ozanne, 2006)), or lead to metabolic perturbations at the cellular level, and potentially tissue and organ level later in development (Feuer and Rinaudo, 2016). While the impact of the metabolic syndrome on the later stages of foetal and placental development have been relatively well established, the impact of metabolic perturbations at the preimplantation stages of development, when the embryo is in the oviduct, have not been thoroughly investigated.

As discussed in Chapter 1, the oviduct provides the environment for gamete maturation, fertilisation and early embryo development (Li and Winuthayanon, 2017). Importantly, the maternal transcriptome, which is essential for the oocyte and early embryo survival

and functions, is gradually degraded and replaced by the embryonic transcriptome, beginning with a process termed Embryonic Genome Activation. Consequently, any modifications to the environment of in which all the above processes occur, may result in severe perturbations in the embryo. Additionally, and considering the Developmental Origins of Health and Disease Hypothesis, discussed in the Introduction (Chapter 1), any diversion from the normal levels of nutrition provided to the embryo, whether it leads to decreased (Barker, 2004; Martin-Gronert and Ozanne, 2006; Rando and Simmons, 2015) or increased nutrient supply, can result in detrimental foetal growth (Martin-Gronert and Ozanne, 2006), and potentially the development of metabolic conditions later in life.

The relationship between body mass index and fertility is J-shaped (Lake, Power and Cole, 1997), Abnormally high or low maternal weight is associated with reduced fertility (Grodstein, Goldman and Cramer, 1994), and obese women may need longer to conceive compared to women of normal weight (Jensen *et al.*, 1999; Van Der Steeg *et al.*, 2008). In mouse models of maternal obesity, obese animals were subfertile, as a result of the animals exhibiting prolonged or absent oestrous cycles and lacking follicular development in the ovary (Bermejo-Alvarez, Rosenfeld and Roberts, 2012). However, little is known about the implications of obesity on preimplantation embryo development.

As mentioned in Chapter 1, the oviduct lumen has an environment that is metabolically distinct to the circulation, due to a combination of the transport of substrates from the circulation and bovine oviduct epithelial cell secretions (Leese, 1988). To date, the oviduct response to hyperglycaemia has not been investigated, however, a number of other studies have provided insight on the oviduct's selective permeability properties. Simintiras et al (2016) reported that the oviduct epithelium is responsive to hormones, in particular to oestradiol and progesterone, which, when basally administered in pathophysiological levels, induce differences in the cells' gene expression, as well as the amino acid composition of the fluid secreted by the cells (*iv*DOF) (Simintiras *et al.*, 2016). A further recent study by Jordaens et al. (2017) has shown that the oviduct epithelium does not allow basally administered non-esterified fatty acids (NEFA) to cross the cells and reach the apical compartment, where the embryo resides for the first 5 days of

development would be; conversely, the monolayer can remove excess NEFA from the apical compartment (Jordaens, Van Hoeck, Maillo, *et al.*, 2017). These data indicate that the oviduct epithelium can regulate the environment for the gametes and the early embryo. Another study by the same group has shown that non-esterified fatty acids (NEFAs) are present in the oviduct, at concentrations reflecting plasma NEFA concentrations, and they are positively correlated with the levels of cholesterol and lactate, and negatively correlated with triglyceride levels in the oviduct fluid (Jordaens, Van Hoeck, De Bie, *et al.*, 2017). These results suggest a role for the oviduct epithelium in buffering the effects of an adverse environment on the preimplantation embryo and in preventing potentially embryotoxic compounds from entering the oviduct lumen.

In order to investigate the impact of a hyperglycaemic challenge on the secretions of the oviduct, the *in vitro* model of the oviduct, described in Materials and Methods, was used (section 2.6). The value of this model is that it mimics the *in vivo* environment in maintaining the oviduct cells as a confluent polarised monolayer enabling compounds of interest to be provided by the basal route with oviduct fluid available in the apical compartment of analysis.

4.2. Hypothesis and aims

The research in this chapter sets out to test the hypothesis that hyperglycaemia impacts on secretions by Bovine Oviduct Epithelial Cells (BOECs) and creates an altered environment for the gametes and early embryo.

Consequently, the aims of this chapter were:

a) To describe the impact of acute and chronic hyperglycaemia, in the presence or absence of insulin on the luminal secretions of the oviduct

b) To explore the impact of acute and chronic hyperglycaemia on the molecular mechanisms of oviduct epithelial cells

4.3. Experimental design

4.3.1. Materials and Methods

Bovine Oviduct Epithelial Cells were harvested from bovine reproductive tracts and cultured in media containing DMEM-F12 as described in Materials and Methods (section 2.1). Epithelial monolayer confluence was determined by TEER measurements as described in section 2.1 of Materials and Methods. After the cells had reached confluence, they were cultured in an air-liquid interphase, under treatments described in section 4.3.2 and 24 hours later, *in vitro* Derived Oviduct Fluid (*iv*DOF) was collected from the apical compartment and stored for further metabolic analyses.

4.3.2. Treatments

In order to investigate the impact of hyperglycaemia with or without the presence of insulin on the composition of *iv*DOF secretions, the following treatments were performed:

1. 7.3mM glucose +20ng/mL insulin
2. 8.5mM glucose +20ng/mL insulin
3. 11mM glucose +20ng/mL insulin
4. 7.3mM glucose (used to describe control conditions)
5. 8.5mM glucose
6. 11mM glucose
7. 20ng/mL insulin experimental control
8. Glucose – free media experimental control

A concentration of 7.3mM glucose was chosen to represent normoglycaemia (i.e normal levels of glucose) as calculated for the media used for standard BOEC culture. This was within the range of normal glucose detected in the bovine circulation (Hugentobler *et al.*, 2008). 8.5mM and 11mM were chosen to describe mild and severe hyperglycaemia, as their levels were higher than those physiologically observed (Hugentobler *et al.*, 2008). The concentration of basal insulin used is that, which, according to the literature produces a close to physiological phenotype for oviduct epithelial cells (20 ng/ml) (Palma-Vera, Einspanier and Schoen, 2014).

4.3.3. Experiments

Two different types of treatment were performed; acute and chronic (Figure 4.1). Acute treatment was performed in order to determine the impact of brief fluctuation in the glucose blood levels, due to, for instance, food consumption on oviduct fluid secretions. Chronic hyperglycaemia aimed to determine how a sustained high glucose challenge, which can be observed in metabolic disorders such as obesity and diabetes, affects the secretions of the oviduct.

Briefly, to determine the impact of acute hyperglycaemia on BOEC secretions and physiology, the cells were grown under standard conditions for BOEC culture (section 2.1, Materials and Methods) and as described in section 4.3.1. TEER was measured to confirm monolayer confluence, and the medium from the apical and basal compartment was aspirated off before the treatments described in section 4.3.2. were applied to the basal compartment. The apical *iv*DOF produced 24 hours later from the apical compartment, was collected and stored at -20^{o}C for further analysis (Figure 4.1.a).

To determine the impact of chronic hyperglycaemia on BOEC secretions and physiology, BOECs were grown to confluence under the treatment conditions described in section 4.3.2. When TEER measurements confirmed the formation of a confluent monolayer, the medium from the top compartment was aspirated, the medium from the bottom compartment was replaced as above and, 24 hours later, the fluid created by the BOECs was collected from the apical compartment for further analysis (Figure 4.1.b).

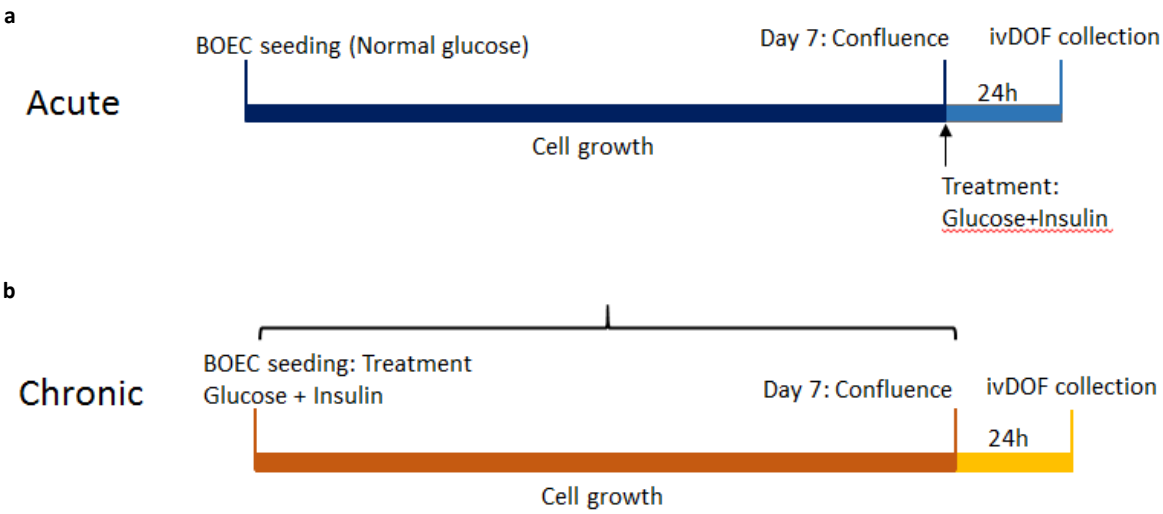


Figure 4.1: Experimental plan outline, for acute (4a) and chronic (4b) hyperglycaemia experiments. See text for details.

4.3.3.1. Volume and composition of *iv*DOF

The volume of *iv*DOF produced by BOECs was collected and glucose, lactate and pyruvate concentrations were determined using ultramicrofluorometric analysis as described in section 2.7. The concentrations of amino acid in the apical fluid were measured as described in section 2.8.

4.3.3.2. Gene expression

The gene expression levels of key genes (*OVGP1, ESR1, SLC2A1, SLC2A3, INSR*) were measured in BOECs in response to normo- and hyperglycaemic challenges, as described in section 2.11.

4.3.4. Data analysis

Details of n numbers and replicates are provided in the respective figure legends. All data were analysed using GraphPad Prism. Data normality was tested using Shapiro-Wilk test. Where data followed to the normal distribution, they were analysed using One-way ANOVA, with Tukey's *post-hoc* analysis and they were presented as mean ± standard deviation. Where data was confirmed as non-parametric, or if the n-number was too small to determine normality, the data were analysed using Kruskal-Wallis with Dunn's *post-hoc* and are presented as median ± interquartile range. Amino acid

concentrations are presented, as mean ± standard deviation, and analysed using Two-way ANOVA with Tukey's post-hoc. Gene expression data are presented as mean fold change ($2^{-\Delta\Delta Ct}$) and were statistically analysed as described above.

4.4. Results

4.4.1. Acute hyperglycaemia does not affect the secretion rates of the oviduct

Exposing BOECs to elevated glucose had no impact on the overall volume of *iv*DOF created over 24 hours. Additionally, the inclusion of insulin did not affect the volume of *iv*DOF created over 24 hours. The volume of fluid secreted by the cells in the 11mM + insulin environment was found to be significantly lower compared to the insulin-only control (Figure 4.2).

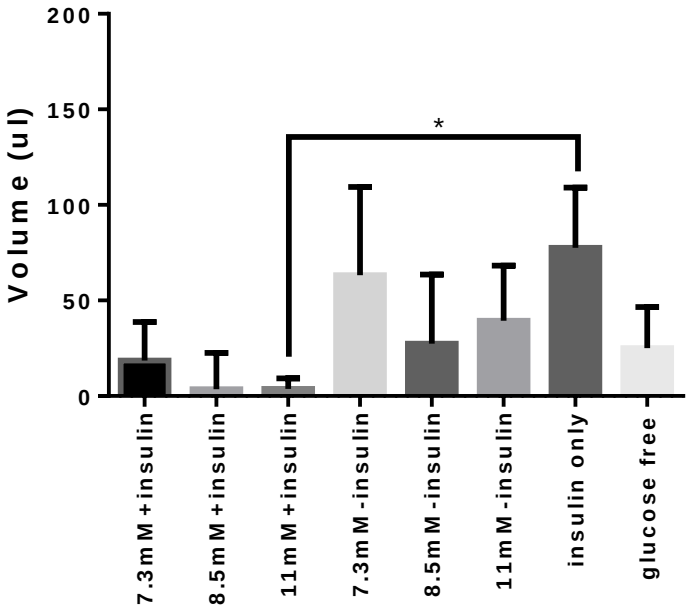


Figure 4.2.: Overall volume of secreted oviduct fluid (*iv*DOF) by BOECs *in vitro*, in response to treatments with normo- and hyper-glycaemic glucose concentrations and in the presence or absence of insulin. n=8-10 biological replicates

4.4.2. Acute hyperglycaemia has minimal effect on the energy substrate concentrations of the oviduct-derived fluid

The *iv*DOF collected after simulation of an acute hyperglycaemic challenge was used to measure the concentration of key energy substrates in the oviduct secretions after a glycaemic challenge. The results indicate that acute hyperglycaemia has minimal impact on the energy substrate content of *iv*DOF secreted by BOECs. However, in the absence of glucose in the basolateral compartment, there was a marked reduction in the amount

of glucose and lactate in the apical compartment, which was significantly lower compared to the 11mM-insulin treatment. Furthermore, the presence of insulin does not appear to alter the concentrations of lactate and pyruvate (Figures 4.3, 4.4, 4.5). Additionally, mild basal hyperglycaemia in the presence of insulin (8.5mM+insulin) resulted in significantly lower glucose concentration compared to severe basal hyperglycaemia in the absence of insulin (11mM-insulin) ($p \leq 0.05$) (Figure 4.3.).

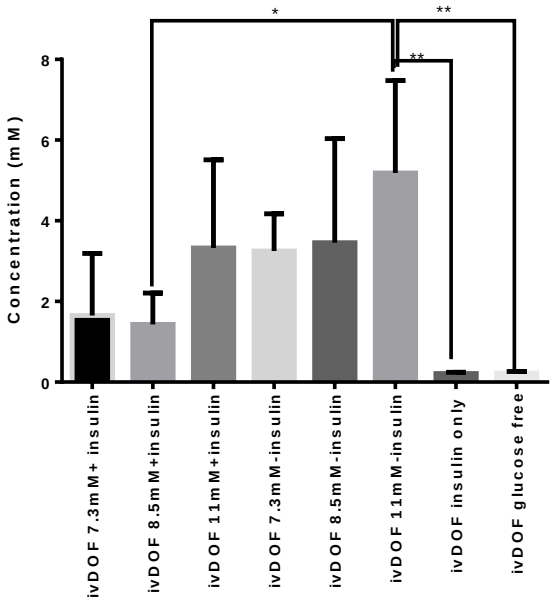


Figure 4.3: Glucose concentration measured in *iv*DOF collected from cells acutely treated under different basal glucose concentrations, in the presence or absence of insulin. n=3-7 biological replicates

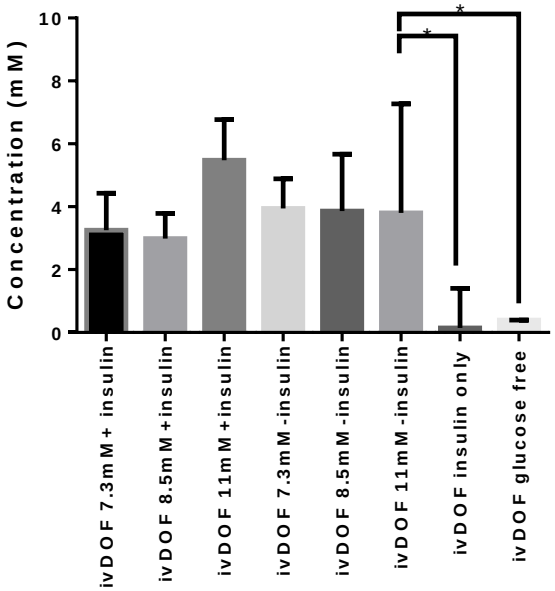


Figure 4.4: Lactate concentration measured in *iv*DOF collected from cells acutely treated under different basal glucose concentrations, in the presence or absence of insulin. n=3-4 biological replicates depending on sample availability

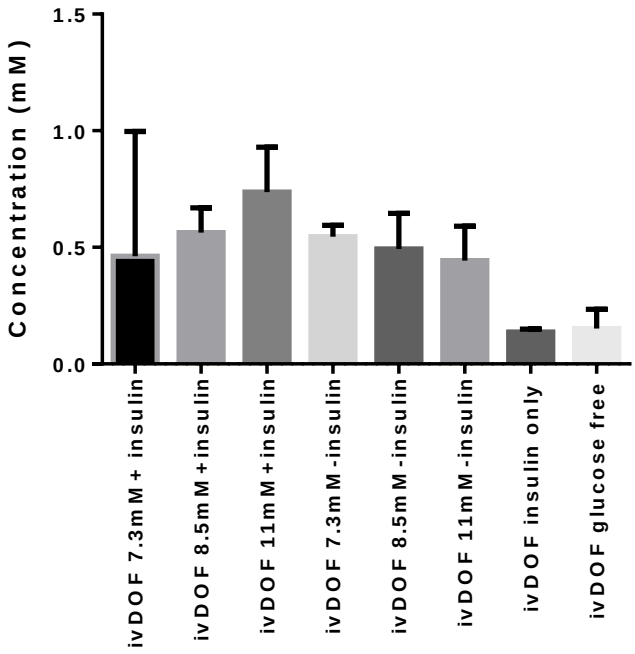


Figure 4.5.: Pyruvate concentration measured in *iv*DOF collected from cells acutely treated under different basal glucose concentrations, in the presence or absence of insulin. n=3 biological replicates

4.4.3. Amino acid composition in *iv*DOF is modified during acute basal hyperglycaemic conditions

Figure 4.6. shows the concentrations of amino acids in *iv*DOF in response to an acute hyperglycaemic challenge. The concentrations of a number of amino acids were altered in response to increasing concentrations of glucose added basolaterally. These are presented in Figure 4.7.

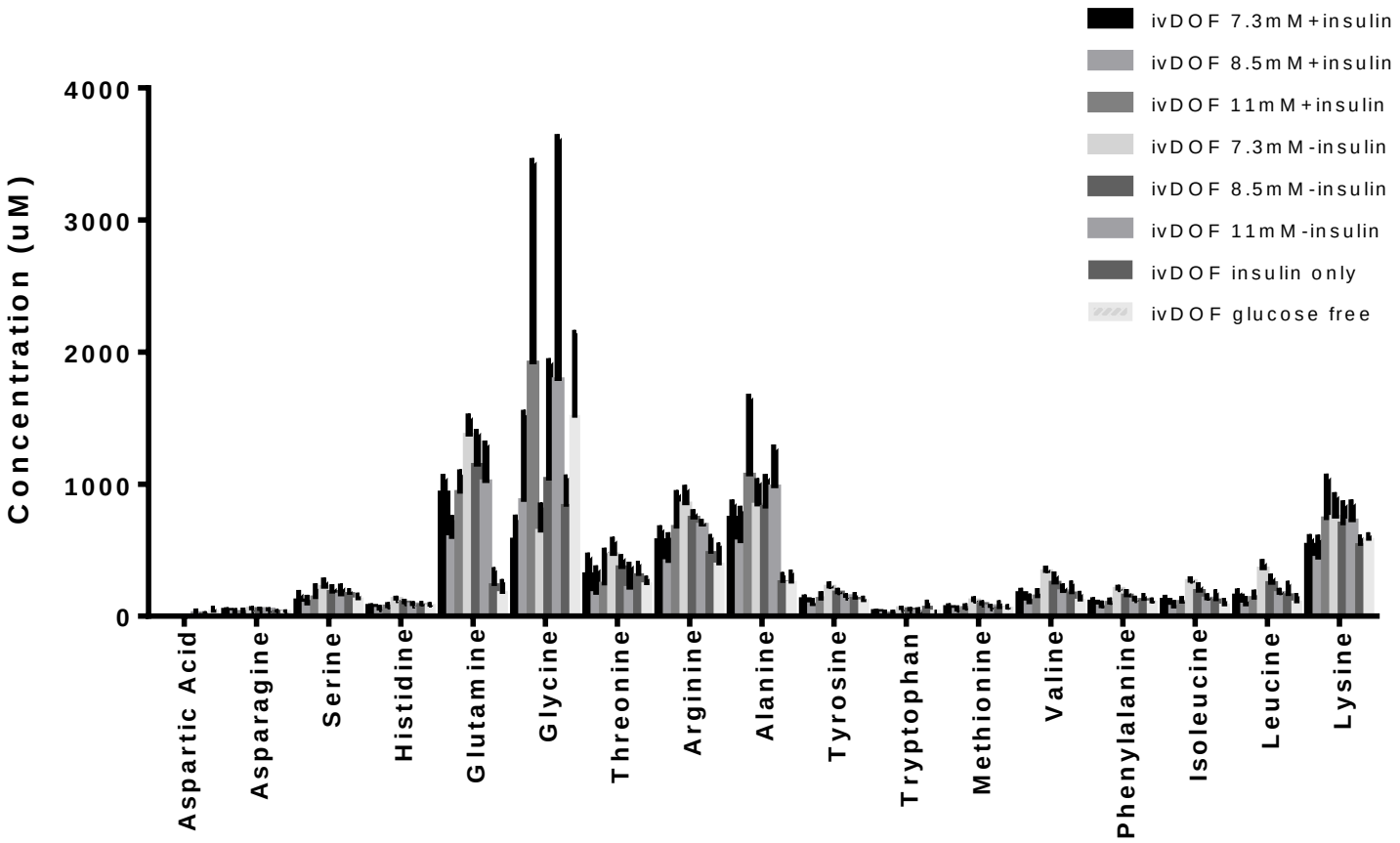
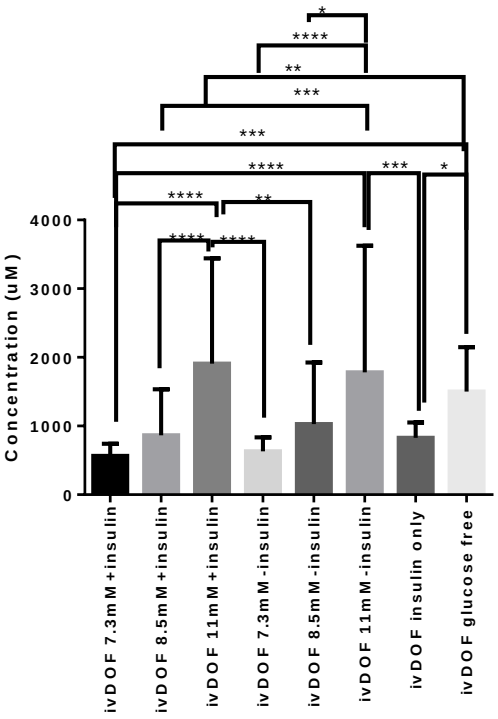
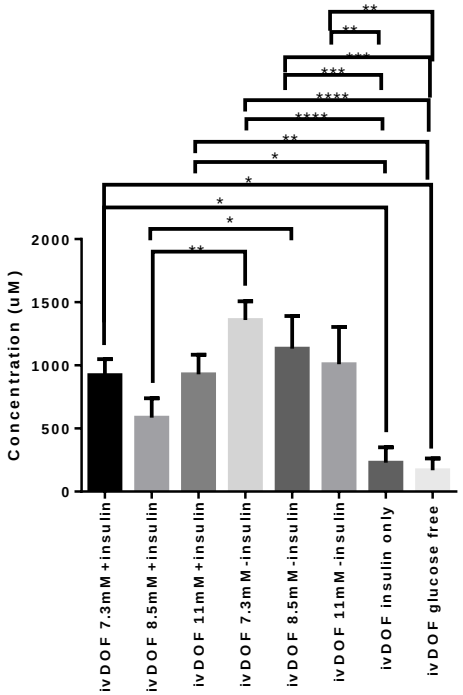


Figure 4.6.: The amino acid composition of *iv*DOF produced by cells treated to simulated hyperglycaemia for 24 hours. Amino acids with significant variance in response to treatment are indicated in subsequent figures, n=3 biological replicates

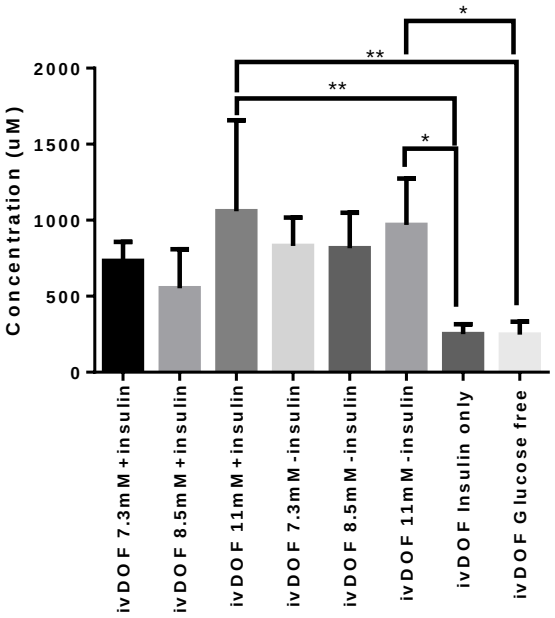
Apical concentrations of glycine increased with increasing basal glucose concentrations, whereas insulin supplementation did not appear to have a significant effect (Figure 4.7.a). On the contrary, glutamine concentrations in *iv*DOF did respond to the presence of insulin; they were significantly higher in normoglycaemia and mild hyperglycaemia in the absence of insulin compared to mild hyperglycaemia in the presence of insulin. Finally, the concentrations of glycine, glutamine and alanine were reduced in the *iv*DOF produced by the insulin-only and glucose-free controls (Figure 4.7.a,b,c).



(a)



(b)



(c)

Figure 4.7.: The amino acid composition of *iv*DOF produced by cells treated to simulated hyperglycaemia for 24 hours, only for the amino acids where significant differences were determined are shown, ie glycine (a), glutamine (b) and alanine (c). n=3 biological replicates

Ion flux experiments for the acute conditions were performed (Figure 4.15_Appendix). However, due to technical difficulties with performance of the voltage clamp, these experiments were not pursued further.

In the next series of experiments, the impact of sustained exposure of hyperglycaemic challenge (i.e. *chronic*) on the creation of ivDOF was investigated.

4.4.4. Chronic hyperglycaemia induces insulin-dependent responses on the volume of *iv*DOF

Figure 4.8 shows that, regardless of the glucose concentration, the presence of insulin for a sustained period results in significantly diminished volumes in the *iv*DOF produced by BOECs. This is further supported by the observation that the volume of *iv*DOF produced by cells treated with insulin in the absence of glucose, is comparable to the volume of insulin-treated samples, whereas volume produced by BOECs treated with the glucose-free control is similar to the insulin-deprived samples.

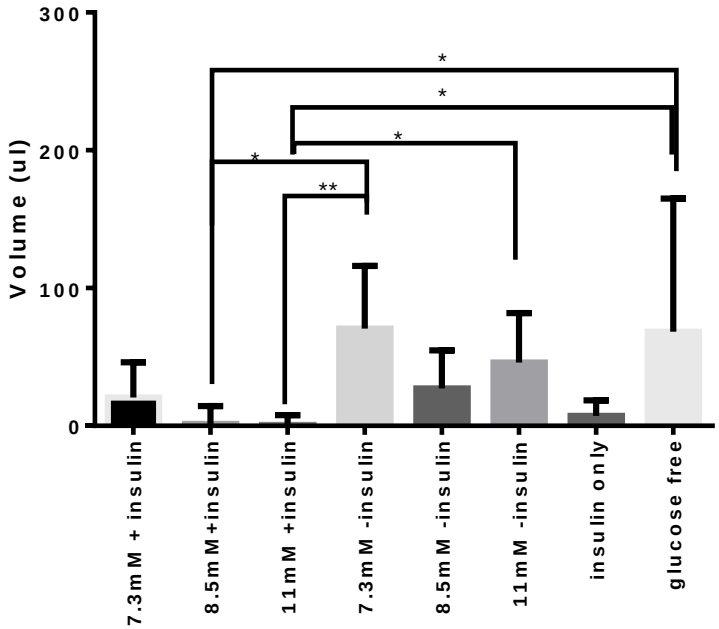


Figure 4.8.: Secretion volumes of BOECs under chronic hyperglycaemic conditions. n=6 for treatments, n=5 for controls, both mean±SD

4.4.5. Chronic hyperglycaemia impacts on glucose concentration in *iv*DOF in an insulin-dependent manner

Figure 4.9 shows that as basal concentrations glucose increase, so does that in the apical chamber. In particular, the concentrations of glucose in *iv*DOF created by cells treated with 7.3mM glucose+insulin and 8.5mM glucose + insulin are 1.1±0.3mM and 1.0±0.54mM respectively. When basal glucose concentration is increased to 11mM+insulin, *iv*DOF was found to contain 3.63±1.24mM glucose; a concentration that did not differ significantly to those concentrations detected under basal glucose concentrations of 7.3mM-insulin and 8.5mM-insulin. Finally, the concentration of glucose detected in *iv*DOF of cells treated under 11mM-insulin was 8.91±1.53mM.

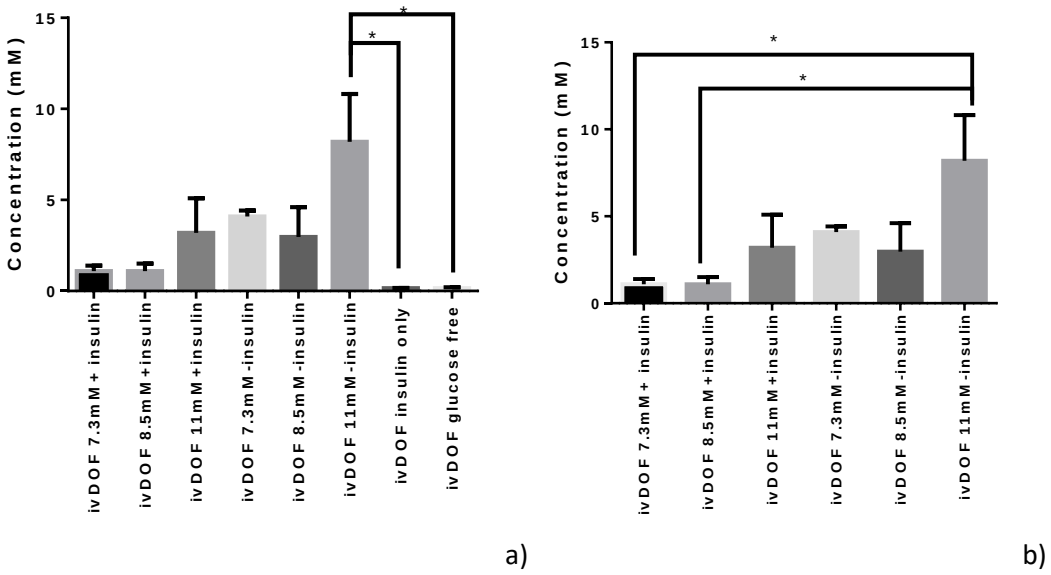


Figure 4.9.: Glucose concentration of *iv*DOF produced by cells treated to simulated hyperglycaemia for 7 days under different treatments a) all treatments, b) no controls included in the statistical analysis, n=3 biological replicates

4.4.6. Chronic hyperglycaemic treatments have no effect on lactate and pyruvate concentrations

The effect of different glucose and insulin conditions added basally for a sustained period of time on lactate and pyruvate composition of *iv*DOF is shown in Figures 4.10, 4.11. Figure 4.10 reveals that the addition of insulin had no significant impact on the concentrations of lactate in *iv*DOF (between 4.41±1.92mM and 5.54±3.61mM). Similarly, the concentrations of pyruvate were comparable among treatments (between 0.29±0.28mM and 0.51±0.1mM). There was a trend for pyruvate concentration to decrease as basal glucose concentration increased from normo- (7.3mM) to hyperglycaemic conditions (8.5mM and 11mM), however this difference was not significant.

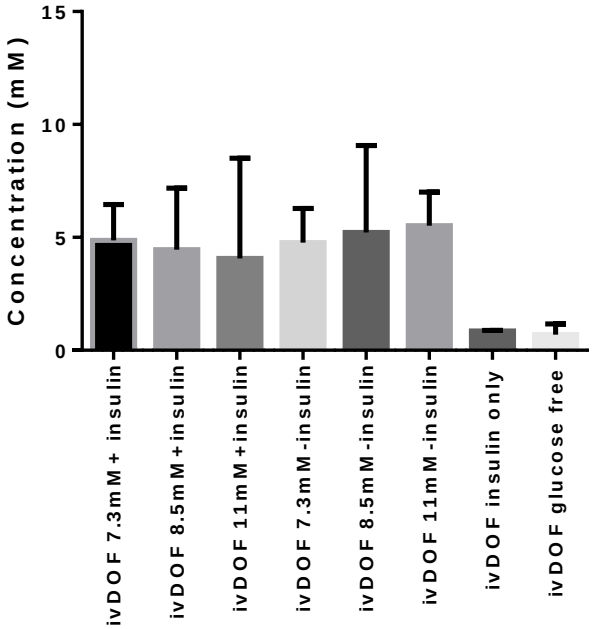


Figure 4.10.: Lactate concentration of *iv*DOF produced by cells treated to simulated hyperglycaemia for 7 days under different treatments. n=3 biological replicates

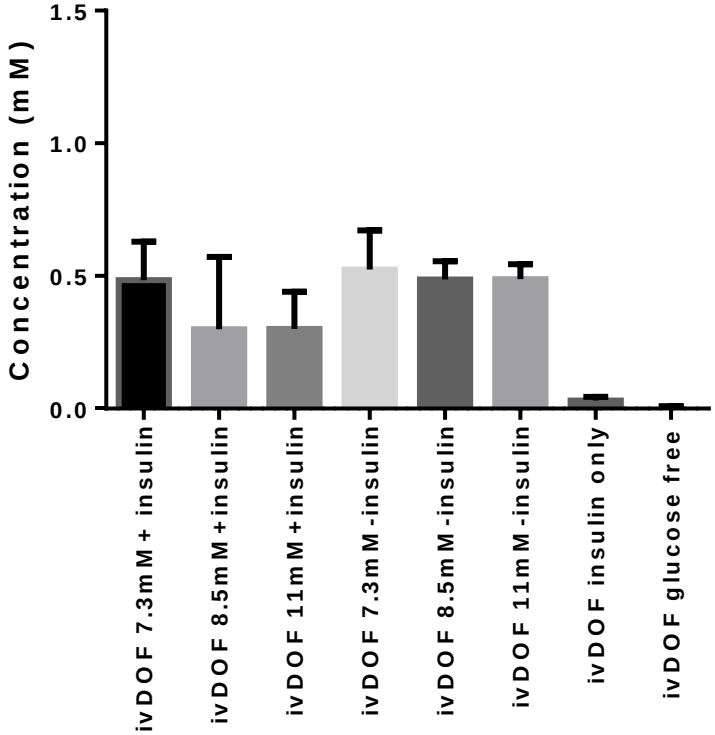


Figure 4.11.: Pyruvate concentration of *iv*DOF produced by cells treated to simulated hyperglycaemia for 7 days under different treatments. n=3 biological replicates

4.4.7. Amino acid composition in *iv*DOF is significantly modified during chronic basal hyperglycaemic conditions

The amino acid composition of basally treated samples under chronic hyperglycaemic conditions is shown in Figure 4.12.

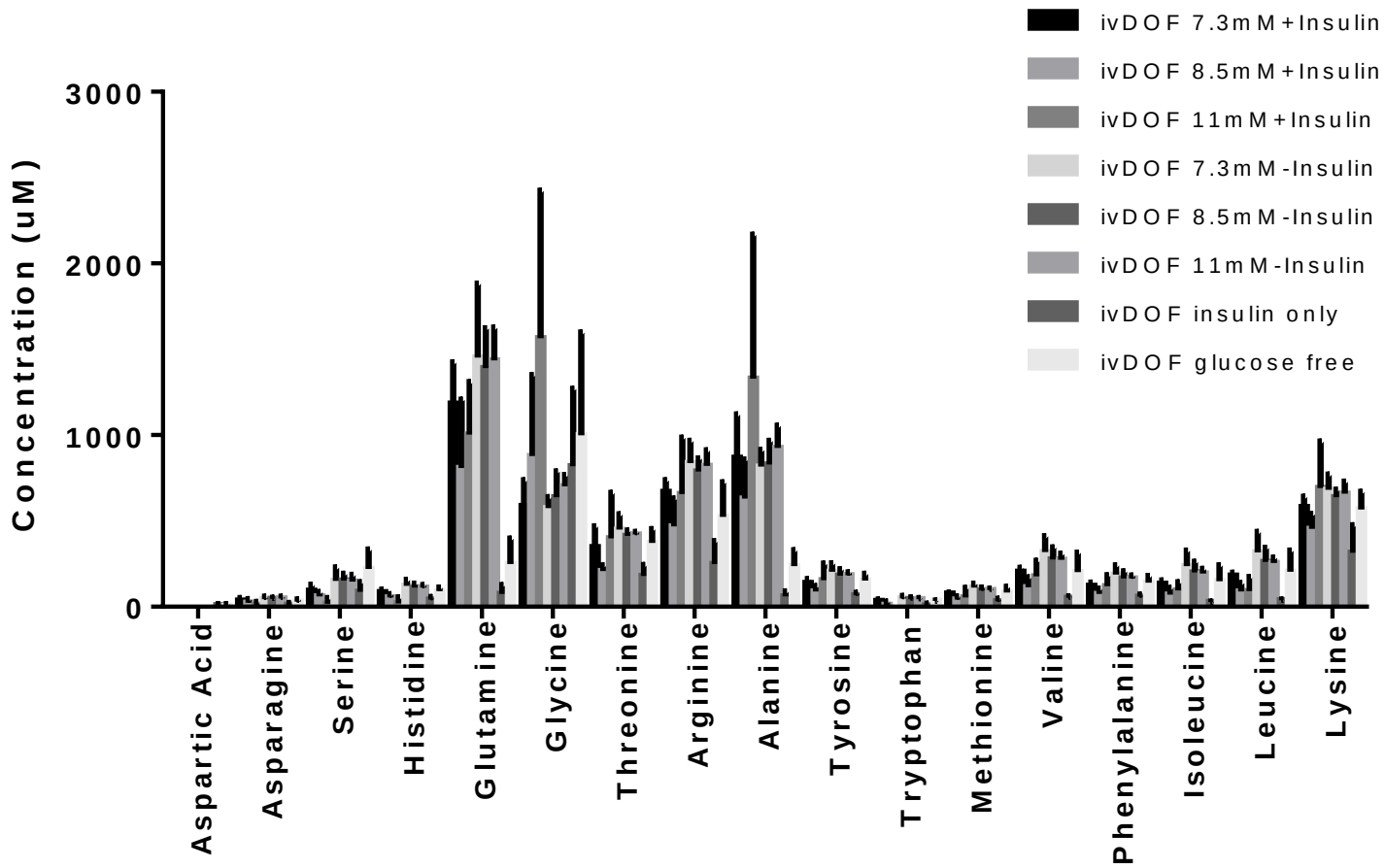


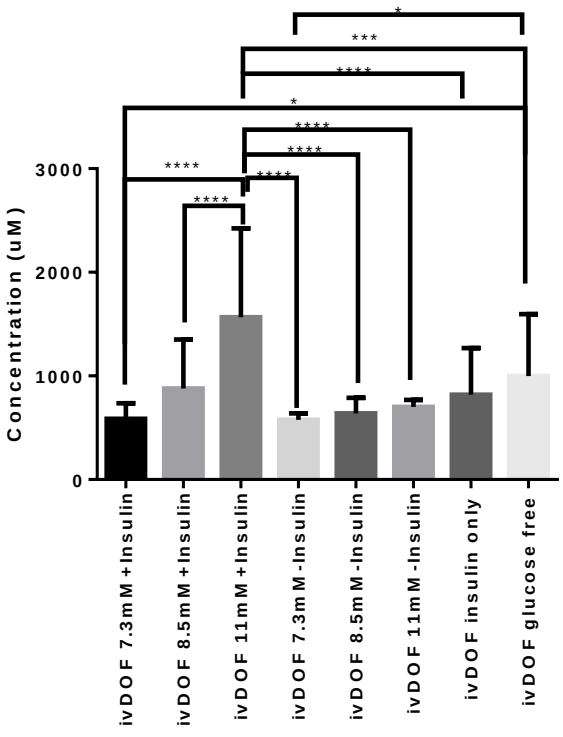
Figure 4.12.: The amino acid composition of ivDOF collected from cells basally treated under chronically hyperglycaemic conditions. n=3 biological replicates

Two-way ANOVA with Tukey's post-hoc revealed that there were significant differences in the concentrations of glycine, alanine, glutamine and arginine between treatments. In particular, increasing basal glucose concentrations led to elevated glycine concentrations detected in the apical compartment. However, this was not the case in the absence of insulin, where glycine concentrations were similar across treatments, at approximately 600µM (Figure 4.13a).

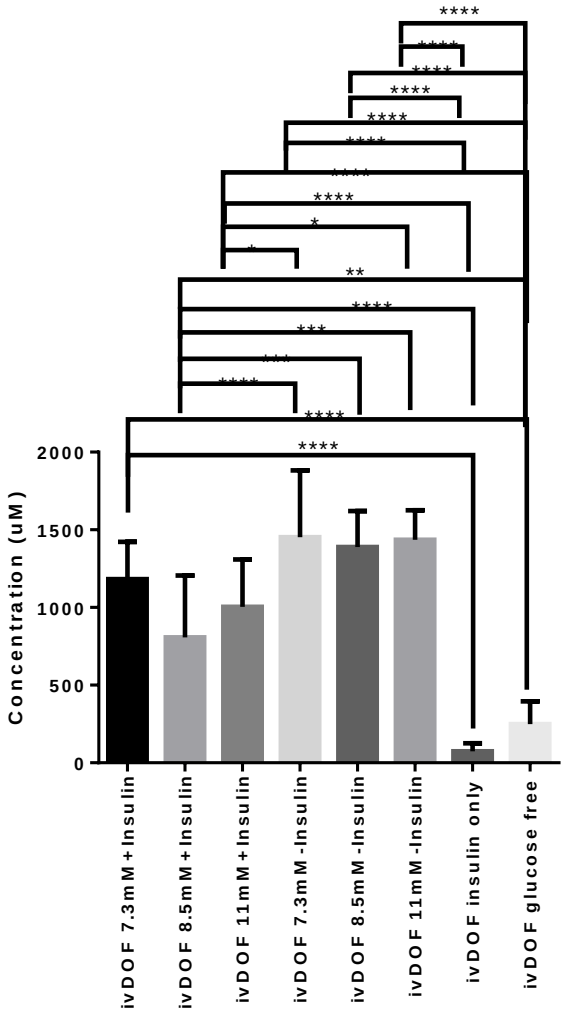
The concentrations of glutamine in *iv*DOF were significantly lower under hyperglycaemic conditions in the absence of insulin, compared to any glucose concentration in the presence of insulin, hinting at an insulin-dependent glutamine secretion (Figure 4.13b).

Chronic treatment of BOECs with glucose had a mild effect on arginine concentration, notably in cells treated basally with 8.5mM glucose with insulin, compared to 7.3mM without insulin. However, the biological significance of this finding remains uncertain (Figure 4.13c).

Finally, alanine concentrations followed a similar pattern to glycine, in that glycine concentration is significantly higher under chronic basal treatment of 11mM in the presence of insulin compared to all other glucose and insulin treatments (Figure 4.13d).



(a)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1000
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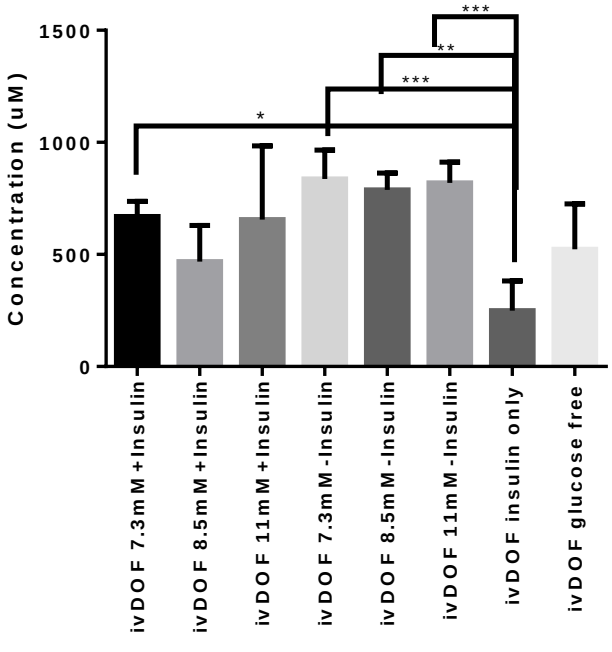
0
Concentration (uM)
ivDOF 7.3mM+Insulin
ivDOF 8.5mM+Insulin
ivDOF 11mM+Insulin
ivDOF 7.3mM-Insulin
ivDOF 8.5mM-Insulin
ivDOF 11mM-Insulin
ivDOF insulin only
ivDOF glucose free

(b)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1500
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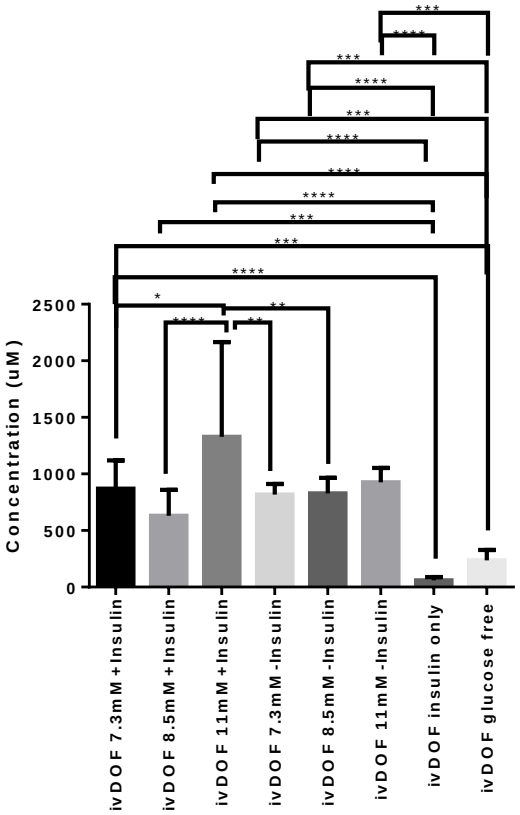
Concentration (uM)
ivDOF 7.3mM+Insulin
ivDOF 8.5mM+Insulin
ivDOF 11mM+Insulin
ivDOF 7.3mM-Insulin
ivDOF 8.5mM-Insulin
ivDOF 11mM-Insulin
ivDOF insulin only
ivDOF glucose free

(c)

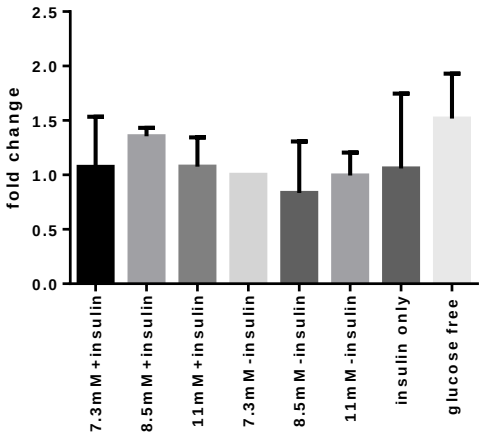


(d)

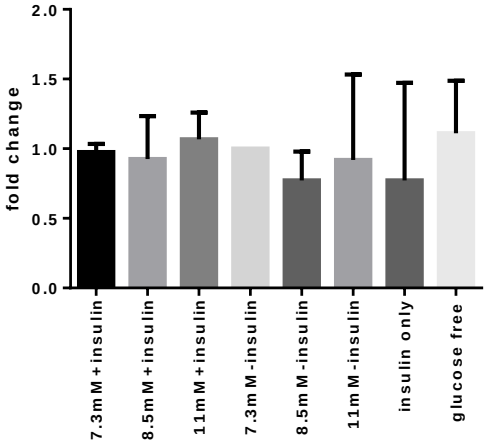
Figure 4.13.: The amino acid concentrations in *iv*DOF produced by cells basally treated under chronic conditions, under different glucose concentrations with or without insulin. a) glycine, b) glutamine, c) arginine, d) alanine, n=3 biological replicates

4.4.8. Gene expression patterns are not affected by the hyperglycaemic treatments

Gene expression studies revealed no changes in the expression patterns of many genes of interest, including those related to oviduct function (oestrogen receptor alpha, oviduct-specific glycoprotein, coded by genes *ESR1, OVGP1* respectively), as well as genes associated with glucose transport in the epithelial cells (glucose transporters 1 and 3, which are insulin-independent, and insulin receptor) (Figure 4.14). The results shown in the figure below, show no impact of any of the treatments on the expression of any of the genes tested, suggesting that the impact on the molecular mechanisms of the cells, if any, is not at the level of gene expression.


fold change
2.5
2.0
1.5
1.0
0.5
0.0
7.3mM+insulin
8.5mM+insulin
11mM+insulin
7.3mM-insulin
8.5mM-insulin
11mM-insulin
insulin only
glucose free

a)


fold change
2.0
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7.3mM+insulin
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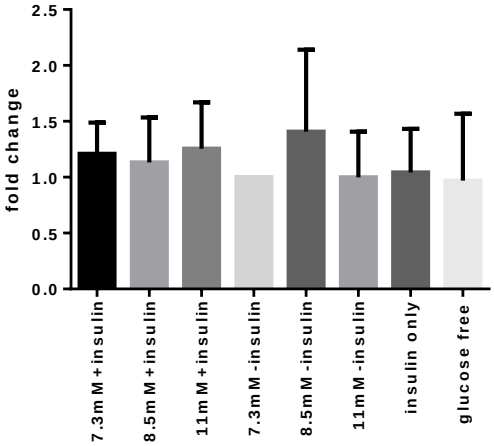
11mM+insulin
7.3mM-insulin
8.5mM-insulin
11mM-insulin
insulin only
glucose free

b)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7.3mM+insulin
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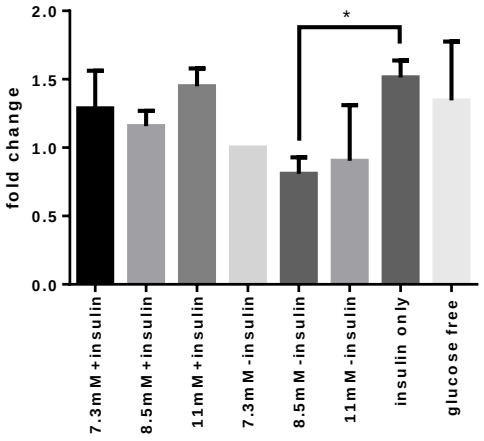
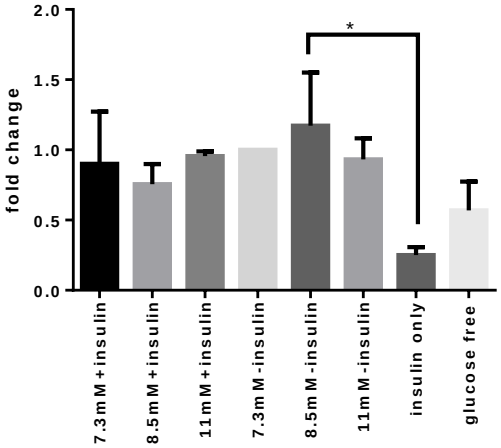
8.5mM-insulin
11mM-insulin
insulin only
glucose free

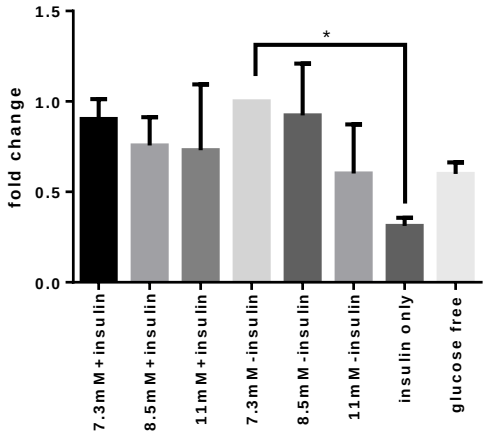
c)


fold change
2.0
1.5
1.0
0.5
0.0
*
7.3mM+insulin
8.5mM+insulin
11mM+insulin
7.3mM-insulin
8.5mM-insulin
11mM-insulin
insulin only
glucose free

d)


fold change
2.0
1.5
1.0
0.5
0.0
*
7.3mM+insulin
8.5mM+insulin
11mM+insulin
7.3mM-insulin
8.5mM-insulin
11mM-insulin
insulin only
glucose free

e)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1.5
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7.3mM+insulin
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11mM+insulin
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11mM-insulin
insulin only
glucose free

f)

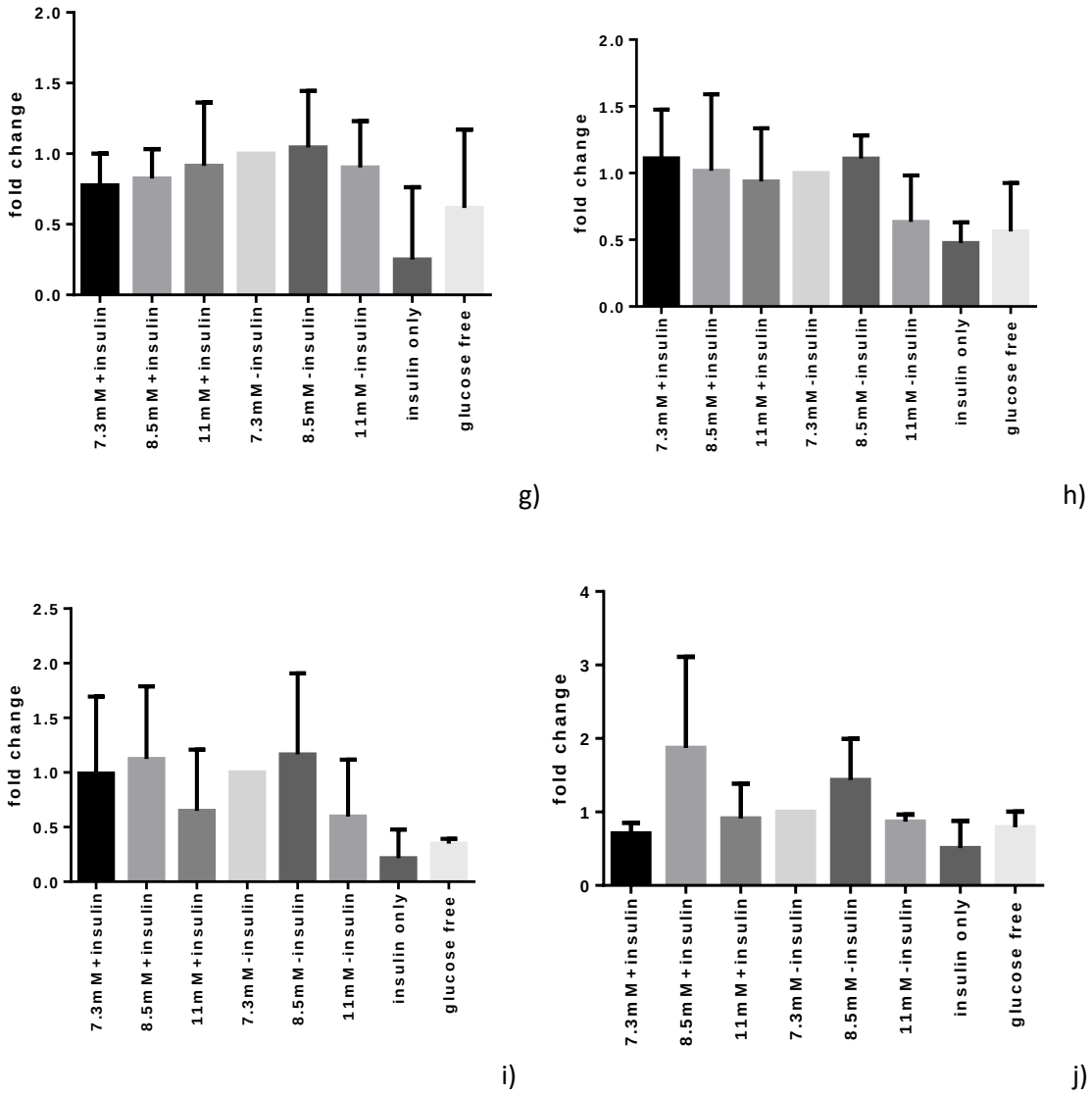


Figure 4.14.: Gene expression patterns of key genes associated with oviduct function (*ESR1, OVGP1*) and glucose transport (*SLC2A1, SLC2A3, INSR*) under different treatments. The above results show no impact of any of the above treatments, regardless of whether they were acute or chronic and regardless of the presence of insulin, on the gene expression patterns of the above genes. a) *SLC2A1* acute treatment, b) *SLC2A3* chronic treatment, c) *ESR1* acute treatment, d) *ESR1* chronic treatment, e) *SLC2A3* acute treatment, f) *SLC2A3* chronic treatment, g) *INSR* acute treatment, h) *INSR* chronic treatment, i) *OVGP1* acute treatment, j) *OVGP1* chronic treatment, n=3-4 biological replicates depending on sample availability

4.5. Discussion

The aim this chapter was to determine the impact of an acute and a chronic simulated hyperglycaemic challenge on the composition of bovine oviduct secretions, and to investigate the extent to which this is regulated at a gene level. The findings suggest that an acute hyperglycaemic challenge has only very modest effects on the physiology of the oviduct, in terms of secretion rates, metabolic substrate composition or the gene expression of key genes associated with the oviduct epithelial cell type or glucose transport into the cells. This is a reassuring finding, which supports the notion that oviduct fluid composition is not easily modified, especially when the levels of glucose or insulin in the circulation fluctuate according to food intake. In other words, the data suggest that the oviduct epithelium is resistant to short-term changes in nutrient availability, possibly to protect the embryo at the early stages of development. As mentioned in the introduction of this chapter, given that important events, such as embryonic genome activation, which occur while the embryo is in the oviduct, its gatekeeper function should be protective, and this work confirms that this is indeed the case.

In contrast to short-term exposure to hyperglycaemia, chronic hyperglycaemia did affect the secretion volumes and the glucose concentration of *iv*DOF in an insulin dependent manner, with more glucose in the apical compartment in the absence of insulin compared to the presence of insulin. The detection of higher glucose concentrations and higher volume for insulin-deprived samples hints at a potentially detrimental situation to embryo development.

Glycine concentrations in the oviduct-derived fluid were highest at the 11mM+insulin treatment. Interestingly, the glycine concentration pattern in the presence of insulin, appeared to increase under increasing glucose concentrations, a pattern similar to the one observed under acute hyperglycaemic conditions regardless of the presence of insulin (Figure 4.7a). However, this pattern was not seen in the absence of insulin, where increasing basal glucose concentrations result in similar apical glycine concentrations. This suggests an impact of high glucose concentrations on glycine secretion at the apical compartment. Combined with the data from acute hyperglycaemia, it appears that

glycine secretion into the apical compartment is glucose-dependent and not insulin-dependent.

Interestingly, the levels of glycine are comparable to the *in vivo* data when the cells are treated with 11mM+insulin (approximately 1500 μM) (Hugentobler et al., 2007), whereas the other concentrations induce lower glycine levels than the ones observed *in vivo* (around 800 μM). Glycine concentrations were previously found to be lower under *in vitro* conditions compared to the *in vivo* conditions (Simintiras et al., 2016), even though the present data suggest a value in between the previously reported *in vitro* and *in vivo* values. Consequently, it may be deduced that hyperglycaemic conditions in the presence of insulin result in abnormally high glycine concentrations, which will then be presented to the gametes and early embryo. Indeed, glycine is known to act as an osmolyte in early embryos (Dawson, Collins and Baltz, 1998; Baltz, 2001), glycine concentrations regulating the cell volume in early-stage embryos (Steeves *et al.*, 2003) and glycine concentrations in the embryo being regulated by glycine transporters, depending on glycine availability in the embryo environment (Steeves *et al.*, 2003; Steeves and Baltz, 2005). Glucose also has the potential to act as an osmolyte in different solutions, however, no osmotic variance was observed in the different media in the present study. Glycine has also been found to be important in human intestinal epithelial cell protection from oxidative damage, by intracellular preservation of glutathione, of which glycine is a constituent amino acid, with cysteine and glutamate (Howard *et al.*, 2010). An association between glucose and glycine transporter SLC6A9 has been described in the nervous system of obese and diabetic rats, where introduction of a glycine transporter inhibitor restored glucose levels, improved glucose tolerance and restored the animals' body weight (Yue *et al.*, 2016).

Glutamine concentrations were significantly lower in samples treated with a hyperglycaemic challenge in the presence of insulin, compared to the respective treatments without insulin. Glutamine may be metabolised by entering the TCA cycle and results in NADPH production, which in turn can modify the ratio of oxidised and reduced glutathione (GSH:GSSG), and therefore in altered cellular redox state (Curi *et al.*, 2005). It is associated with insulin secretion by the pancreas, via the stimulation of glucose oxidation leading to increased ATP production (Newsholme *et al.*, 2003), which results in membrane depolarisation and consequently insulin release (McClenaghan and

Flatt, 1999). An association between glutamine transporters and glucose has been described in cancer cell lines where glucose deprivation resulted in glutamine transporter ASCT2 deglycosylation, and associated induction of other compensatory mechanisms of glutamine transport, mainly via LAT1 (Polet *et al.*, 2016). Furthermore, reduced expression of SLC6A19, which is a glutamine transporter, as well as a more general neutral amino acid transporter, led to improved glycaemic control in mice, with the potential to be addressed as a therapeutic target for diabetes (Liu *et al.*, 2018). Additionally, the gene expression of SNAT3, which is another glutamine transporter, increased significantly in obese and glucose-intolerant mice, where ammonium excretion was severely impaired (Busque, Stange and Wagner, 2014). Interestingly, Leary *et al* (2015) reported that glutamine depletion was higher in embryos produced by fertilisation of oocytes from obese women compared to women with normal BMI (Leary, Leese and Sturmey, 2015). Moreover, high glutamine uptake by the embryos have been associated with increased DNA damage (Sturmey *et al.*, 2009), which in turn could be linked to the appearance of high glutamine levels in the apical fluid of insulin-deprived cells. Overall, there is a strong link between glutamine expression and glucose levels, as determined in various experimental conditions.

Alanine concentrations were also found to be significantly higher in the 11mM glucose +insulin treatment compared to any other treatment. The concentrations of all other treatments are comparable to those observed *in vivo* (Hugentobler, Diskin, *et al.*, 2007). Previously, alanine in the follicular fluid has been positively correlated with blastocyst formation (Matoba *et al.*, 2014). Alanine is produced by embryos (Sturmey, Brison and Leese, 2008), possibly through pyruvate transamination for the purpose of recycling of ammonium ions (Donnay, Partridge and Leese, 1999; Orsi and Leese, 2004). Finally, arginine concentrations were significantly lower in the insulin-only treatment, but not the glucose-free treatment, compared to all other treatments. Elevated glucose has previously been associated with increased arginine transport in human umbilical vein endothelial cells (Flores *et al.*, 2003); however, this was not observed in the present experiments.

Overall, the different glucose and insulin conditions basally applied to BOECs lead to the secretion of modified apical fluid, suggesting that these conditions may impact on embryo development. The impact of amino acids on the early embryo has been

previously established, some amino acids having a stimulatory and others having an inhibitory effect on the mouse early embryo (Gardner and Lane, 1993). In the next chapter, the impact of the above conditions on early embryo development will be further explored.

Chapter 5

The embryo in the hyperglycaemic oviduct

5.1. Introduction

A major health risk of prolonged overweight and obesity is the development of insulin insensitivity leading to the emergence of type 2 diabetes mellitus (T2DM). A notable characteristic of T2DM is hyperglycaemia (Martyn, Kaneki, & Yasuhara, 2008). T2DM has additional health impacts, including affecting fertility, however, even in the absence of T2DM, or in a so-called "pre-diabetic" state, obesity per se can have a dramatic impact on reproductive function. The relationship between obesity and reduced fertility has been attributed to dysregulation of steroidogenesis (Azziz, 1989; Robker *et al.*, 2009) and/or production of poor quality oocytes (Metwally, Li and Ledger, 2007; Grindler and Moley, 2013). Indeed, the follicular microenvironment of women with abnormally high BMI shows signs of dyslipidaemia, hyperinsulinaemia and inflammatory response (Robker *et al.*, 2009), due to the metabolic perturbations in the serum of obese women (Valckx *et al.*, 2012). Excess body fat is more strongly associated with the appearance of ovulatory disturbances, when obesity onset occurs earlier in life (Azziz, 1989). High BMI (greater than 25kg/m^2) reduces the number (Robker *et al.*, 2009; Valckx *et al.*, 2012) and size (Leary, Leese and Sturmey, 2015) of human oocytes. Since obesity and diabetes often coexist, and in many cases, T2DM remains undiagnosed, it is still unclear whether the poor quality of oocytes and consequently with poor embryo development is associated with hyperglycaemia, hyperinsulinaemia or high adiposity or with some combination of these conditions (Lane et al., 2015).

Startlingly, fewer embryos are produced after fertilisation of oocytes of obese women compared to women with a BMI <30 kg/m^2 (Robker *et al.*, 2009; Valckx *et al.*, 2012). Moreover, the blastocysts produced after *in vitro* fertilisation of oocytes of women with healthy BMI differ from the embryos of women with high BMI, in terms of developmental timing, glucose consumption, amino acid turnover and triglyceride levels (Leary, Leese and Sturmey, 2015). However, there is evidence that suggests that fertilisation of oocytes donated by women of healthy BMI does not impact on the rates of implantation of the resulting blastocysts or on spontaneous miscarriage, regardless of the BMI of the recipient (Styne-Gross, Elkind-Hirsch and Scott, 2005). This suggests an impact of obesity on fertility at the pre-implantation stages, in the ovary and the oviduct.

The extent to which the oviduct environment is modified in a hyperglycaemic response, and the subsequent impact on embryo physiology have not been investigated. This is crucial, given that the oviduct provides the environment for key developmental events, as discussed earlier in Chapter 1, including fertilisation (Ulbrich *et al.*, 2010; Li and Winuthayanon, 2017), embryonic genome activation (Duranthon, Watson and Lonergan, 2008), early embryo epigenetic remodelling (Ulbrich *et al.*, 2010; Li and Winuthayanon, 2017), cell lineage allocation (Wang and Dey, 2006) and development of the blastocyst (Wang and Dey, 2006).

As described in Chapter 1, embryo metabolism is dynamic as development progresses from zygote to blastocyst. During the first stages of cleavage, glucose consumption is negligible, and although oxygen consumption is low, oxidation of substrates such as pyruvate and endogenous fatty acids provide the main source of ATP production. As development progresses, glucose consumption increases and so does oxygen consumption. Since glycolysis generates much less ATP than oxidative phosphorylation (2 versus 30 ATP molecules), it has been hypothesised that the increased glucose consumption is necessary for biosynthetic processes, through generation of carbon intermediates, which then feed into the pentose-phosphate pathway (Smith and Sturmey, 2013; Lewis and Sturmey, 2015). As reviewed by Leese (2012), and the increase in oxygen consumption at the blastocyst stage is associated with increased embryo requirement for ATP, for the formation of blastocoel cavity, as well as protein synthesis (Leese, 2012). Importantly, it has been shown that there is a link between glucose consumption (Gardner *et al.*, 2011) or amino acid turnover (Houghton *et al.*, 2002; Brison *et al.*, 2004) and subsequent embryo viability to the blastocyst stage and beyond.

Given the relationship between embryo metabolism and embryo viability (Houghton *et al.*, 2002; Leese, 2002; Brison *et al.*, 2004), it is striking that little is known about how an altered oviduct environment, in response to maternal conditions such as diabetes and obesity might impact the biochemistry and subsequent development of the preimplantation embryo. Thus, the overall aim of the research in this chapter, was to determine the impact of an embryo environment, modified according to the secretions of the oviduct, as described in Chapter 4 for normoglycaemia and hyperglycaemia, on

the developmental progression and metabolism of embryos at the cleavage and blastocyst stage.

5.2. Hypothesis and aims

This chapter sets out to test the hypothesis that a hyperglycaemia-modified embryo culture environment can affect the developmental progression and metabolism of embryos at the cleavage and blastocyst stage.

Consequently, the specific aims of this chapter were:

a) To determine the impact of the modified environment on the cleavage and blastocyst rates of embryos

b) To determine how the modified environment impacts on the metabolism of the embryos in terms of their rates of energy substrate consumption/production

5.3. Experimental design

5.3.1. Treatments

Informed by the results of Chapter 4, in which it was demonstrated that the secretions from bovine oviduct epithelial cells responded to the presence of elevated glucose and insulin, it was decided to test the response of the preimplantation embryo to four experimental conditions. The data from Chapter 4 demonstrated that the presence of insulin had no significant effect on the concentrations of nutrients measured in oviduct cell secretions of cells grown in conditions designed to mimic normo- (7.3mM glucose) or mildly hyperglycaemic (8.5mM glucose). Therefore, for the purposes of the experiments described in this chapter, the composition of *iv*DOF generated from cells grown in 7.3mM of glucose in the absence of insulin were selected as a baseline physiological comparator. Additionally, for the 8.5-modified environment, the 8.5mM+insulin concentrations were used, as these were considered as more likely to be encountered physiologically. The 11mM+insulin and 11mM-insulin will be referred to as '11+' and '11-', respectively. The overall concentrations of glucose, lactate, pyruvate and amino acids used for this experiment are shown in Tables 5.1, 5.2 and 5.3, whereas the remaining components of embryo culture media were not modified.

Table 5.1.: Glucose, lactate and pyruvate concentrations for embryo culture, as detected in ivDOF.

	7.3mM	8.5mM	11mM+insulin	11mM-insulin	Control
Glucose (mM)	3.6	1.0	3.6	8.9	1.5
Lactate(mM)	4.5	4.7	5.2	4.9	3.3
Pyruvate (mM)	0.6	0.3	0.3	0.5	0.3

Table 5.2.: Amino acid concentrations used for embryo culture, as detected in ivDOF.

Concentration (µM)	7.3mM	8.5mM	11mM+I	11mM-I
Asparagine	44.6	35.2	31.2	49.1
Serine	150.4	60.0	54.2	145.9
Histidine	122.1	55.3	58.7	112.4
Glutamine	1452.4	808.2	1004.0	1435.9
Glycine	575.5	877.7	1565.4	701.1
Threonine	450.4	207.0	399.1	420.2
Arginine	836.5	467.8	655.9	819.9
Alanine	817.5	630.6	1329.0	925.9
Tyrosine	203.7	89.8	155.3	182.7
Tryptophan	48.8	16.1	0.0	44.0
Methionine	110.7	41.3	80.9	97.9
Valine	319.0	114.2	176.7	273.5
Phenylalanine	187.7	75.2	120.0	164.7
Isoleucine	236.3	70.1	96.1	193.4
Leucine	315.7	88.6	138.2	254.6
Lysine	682.2	453.7	691.0	658.1

Individual embryo culture was performed in order to assess the impact of the different culture conditions on embryo metabolism. For the individual culture, the respective concentrations of the controls were 0.5mM for glucose, 0.3mM for pyruvate and 0 for lactate. The amino acid concentrations of control conditions were as shown in Table 5.3:

Table 5.3.: Amino acid concentrations used for control individual culture conditions.

Amino acid	Concentration (μM)
Asparagine	50.0
Serine	40.0
Histidine	40.0
Glutamine	1984.9
Glycine	40.0
Threonine	50.0
Arginine	200.0
Alanine	110.0
Tyrosine	40.0
DABA	50.0
Tryptophan	20.0
Methionine	20.0
Valine	30.0
Phenylalanine	20.0
Isoleucine	20.0
Leucine	60.0
Lysine	60.0
Cysteine	26.0
Proline	20.0
Glutamate	100.0

5.3.2. Embryo culture experiments

Oocyte maturation, fertilisation and embryo culture were performed as described in Materials and Methods (Section 2.13). Embryos were allocated randomly to one of the five treatment conditions ('7.3'-modified, '8.5'-modified, '11+'-modified, '11-' -modified, control). Three different experiments were performed:

-The impact of modified embryo environment on embryo developmental progression: Embryos were cultured in groups of 15-25, according to embryo

availability, under the five conditions. Embryo cleavage and progression to blastocyst were monitored.

-The impact of modified embryo environment on the metabolism of cleavage-stage embryos: For this experiment, the embryos were placed into individual culture immediately after fertilisation, and incubated for 20-26 hours. The embryos were then removed from individual culture and the drops stored in -80^{o}C for further metabolic analysis. All zygotes which had cleaved to create embryos with two (or more) blastomeres were classified as “cleaved”.

-The impact of modified embryo environment on the metabolism of blastocyst-stage embryos: For this experiment, the embryos were cultured in groups of 15-25 embryos, according to embryo availability, under the five treatment culture conditions. On day 7 of embryo culture, embryos that had progressed to blastocyst were placed into individual culture conditions, and incubated under these conditions for 20-26 hours. The embryos were then removed from the individual culture conditions and the drops stored in -80^{o}C for further metabolic analysis.

5.3.3. Metabolic analyses

As described in section 5.3.2, cleavage and blastocyst stage embryos were incubated for 20-26 hours in individual culture drops. The embryos used for evaluation of cleavage stage embryo metabolism were returned into group culture conditions, but were not included in the cleavage and blastocyst rates or in further metabolic analyses. After completion of the experiments, the embryos were discarded and the individual culture drops collected and stored in -80^{o}C.

Enzymatic analyses of glucose, lactate and pyruvate were carried out as described in section 2.7 of Materials and methods. HPLC analysis of the amino acid composition of the spent medium was performed as described in section 2.8.

Samples were thawed and diluted as appropriate for the assays in question (Table 5.4); for the purposes of glucose, lactate and pyruvate production, dilutions were made to ensure that values fitted the standard curve that was optimised for the ultramicrofluorometric assays. This was necessary, since the respective standard curves were not linear above the concentrations already established (Figure 5.25_Appendix).

In cases where the samples were expected to be within the standard curve, a dilution of 1/2 was applied, to ensure the availability of duplicate samples for assay.

Samples were diluted 1:12.5 for amino acid detection. The concentration of substrates in spent media were measured and compared to blank drops, thus allowing the overall change in concentrations to be determined. These values were converted into rates of consumption/production of the substrates by dividing the change in the moles of substrate by the duration of the assay.

Table 5.4: Dilutions of spent media and blank media for ultramicrofluorometric assays

	Glucose	**Lactate**	**Pyruvate**
'7.3'	1/6	1/4	1/4
'8.5'	1/3	1/4	1/2
'11+'	1/6	1/4	1/2
'11-'	1/15	1/4	1/4
control	1/2	1/2	1/2

5.3.4. Statistical analysis

Data on cleavage and blastocyst rates are presented as percentages, and all percentage data were arcsine-transformed prior to further analysis. A Shapiro-Wilk normality test was performed on the arcsine-transformed data of percentages, and on the rates of consumption/production of glucose, lactate and pyruvate. This analysis was not performed on amino acid data, as Prism does not offer a non-parametric analysis and a respective normality test, as there is no high power non-parametric alternative to the two-way ANOVA. Wherever all groups were found to be normally distributed, a One-Way ANOVA was performed, with Tukey's multiple comparisons post-hoc. In these cases, data are presented as mean ± standard deviation. If at least one of the groups was not normally distributed or had an n- number too small to determine normality, the Kruskal-Wallis test was performed, with Dunn's multiple comparison post hoc; in such situations, data are presented as median ± interquartile range. For analysis of amino acid metabolism values, a Two-way ANOVA was performed, with Tukey's multiple comparisons was performed. The amino acid data are presented as mean ± standard

deviation. In all figures of substrate consumption/production presented, positive values suggest substrate production and negative values suggest substrate consumption by the embryos. A One sample t-test was performed for individual amino acid data and a Wilcoxon Signed Rank Test was performed for the glucose, lactate and pyruvate data, in order to determine whether the mean and median values respectively differed significantly from zero.

5.4. Results

5.4.1. Modified embryo environment does not impact on the cleavage rates of the embryos

The cleavage rates of the embryos grown under modified group culture conditions are shown in Figure 5.1. Cleavage rates were unaffected by the treatment conditions, although the '7.3' environment hinted towards slightly higher cleavage rates.

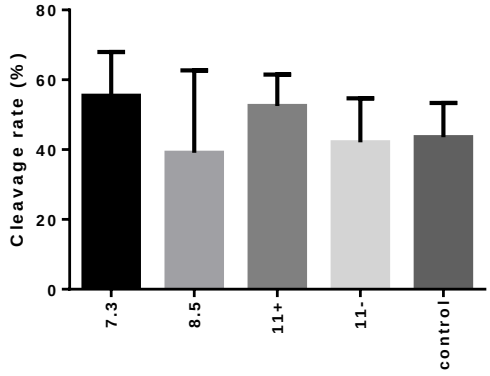


Figure 5.1.: Cleavage rates in modified and control embryo environments. n=5

5.4.2. Modified embryo environments significantly modify lactate and pyruvate production/consumption in cleavage-stage embryos

Glucose depletion by cleavage-stage embryos was marginal under control conditions, as well as the '7.3' and '8.5' environment and did not differ between these groups. Surprisingly, embryos grown in medium that mimicked the oviduct fluid generated by severe hyperglycaemia consumed glucose (Figure 5.2). However, when only those embryos that cleaved were analysed, those embryos exposed to hyperglycaemia in combination with insulin consumed significant quantities of glucose, whereas in embryos exposed to hyperglycaemia without insulin, glucose was marginally produced (Fig 5.3a). The very low rates of glucose consumption/production were further supported by the fact that the median values for each culture environment were significantly different from zero only under the '11+' conditions, when all embryos and cleaved embryos were assessed. Similar to pyruvate, when glucose consumption/production was compared in each of the environments, there was no

statistically significant difference between cleaved and uncleaved embryos (Figure 5.27_Appendix).

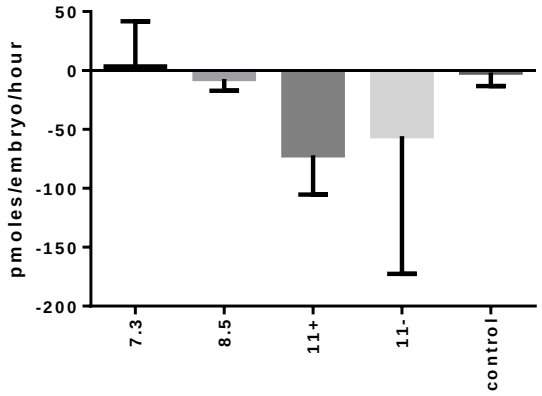
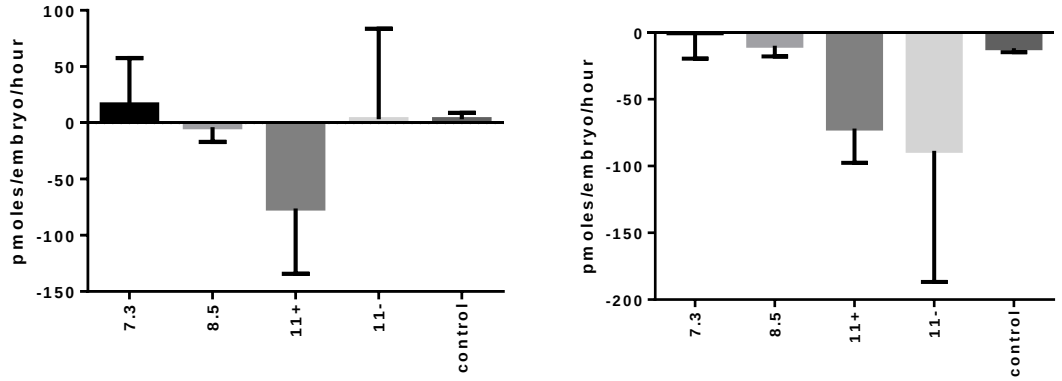


Figure 5.2.: Glucose consumption/production for cleavage stage embryos. n=16-18 depending on the availability of the drop at the time of the assay.



a) b)

Figure 5.3.: Glucose consumption/production for cleavage stage embryos a) embryos that cleaved (n=8-13), b) embryos that failed to cleave (n=5-10).

Embryos grown under control conditions produced small amounts of lactate, whereas the embryos grown in the '8.5' and '11-' modified environments marginally produced this substrate, suggesting very low rates of the substrate utilisation or production by the embryos, which did not differ significantly from zero. The embryos grown under the '7.3' conditions consumed lactate, whereas the embryos grown under the '11+' conditions produced it. In particular, the embryos grown under the '7.3' conditions showed a

statistically significant lactate consumption compared to '11+' conditions, both when all embryos, and when cleaved embryos only were assessed. Additionally, lactate consumption was statistically significant compared to the control conditions when all embryos were assessed (Figures 5.4, 5.5). Similar to what was observed for the other two substrates, there was no statistically significant difference in lactate consumption/production when cleaved embryos were compared to uncleaved in each of the embryo environments (Figure 5.28_Appendix).

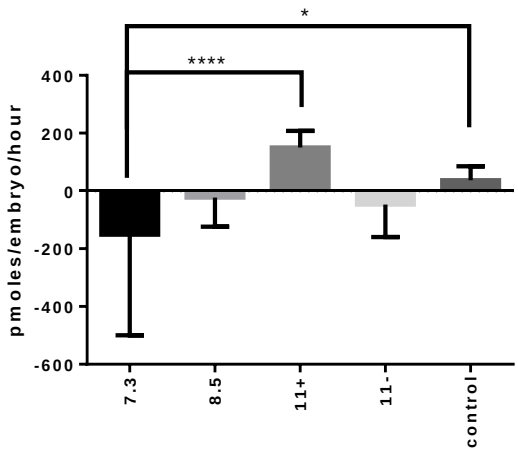


Figure 5.4.: Lactate consumption/production for cleavage stage embryos. n=12-16 depending on the availability of the drop at the time of the assay.

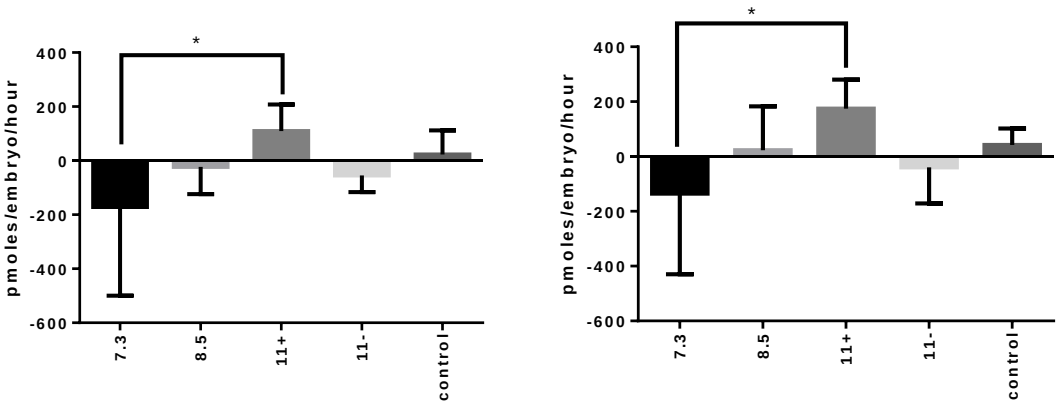


Figure 5.5.: Lactate consumption/production for cleavage stage embryos a) for embryos that cleaved (n=6-11), b) for embryos that did not cleave (n=4-8)

Pyruvate tended to be consumed by embryos of the control group, as well as the '7.3' and '8.5' groups; however, pyruvate consumption of the two latter groups did not differ significantly from zero. Similarly, pyruvate production by the embryos cultured under

the ‘11+’ conditions was not significantly different from zero. Conversely, the concentration of pyruvate in spent medium of embryos grown in the ‘11-’ groups had increased and pyruvate production under the ‘11-’ conditions was significantly higher compared to the 8.5-modified and control groups. Interestingly, the same pattern of pyruvate consumption/production was observed when analysis was restricted to only those embryos that had cleaved, (Figure 5.4a). When pyruvate consumption/production was examined in embryos that had failed to cleave, embryos derived from the 7.3mM treatments appeared to produce pyruvate and the embryos from ‘8.5’, ‘11+’ and control treatments consumed pyruvate; however, it is worth noting that pyruvate production was different from zero in only the ‘11-’ environment. Comparison of pyruvate consumption between cleaved and uncleaved embryos in each of the different environments was not statistically significantly different (Figure 5.29_Appendix).

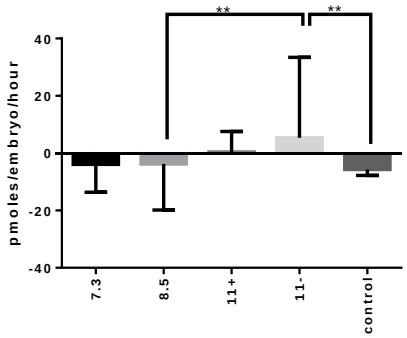
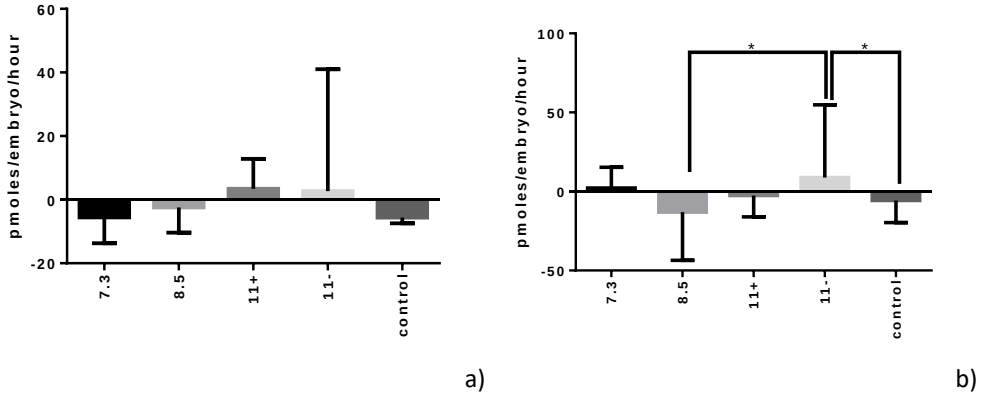


Figure 5.6.: Pyruvate consumption/production by cleavage stage embryos. n=16-18 depending on the availability of the drop at the time of the assay.



a) b)

Figure 5.7.: Pyruvate consumption/production by cleavage stage embryos a) embryos that cleaved (n=9-13), b) embryos that didn’t cleave (n=5-9).

5.4.3. Hyperglycaemic embryo culture conditions significantly modify the rates of amino acid consumption/production at the cleavage stage

The rates of amino acid consumption/production by embryos generated under normo- and hyperglycaemic embryo culture conditions are summarised in Figure 5.8. Due to the volume of data generated, these are presented in subsequent figures to highlight findings of note.

Figure 5.9 shows the mean amino acid profile of embryos from the control group. These data showed that the embryos under control conditions consumed arginine and produced alanine, which, as discussed later, is in agreement with previous studies, and therefore reinforces the validity of the data produced and the subsequent comparisons.

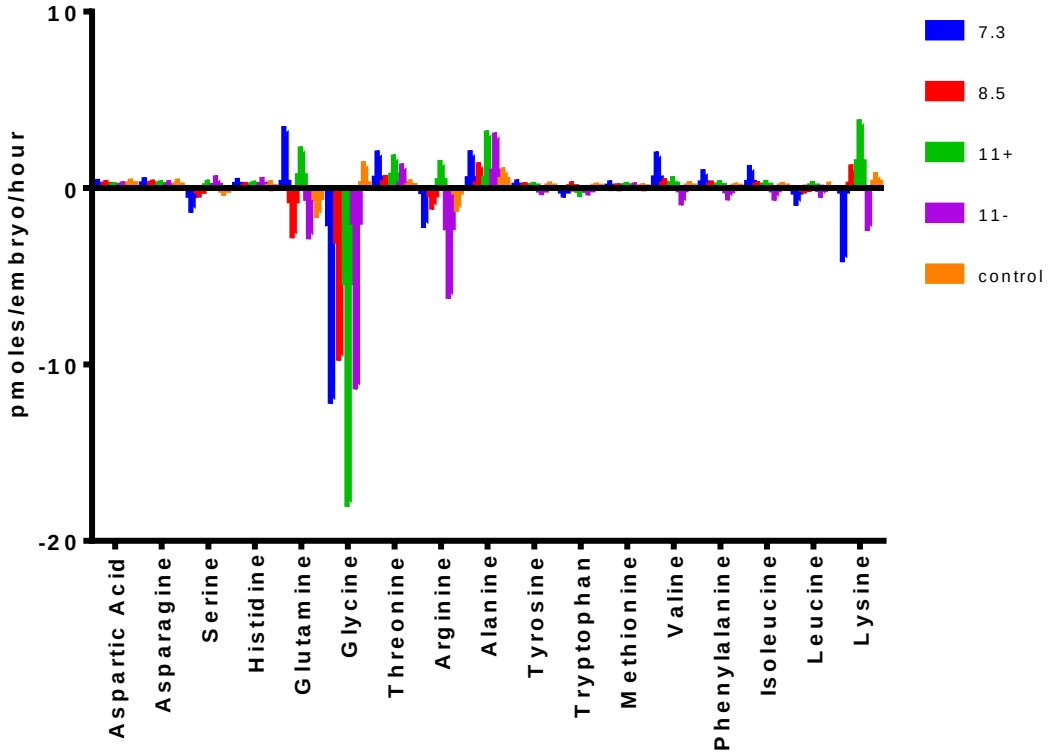


Figure 5.8.: Amino acid consumption/production rates for modified individual culture conditions at the cleavage stage. n=16-18 according to culture drop availability.

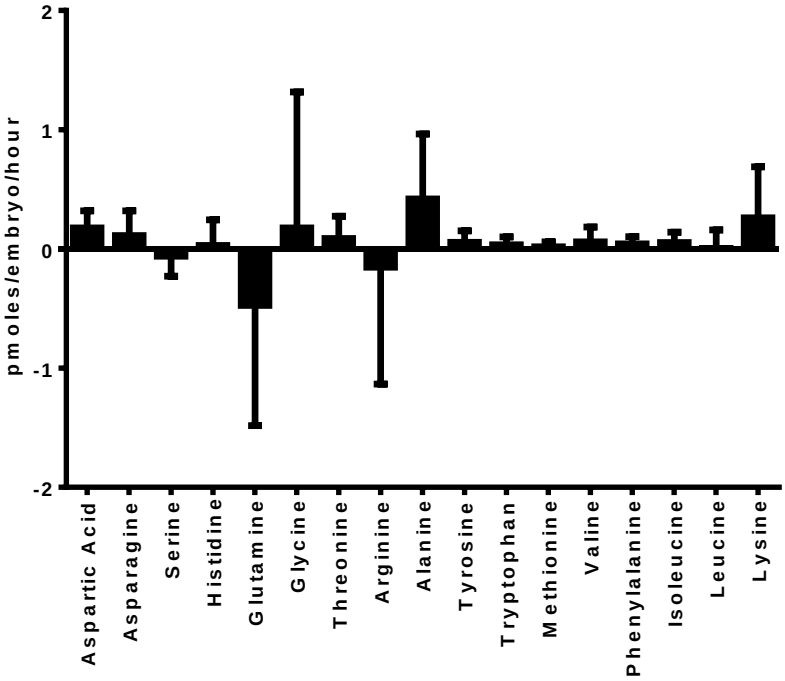
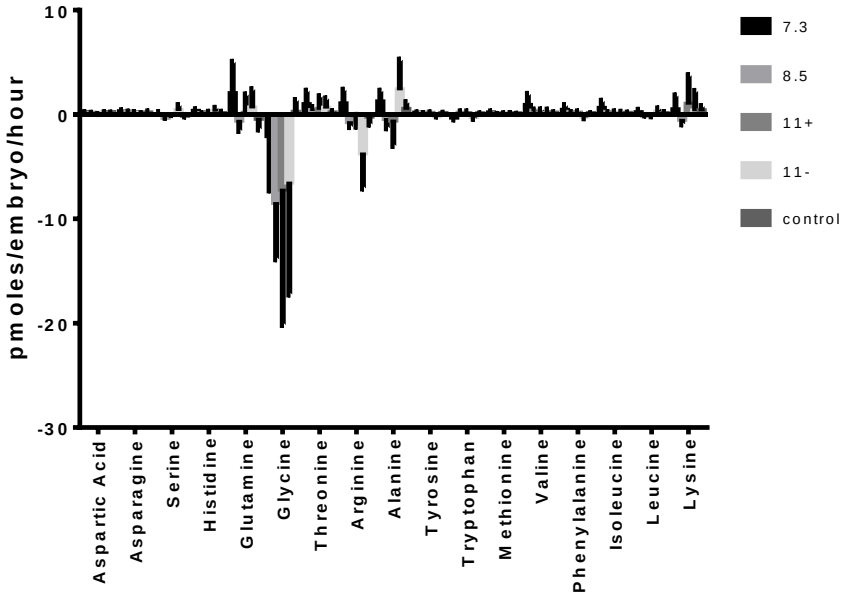
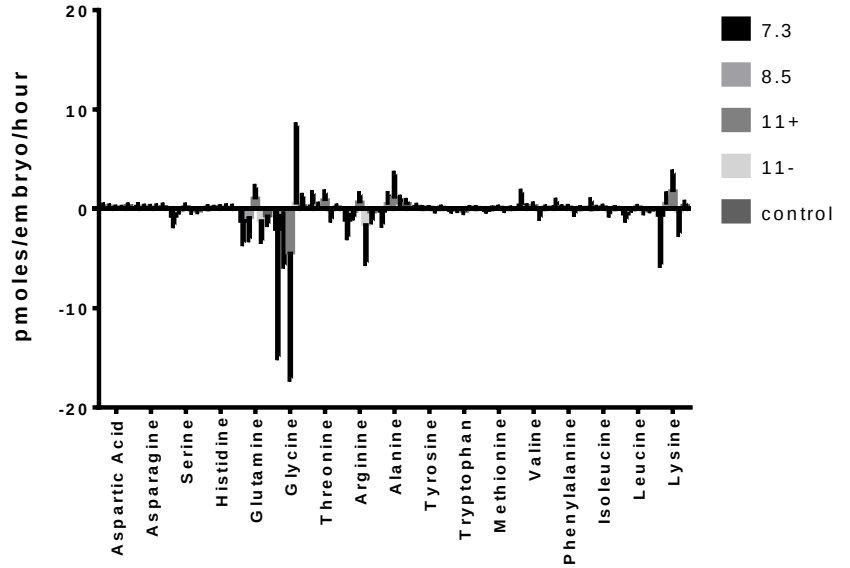


Figure 5.9.: Rates of amino acid consumption/production of embryos grown under control conditions at cleavage stage (n=18)

The embryo populations were further divided into cleaved and uncleaved embryos (Figures 5.10, 5.11). However, there were no differences in the amino acid consumption/production between the embryos that cleaved and the embryos that did not cleave (Figure 5.11), with the exception of glycine, which showed different rates of consumption in the '8.5' and '11-' environments (Figure 5.11.b,d). Assessment of glycine should therefore be performed both in the pooled embryo population and when embryos are split into cleaved and uncleaved. Statistical evaluation of all other amino acids was performed using the pooled embryo population.

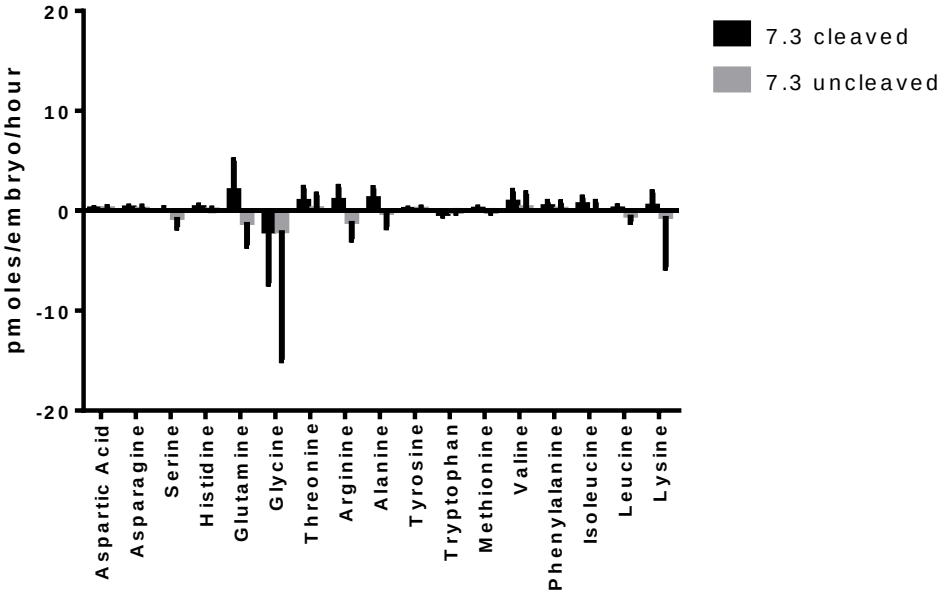


a)

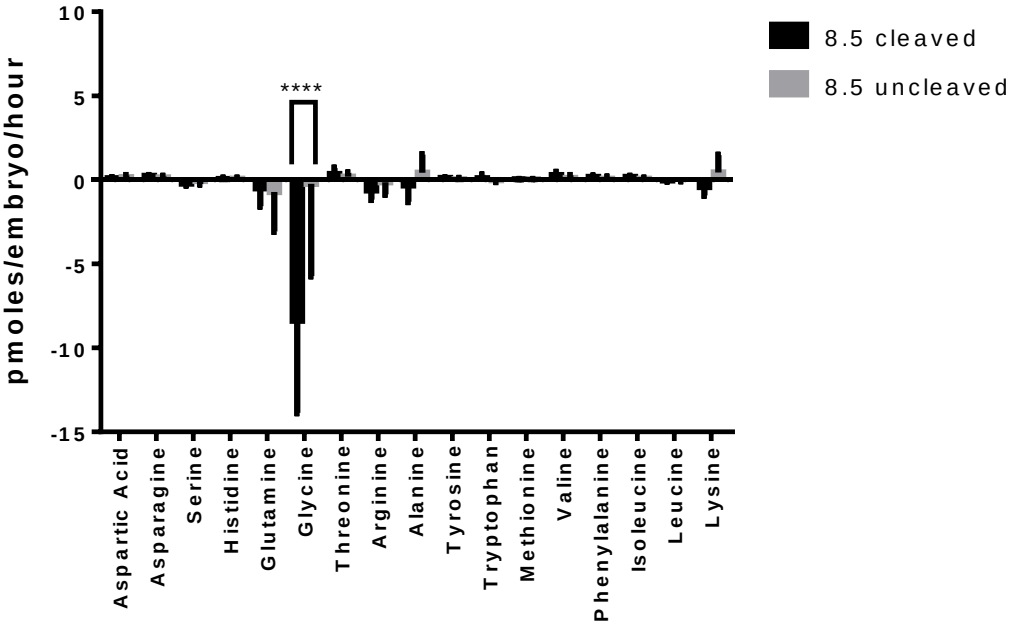


b)

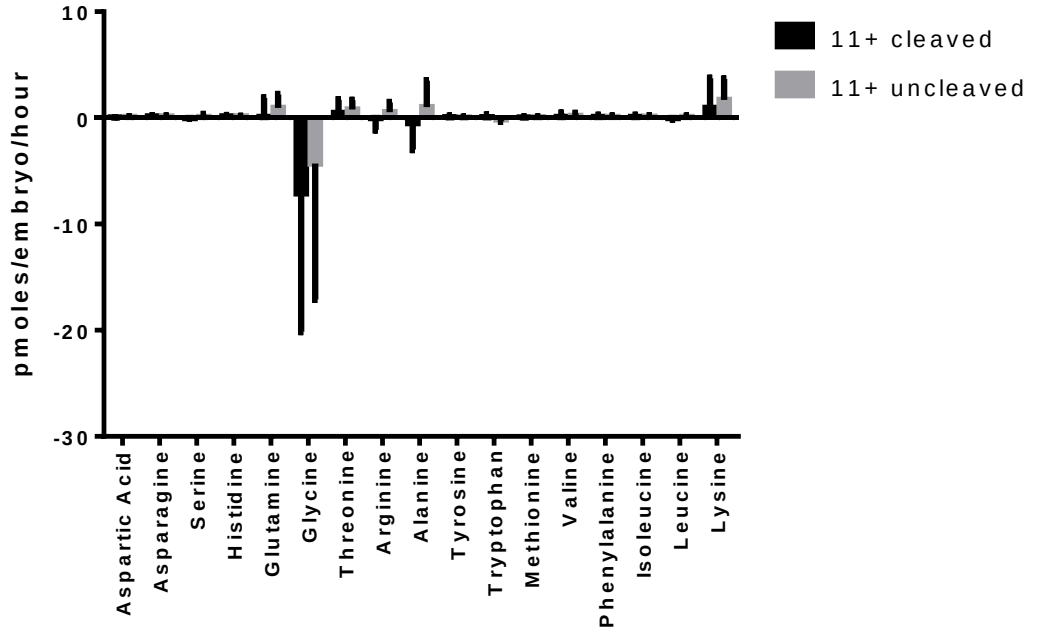
Figure 5.10.: Amino acid consumption/production rates for embryos grown in medium based on the secretions of cells exposed to normo- and hyperglycaemic conditions, for cleavage-stage embryos, a) for embryos that cleaved (n=5-8 depending on culture drop availability), b) for embryos that did not cleave (n=10-11 depending on culture drop availability)


7.3 cleaved
7.3 uncleaved
pmoles/embryo/hour
20
10
0
-10
-20
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

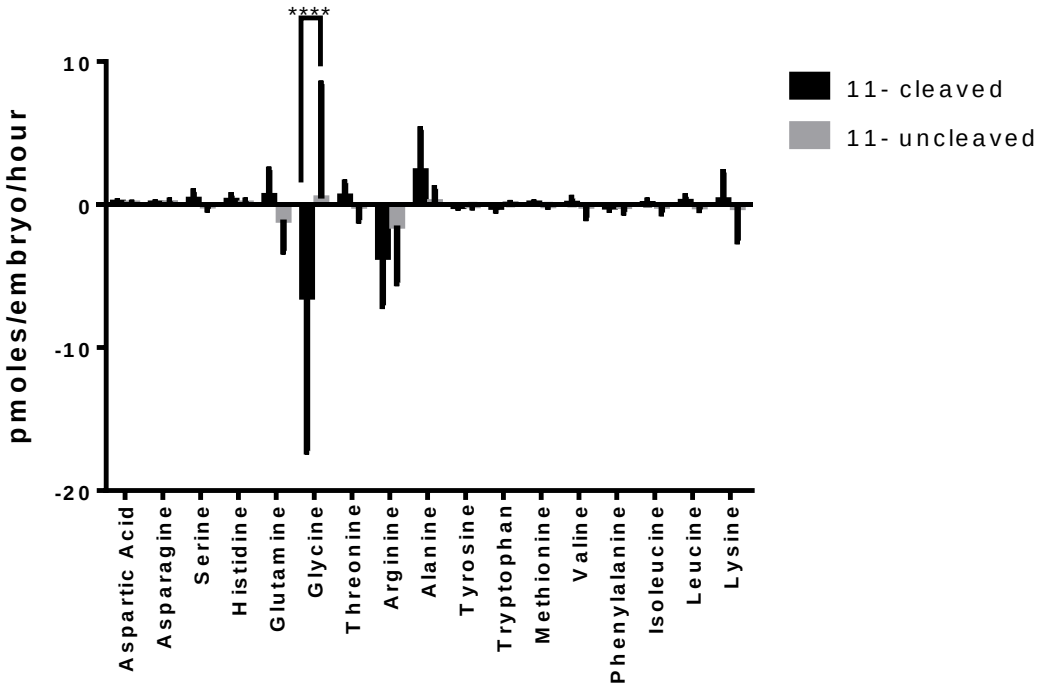
a)


8.5 cleaved
8.5 uncleaved
pmoles/embryo/hour
****
10
5
0
-5
-10
-15
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

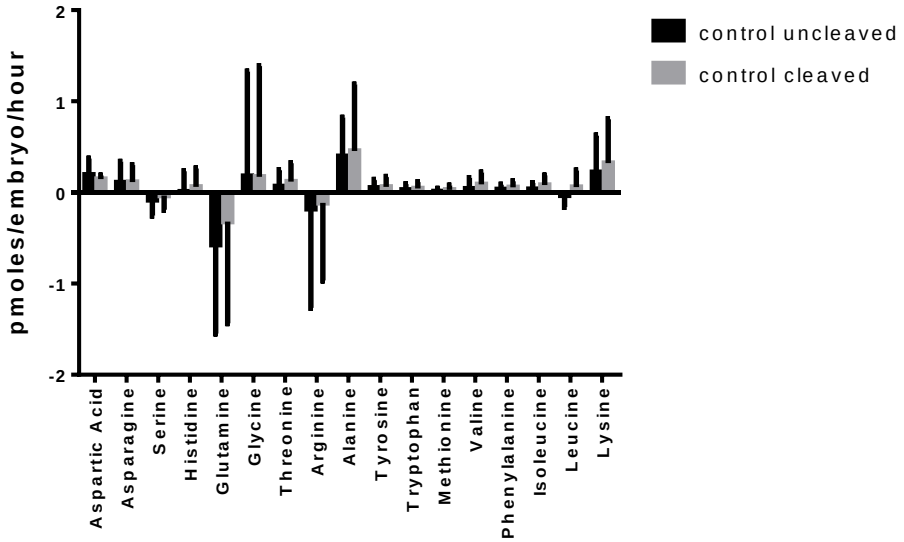
b)


10
0
-10
-20
-30
pmoles/embryo/hour
11+ cleaved
11+ uncleaved
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

c)


****
10
0
-10
-20
pmoles/embryo/hour
11- cleaved
11- uncleaved
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

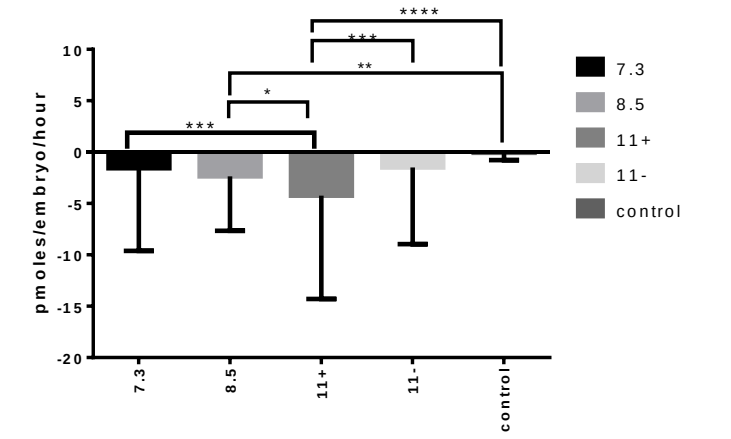
d)



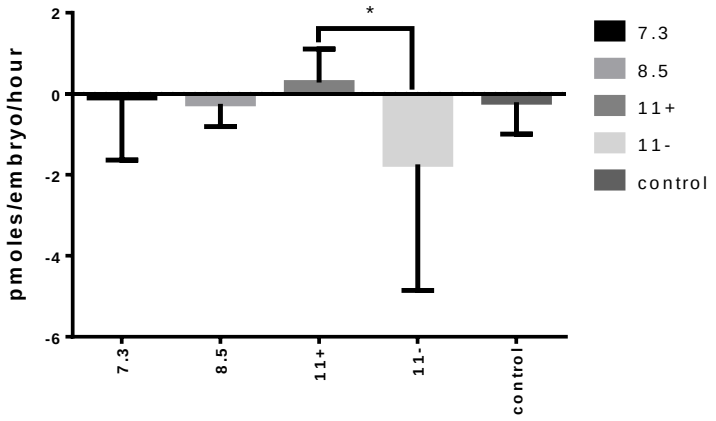
e)

Figure 5.11: Embryo population segregation into embryos that cleaved (n=5-8 depending on culture drop availability) and embryos that did not cleave (n=10-11 depending on culture drop availability) for a) '7.3', b) '8.5', c) '11+' d) '11-' and e) control embryo environments.

The impact on amino acid consumption/production of growing embryos in media whose composition was informed by the action of hyperglycaemia and insulin on BOEC secretions was next explored (Figures 5.12-5.13). Of note, when the whole embryo cohort was assessed, depletion of glycine was higher in the '11+' conditions compared to all other conditions (Figure 5.12a). Similarly, glycine consumption was increased in the 8.5 group compared to control (Figure 5.12a). Arginine was produced by embryos grown under '11+' conditions, as opposed to all other conditions, and this difference was statistically significant compared to the '11-' environment (Figure 5.12b).



a)

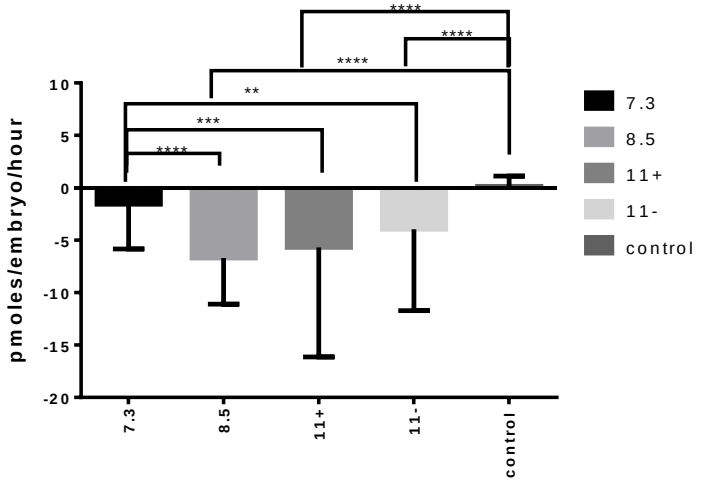


b)

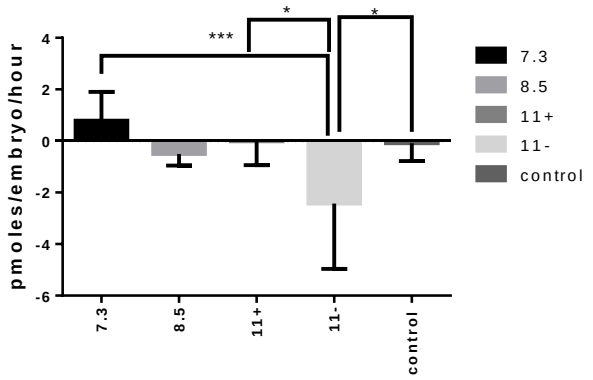
Figure 5.12.: Glycine (a) and arginine (b) consumption/production rates under modified embryo culture conditions at cleavage stage. (n=16-18 depending on culture drop availability)

Glycine showed net depletion and had an interesting profile. The overall mean profile for glycine depletion did not appear to change when embryos that had arrested were excluded from analysis (Fig 5.13a). An exception to this was seen in the control group. When all embryos were included, there was a mean depletion of glycine; by contrast when only those embryos that cleaved during the culture duration were analysed there appeared to be a net mean production of glycine. Overall, embryos exposed to culture conditions based on the secretions from BOECs exposed to elevated glucose depleted glycine in the greatest amounts (Fig 5.12,5.13). No difference was found between cleaved and uncleaved embryos in any of the conditions examined. However, the results of this analysis must be interpreted with caution, as most of the values were not significantly different from zero, regardless of whether the entire embryo population or the segregated populations were assessed, with the exception of the arginine

consumption in the ‘11-’ conditions and glycine consumption in cleaved embryos in the ‘8.5+’ environment.



a)



b)

Figure 5.13.: Rates of glycine (a) and arginine (b) consumption/production under normo- and hyperglycaemic group embryo culture conditions for cleavage stage embryos, when only cleaved embryos are assessed. (n=5-8 depending on culture drop availability)

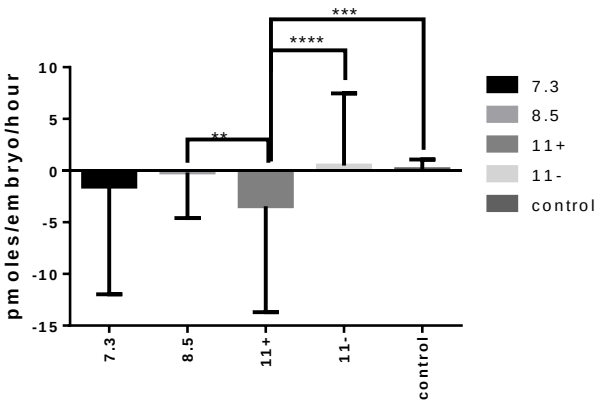


Figure 5.14.: Rates of glycine consumption/production under normo- and hyperglycaemic group embryo culture conditions for cleavage-stage embryos, when only uncleaved embryos are assessed. (n=10-11, depending on culture drop availability)

Arginine also presented an interesting profile. When all embryos were assessed, this amino acid was marginally consumed/produced in all treatments with the exception of '11-', where it as depleted in comparatively high amounts (Figure 5.12b). However, when only cleaved embryos were assessed, arginine was produced by embryos in the '7.3' conditions, whereas it was consumed in all other conditions (Figure 5.13b). This is similar to what was observed when all embryos were included, with the exception of the '11+', which no longer showed a small net arginine production when only cleaved embryos were assessed. Similar to glycine, the results of this analysis must be interpreted with caution, as only the 11- was significantly different from zero in the overall embryo population, as well as in the cleaved embryos.

Finally, the amino acid profiles of those embryos that did not cleave during the period were scrutinised. Only glycine consumption/production differed across the treatments, where embryos that remained developmentally static in medium based on secretions from BOECs exposed to 11mM glucose, as well as controls, secreted small amounts of glycine. This amino acid was consumed by embryos in all other treatments (Figure 5.14). Glycine consumption by the 11+ modified group was significantly different compared to the '8.5' ($p<0.01$), '11-' ($p<0.0001$) and control ($p<0.001$) treatments.

5.4.4. Hyperglycaemic culture conditions do not affect the blastocyst rates of the embryos

The blastocyst rates were unaffected by the culture conditions (Figure 5.15). Exposure to the '8.5' and '11+' environments appeared to lead to improved blastocyst rates, however, these did not reach significance. Notably, blastocyst production was highly variable. In an attempt to mitigate variability, the effect of adding of FBS to the embryo culture media was tested, however, this did not have a notable effect on outcomes (Figure 5.26_Appendix). This experimental approach was therefore not pursued.

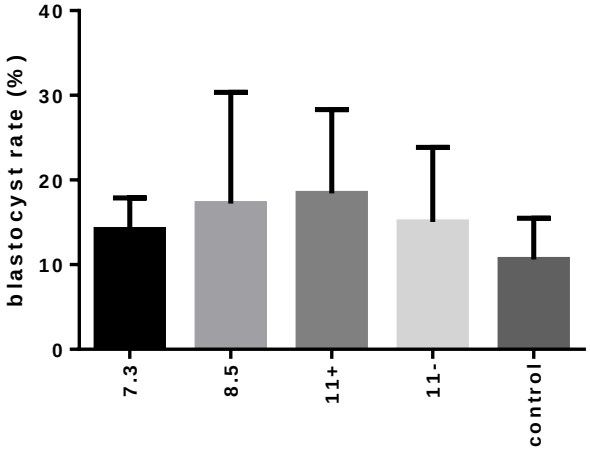


Figure 5.15.: Blastocyst rates under normo- and hyperglycaemic modified embryo culture conditions. n=4-5, depending on embryo availability

In addition, owing to tissue limitations, it was decided to focus on amino acid consumption/ production for blastocyst-stage embryos, the formation of which is an accepted marker of embryo quality (Sturmey *et al.*, 2010).

5.4.5. Amino acid consumption/production by blastocysts is significantly modified in response to a modified embryo culture environment

The pattern of amino acid consumption/production of embryos grown in an environment based on the secretions of BOECs exposed to normo- and hyperglycaemic conditions was assessed (Figures 5.16, 5.17).

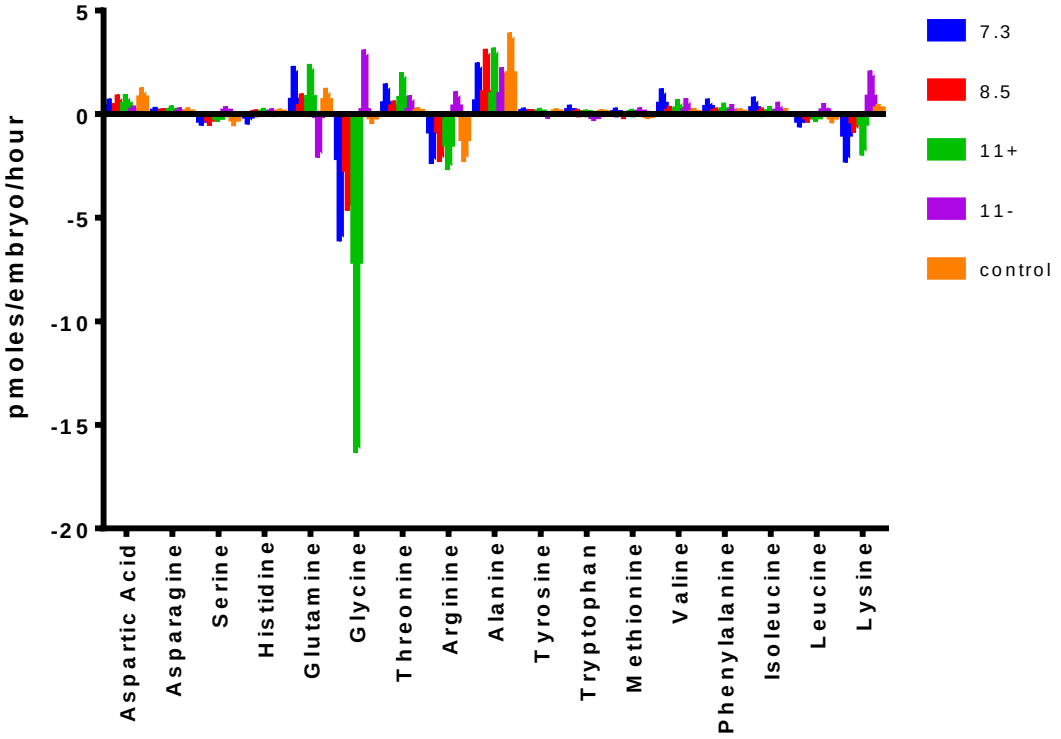


Figure 5.16.: Amino acid consumption/production of blastocyst stage embryos grown in media whose composition was based on the secretions of BOECs cultured in conditions designed to mimic normo- and hyperglycaemia. n=8-12 depending on number of blastocyst stage embryos.

As with the data from cleavage stage embryos, arginine was consumed and alanine was produced by the embryos under control conditions (Figure 5.17).

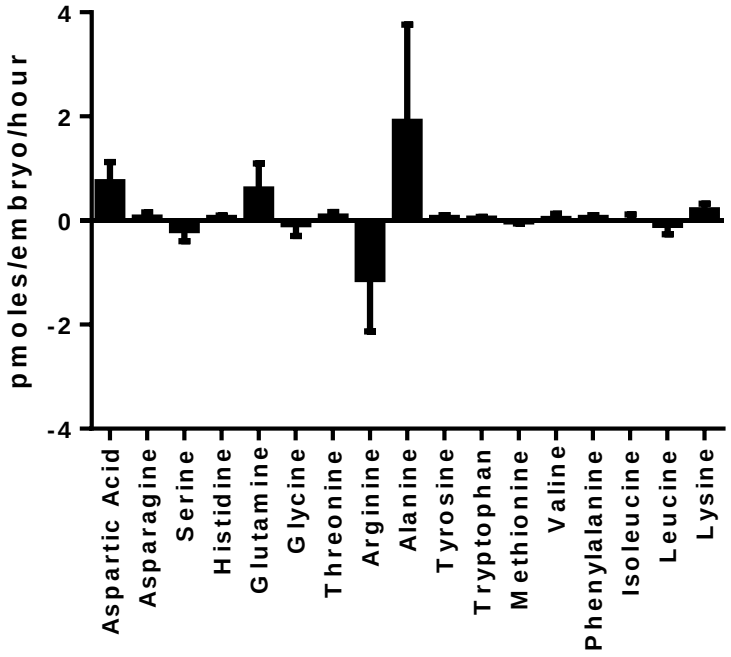
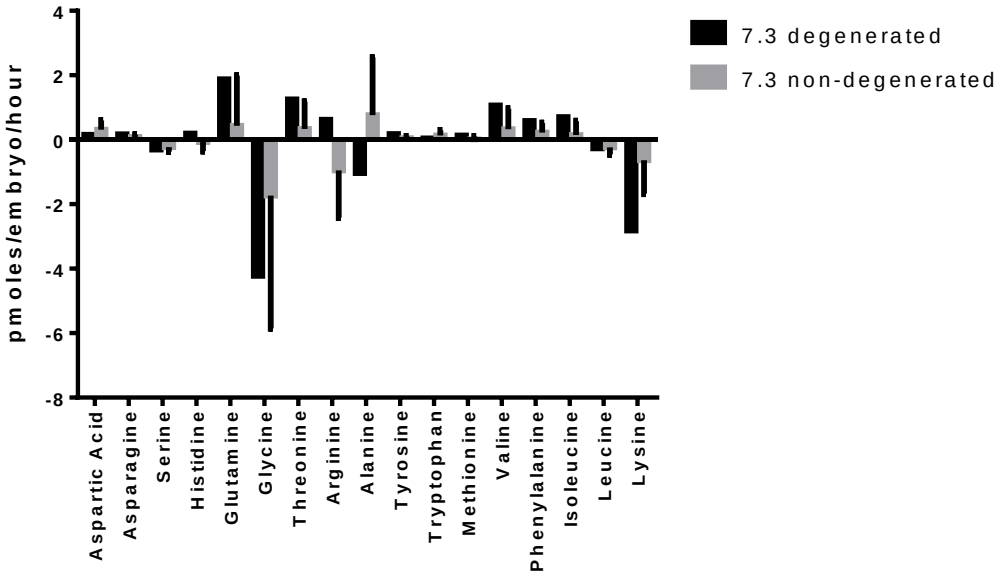
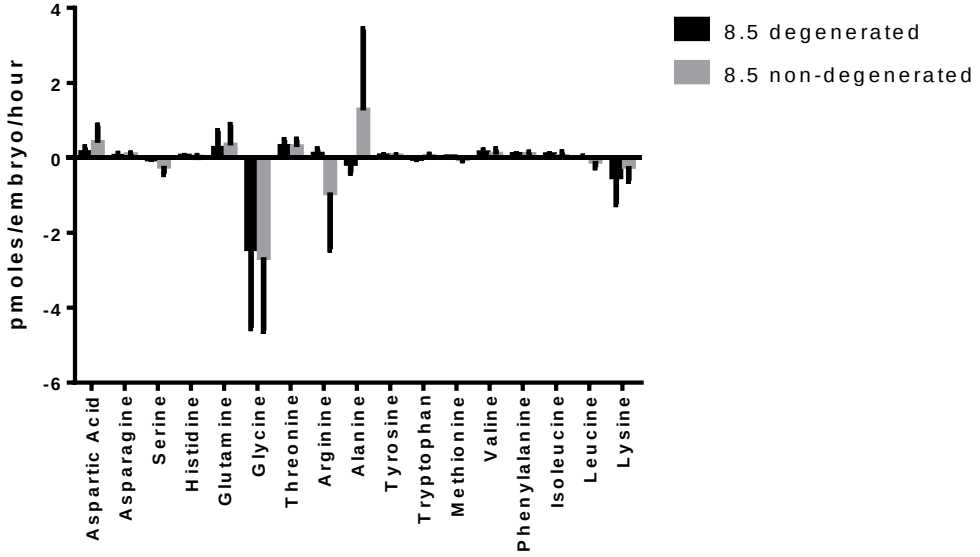


Figure 5.17.: Amino acid consumption/production rates under control conditions in blastocyst stage embryos. n=12 embryos

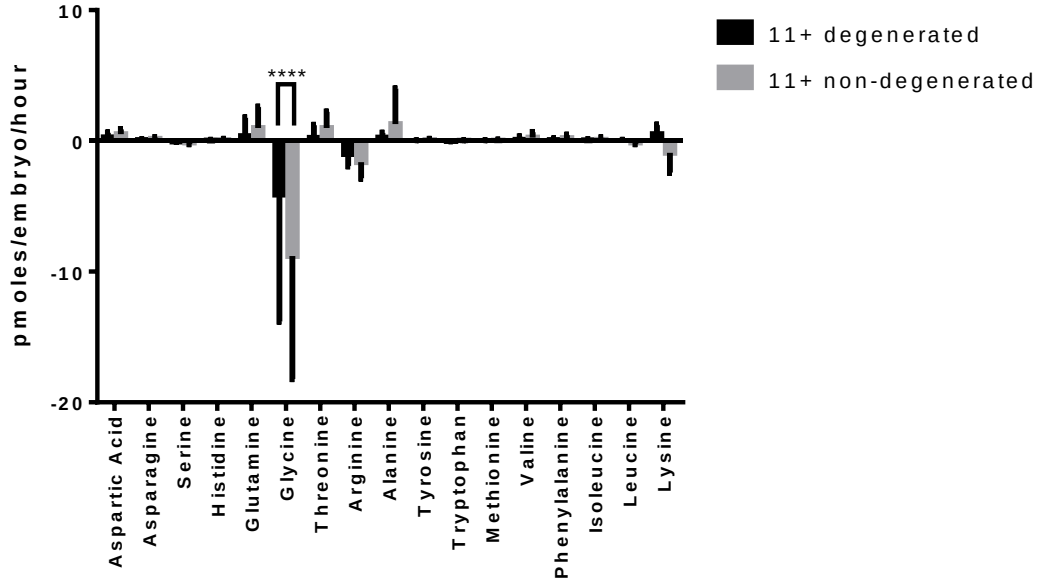
Similar patterns of amino acid consumption were observed when blastocysts that either progressed or remained developmentally static, as well as when blastocysts remained or did not remain viable, were assessed (Figures 5.18, 5.19). Since glycine was significantly modified for the '11+' treatment when embryos were segmented to degenerate and non-degenerate (Figure 5.18c) and embryos that progressed and did not progress (Figure 5.19c), this amino acid was assessed separately between treatments in these populations. Alanine was also observed to be different between embryos that progressed and did not progress in the '8.5' environment, however it was not assessed separately because it was not found to be significant when all treatments were compared (Figure 5.19b).


7.3 degenerated
7.3 non-degenerated
pmoles/embryo/hour
4
2
0
-2
-4
-6
-8
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

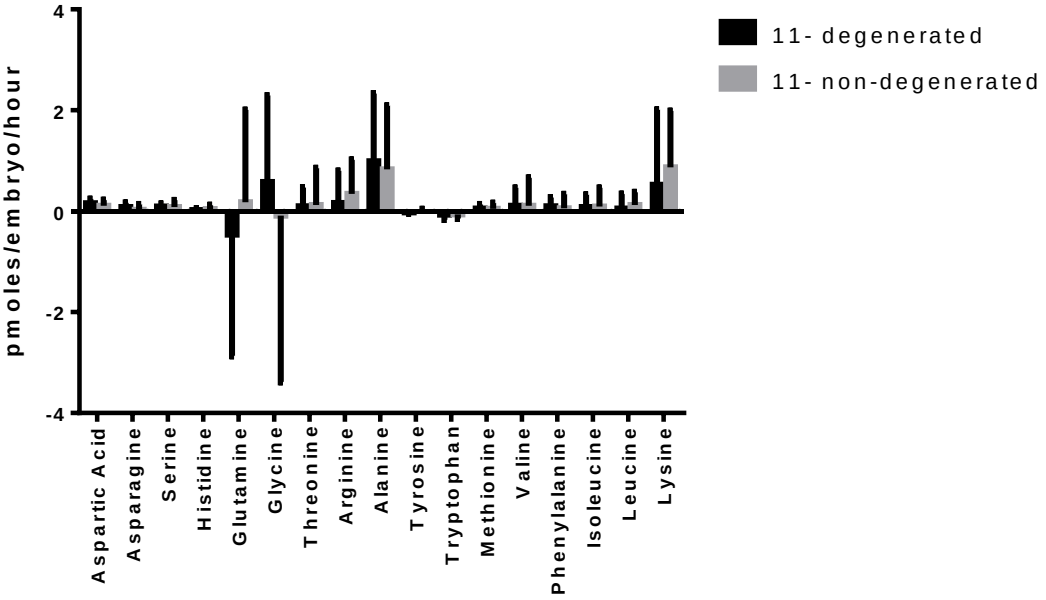
a)


8.5 degenerated
8.5 non-degenerated
pmoles/embryo/hour
4
2
0
-2
-4
-6
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

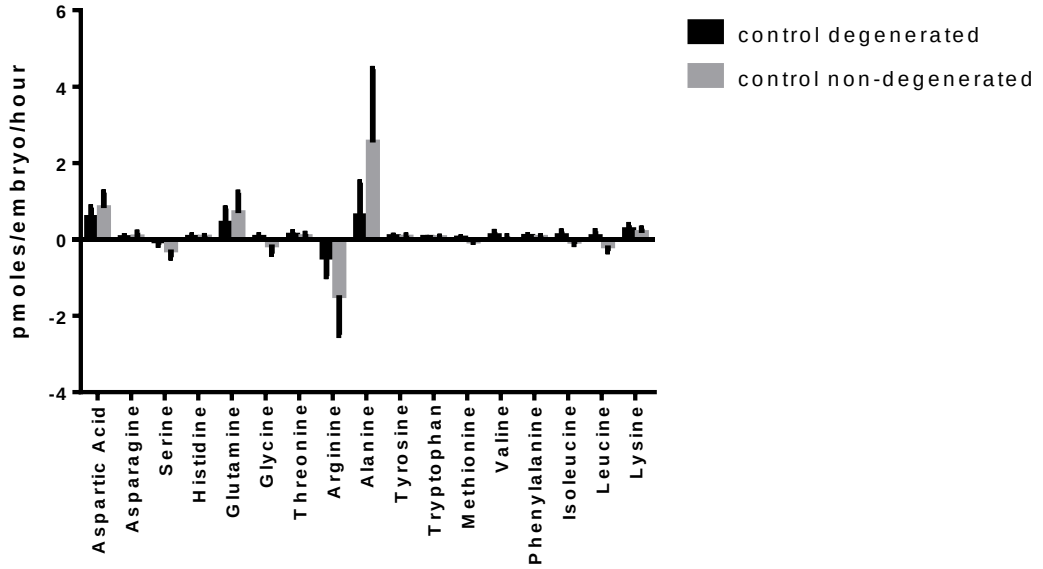
b)


10
0
-10
-20
pmoles/embryo/hour
****
11+ degenerated
11+ non-degenerated
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

c)

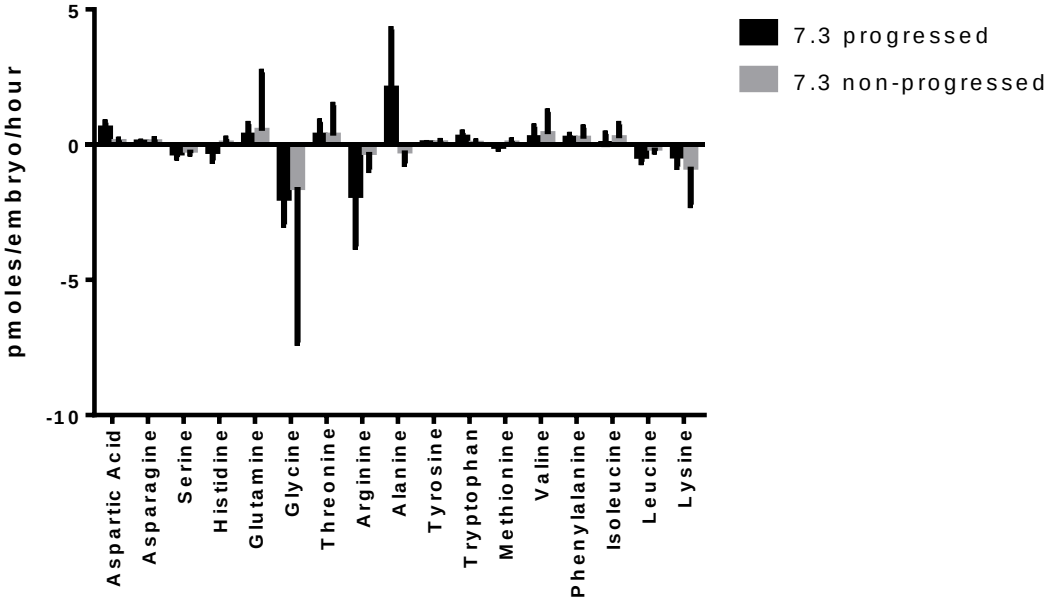

4
2
0
-2
-4
pmoles/embryo/hour
11- degenerated
11- non-degenerated
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

d)

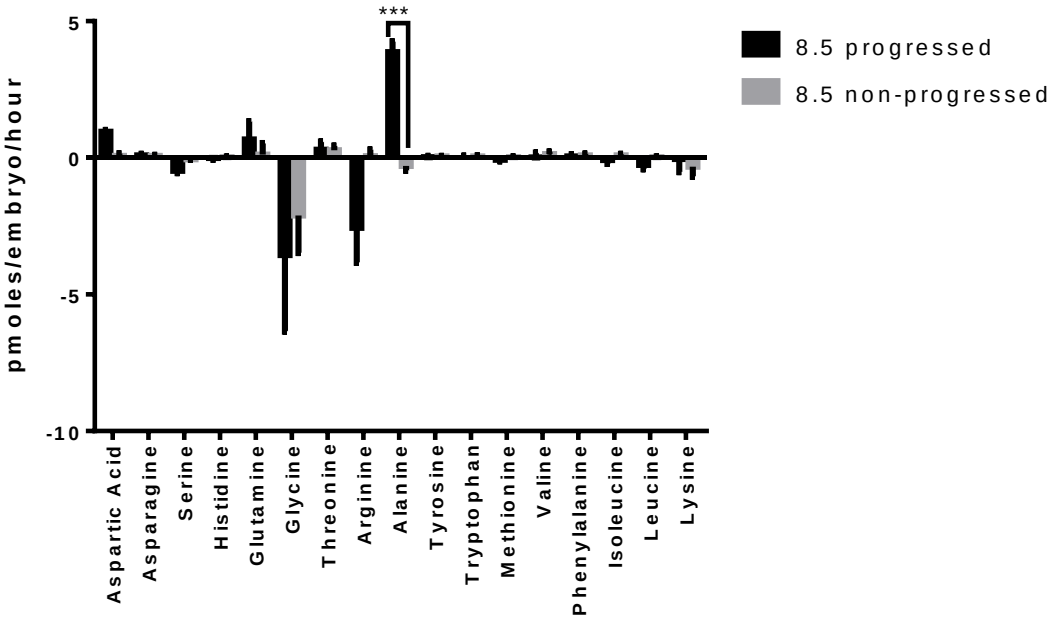


e)

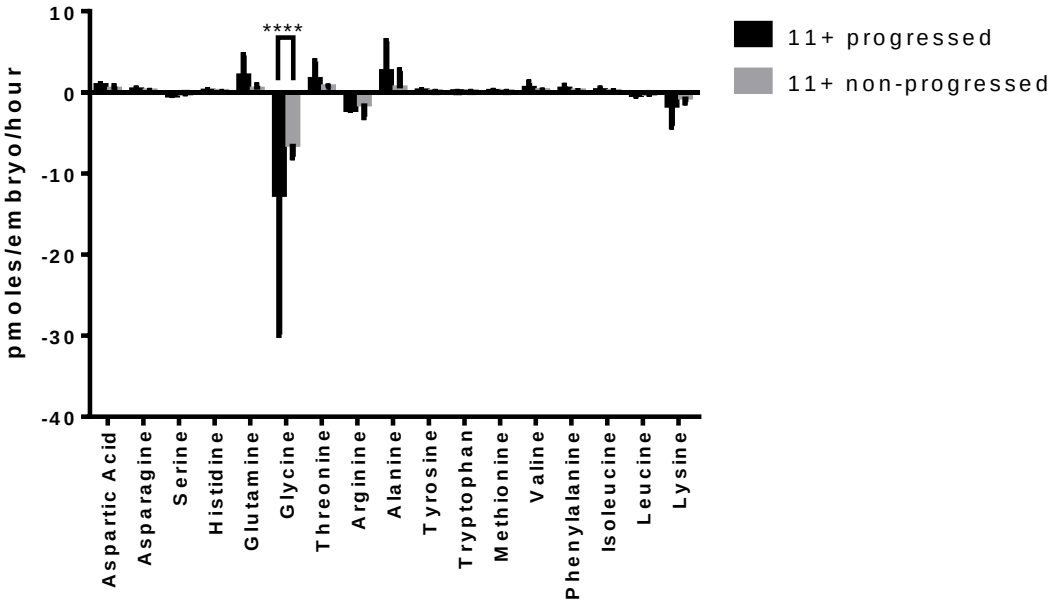
Figure 5.18: Embryo population segregation into blastocysts that degenerated (n=1-4 depending on embryo availability) and blastocysts that did not degenerate (n=5-8 depending on culture drop availability) for a) '7.3', b) '8.5', c) '11+' d) '11-' and e) control embryo environments.



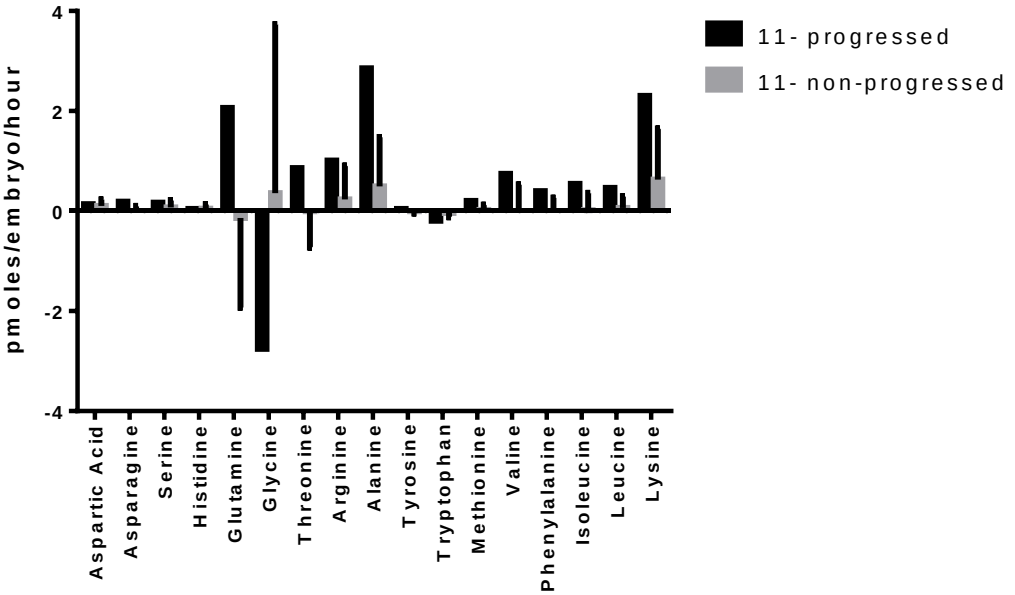
a)


***
5
0
-5
-10
pmoles/embryo/hour
8.5 progressed
8.5 non-progressed
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

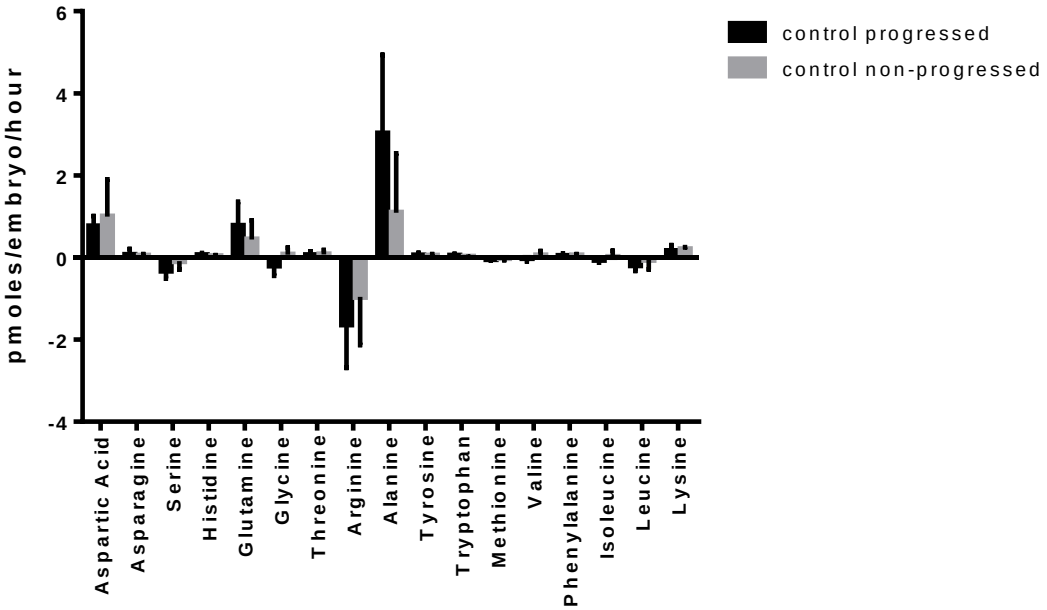
b)


****
10
0
-10
-20
-30
-40
pmoles/embryo/hour
11+ progressed
11+ non-progressed
Aspartic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine

c)



d)



e)

Figure 5.19.: Embryo population segregation into blastocysts that progressed (n=1-6 depending on embryo availability) and blastocysts that did not progress (n=2-6 depending on culture drop availability) for a) '7.3', b) '8.5', c) '11+' d) '11-' and e) control embryo environments.

The depletion/ appearance of glycine, arginine and lysine was influenced in blastocysts cultured in embryo culture modified according to oviduct secretions in hyperglycaemic conditions and in the presence or absence of insulin (Figure 5.20). Specifically, glycine was found to be marginally consumed by control embryos and marginally produced by embryos cultured in the '11-' environment, both of which differed significantly to '7.3',

'8.5' and '11+' conditions, where glycine was consumed. Embryos grown under the '11+' conditions, depleted glycine in high amounts and was significantly different to all other conditions ($p<0.0001$, Figure 5.20a). On the other hand, arginine was consumed in all treatments, apart from the '11-' conditions, where it was produced, a difference which was statistically significant to the '11+' conditions ($p<0.05$, Figure 5.20b). Lysine depletion was significantly different from zero in the '11+' and control environments respectively, however this was not the case in the rest of the treatments. Finally, lysine production was observed for the '11-' - modified and control treatments, an inverse of the pattern observed for the '7.3', '8.5' and '11+' -modified treatments (Figure 5.20c). Interestingly, the rates of consumption/ production of all three amino acids presented a similar pattern, in that, in all cases, the amino acids were produced in the '11-' treatment, but consumed in all other treatments, with the exception of lysine, which was also produced in the control treatment.

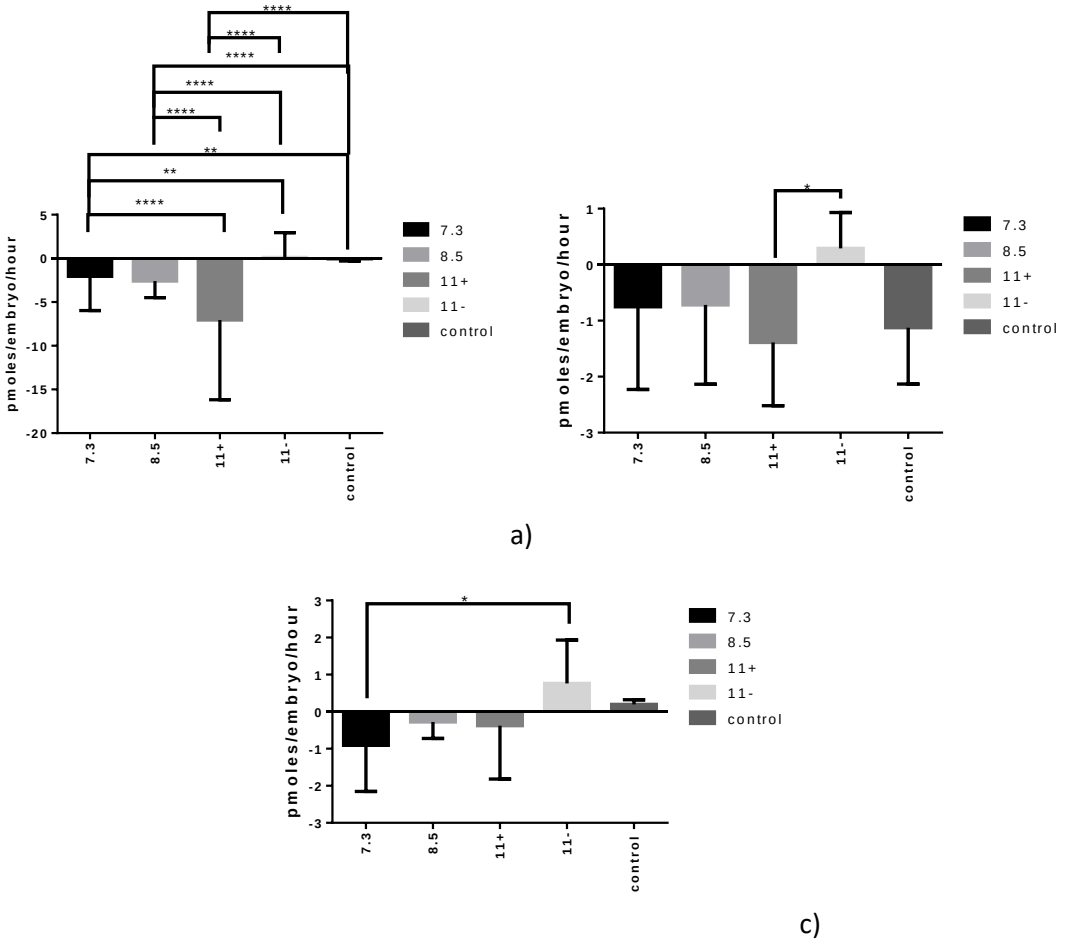
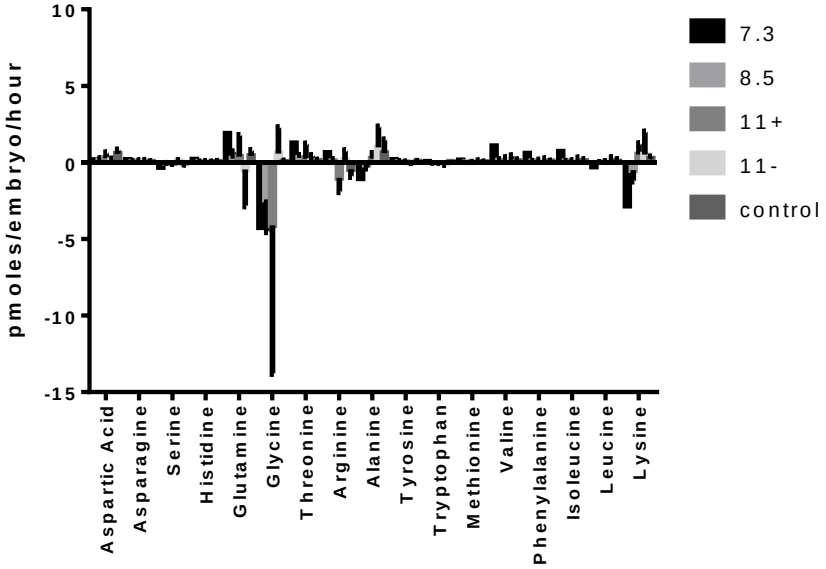
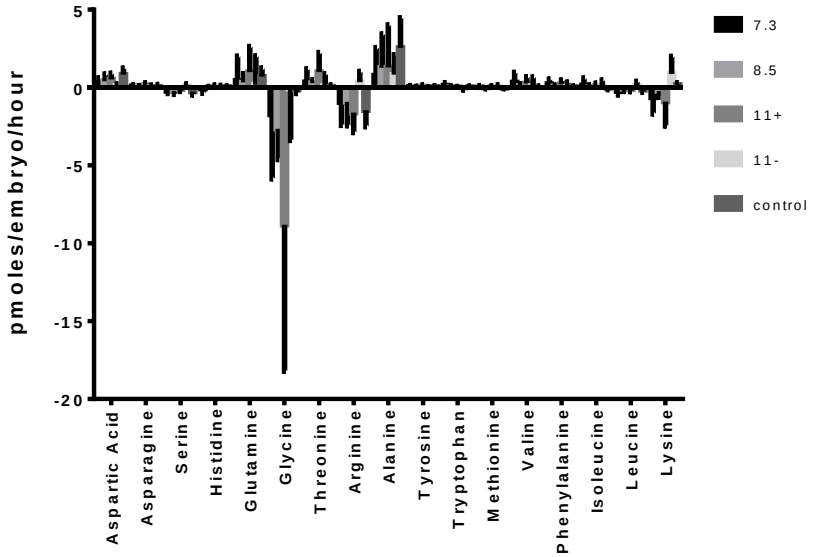


Figure 5.20: Glycine (a), arginine (b) and lysine (c) consumption/production rates under modified embryo environment. n=8-12 depending on number of blastocyst-stage embryos

The amino acid profiles of embryos that had degenerated differed to those developed in culture (Figures 5.21,5.22). In embryos that degenerated during the assay period, glycine consumption was significantly different in the '7.3' ($p<0.05$) and '11+' ($p<0.001$) conditions from glycine production in the '11-' and control conditions, even though glycine production in these two latter cases was marginal. The rates of glycine consumption/ production were not significantly different from zero in degenerated embryos (Figure 5.22a). Similarly, in embryos that developed in culture, the patterns of amino acid consumption/production were similar to the overall results. Glycine was marginally consumed by all viable embryos, and the only difference observed compared to Figure 5.21 was the marginal glycine embryo consumption rather than production by the 11- -modified treatment (Figure 5.22b). Glycine consumption was significantly different from zero in the '8.5' environment. Arginine followed the same pattern as in Figure 5.20, and consumption under control conditions were significantly different from zero (Figure 5.22c).

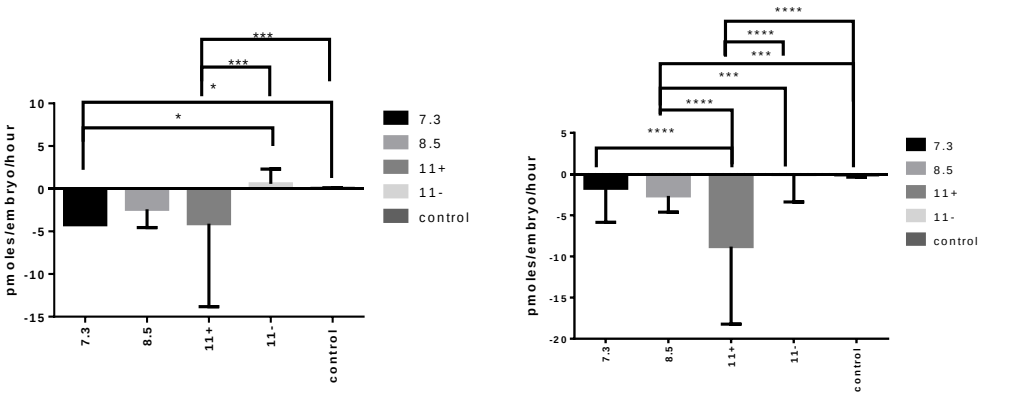


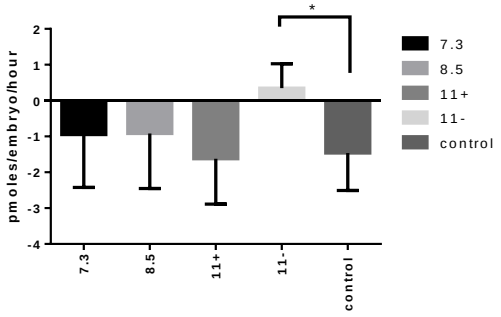
a)



b)

Figure 5.21.: Rates of amino acid consumption/production for blastocyst stage embryos. a) degenerate embryos- all amino acids b) non-degenerate embryos – all amino acids, n=1-4 for degenerate embryos, n=5-8 for non-degenerate, depending on embryo availability

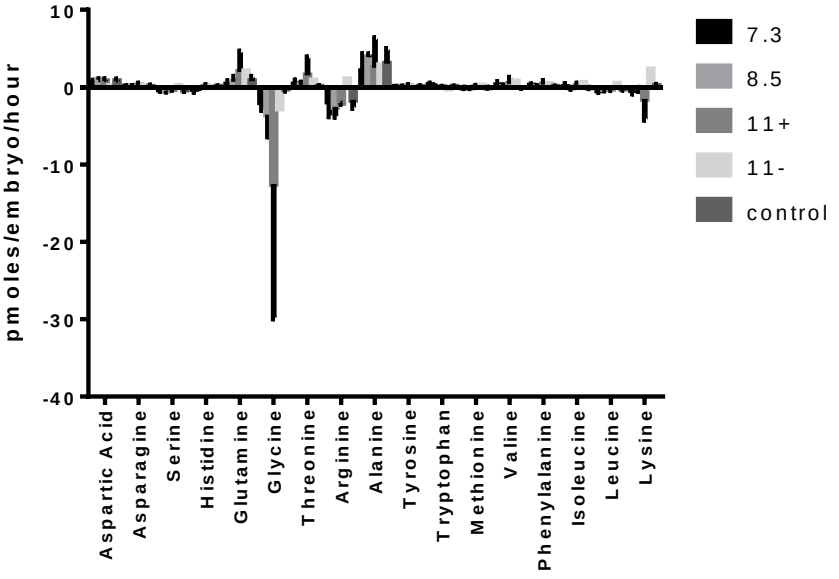




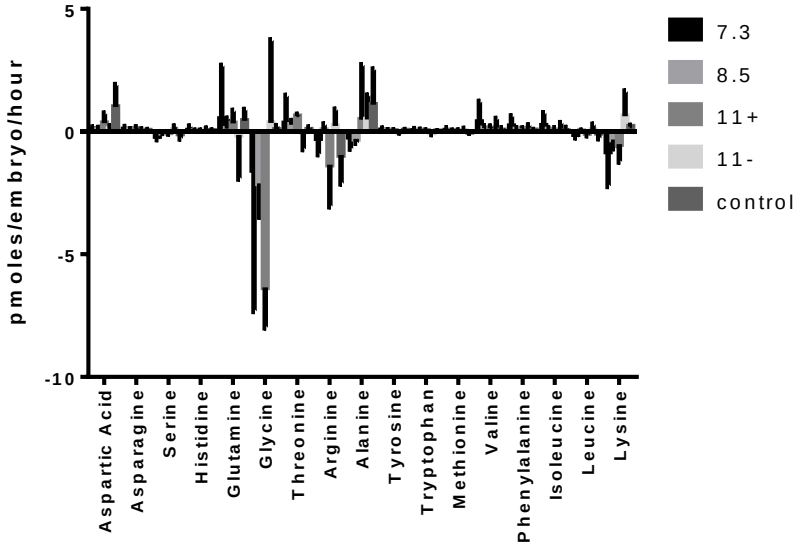
c)

Figure 5.22.: Rates of amino acid consumption/production for blastocyst-stage embryos. a) glycine rates for degenerated embryos, b) glycine rates for non-degenerated embryos, c) arginine rates for non-degenerated embryos. n=1-4 for degenerated embryos, n=5-8 for non-degenerated, depending on embryo availability

Finally, when the embryos that did not degenerate were assessed in terms of developmental progression (Figures 5.23, 5.24), the only amino acids that appeared to be significantly affected was glycine, which was depleted in embryos in the '11+' conditions compared to the other conditions. However, in most cases, with the exception of glycine consumption in embryos that did not progress in the '8.5' and '11+' environments, the values were not significantly different from zero.



a)



b)

Figure 5.23.: Rates of amino acid consumption/production of blastocyst-stage embryos that survived for the duration of the culture: a) embryos that progressed – all amino acids, b) embryos that did not progress – all amino acids. n=2-6 for embryos that did not progress, depending on embryo availability.

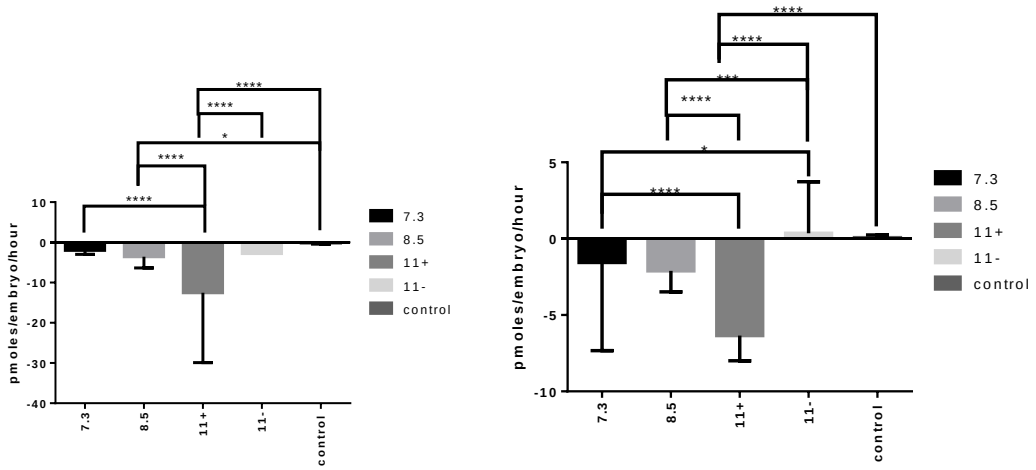


Figure 5.24.: Rates of glycine consumption/production of blastocyst-stage embryos that survived for the duration of the culture: a) embryos that progressed, embryos that did non progress, n=1-6 for embryos that progressed, n=2-6 for embryos that did not progress, depending on embryo availability.

Glycine was depleted from the medium in cases when the embryo progressed, with highest rates observed for embryos grown in the '11+' conditions. Additionally, glycine consumption by these embryos was higher than that of controls. On the other hand, in embryos that did not progress, glycine was marginally produced by embryos in the '11-' and control conditions, whereas it was consumed in the '7.3', '8.5' and '11+' conditions, and these differences in the patterns of glycine consumption/production were statistically significant.

5.5. Discussion

Culturing BOEC cells in conditions designed to mimic mild and severe hyperglycaemia has an impact on the nutrient composition of the fluid created (Chapter 4). However, to understand the importance of these data, it was necessary to explore the effect of exposing the early embryo to such conditions. Endpoints were the ability to reach the key developmental stages of 2-cell and blastocysts, and consumption and production of nutrients under different embryo culture conditions. Overall, the modified environments did not have a major effect on embryo cleavage or blastocyst formation, however the nutrient turnover was significantly affected by the modified embryo culture conditions, mainly in terms of the rates of glucose, pyruvate, lactate and glycine consumption/production.

5.5.1. Cleavage rates and blastocyst rates were unaffected by modified embryo environment

Embryos were exposed to 5 different environments, the composition of 4 of which were based on BOEC secretory activity. None of the modified environments led to a change in the rates of cleavage or blastocyst formation. Interestingly, embryos grown in a medium based on the secretions of cells grown with 7.3mM glucose in the absence of insulin tended towards higher cleavage rate than the control conditions, however, these did not reach significance; therefore, repetition of this experiment at an appropriate power would clarify whether this difference observed was statistically valid.

The cleavage and blastocyst rates were lower than those normally expected. One explanation for this might be the timing of the experiment, which was performed during summer 2018, which was unseasonably warm (MetOffice, 2018). It is therefore plausible that the oocytes used for these experiments had been collected from animals that were heat stressed. There is literature studying the effects of heat stress on high production cattle (Alves *et al.*, 2013; Ahmed *et al.*, 2017; Abdelatty *et al.*, 2018). As well as the impacts on the animal, there are well-described effects of heat stress on the developmental competence of the oocyte (Ahmed *et al.*, 2017), and the overall embryonic development, in terms of progression to blastocyst (Alves *et al.*, 2013).

Despite this, the fact that there were no differences observed between treatment groups and study groups, leads us to conclude with some confidence that the conditions under investigation do not noticeably affect embryo development.

5.5.2. Modified embryo environment altered the rates of glucose, lactate and pyruvate consumption and production in cleavage-stage embryos

The patterns of glucose, lactate and pyruvate consumption/production by cleavage stage embryos were affected as a result of culture in modified environments. Embryos in the control group consumed pyruvate and glucose and produced lactate; a pattern reported in a number of studies for the pre-implantation embryo (Thompson *et al.*, 1996; Leese *et al.*, 2007; Sturmey, Brison and Leese, 2008; Gardner *et al.*, 2011; Guerif *et al.*, 2013; Krisher *et al.*, 2015). To understand whether these data were influenced by the activity of embryos that did not cleave, the metabolic profile of embryos that failed to cleave with those that progressed was compared. In both categories the patterns of metabolic substrate consumption and production were similar, although in the case of glucose, there was a switch from marginal consumption to marginal production for cleaved embryos, whereas uncleaved embryos consumed significantly more glucose. However, as discussed in the results of this chapter, in all cases glucose consumption/production was not significantly different from zero and it would be difficult to account for a glucose secretion physiologically. This switch can also be attributed to the limitations of separating the embryos into categories, discussed later, in section 5.5.5.

Pyruvate consumption is associated with embryo viability (Conaghan *et al.*, 1993), and optimal embryo viability is associated with a limited range of pyruvate consumption (Guerif *et al.*, 2013). This is also illustrated in the *Goldilocks hypothesis*, described by Leese *et al.* for the preimplantation embryo (Leese *et al.*, 2016), where highest rates of developmental potential in the early embryo are observed within a specific range of nutrient profiles, which are neither too low nor too high. This hypothesis is a modified version of the previously described Quiet Embryo hypothesis, which suggested highest viability for embryos exhibiting lowest metabolism (Leese, 2002). Pyruvate consumption in the present study control group was very close to the range of pyruvate consumption

associated with increased embryo viability, however this was not the case for any of the other treatments. Even so, pyruvate consumption in the '7.3' and '8.5' environments followed very similar patterns to control conditions, although at lower levels, and the switch to pyruvate production observed in the '11+' and '11-' was not significantly different from zero. Consequently, repetition of this experiment with an increased number of embryos could determine whether the trends observed in pyruvate consumption/production by embryos cultured in environments based on the oviduct secretions are statistically significant.

Glucose consumption has also been linked with embryo viability; higher glucose consumption has been associated with a greater higher chance of a successful pregnancy compared to lower (Gardner and Leese, 1987; Gardner *et al.*, 2011). However, both studies reporting on this data measured the glucose uptake in blastocyst-stage embryos, and therefore this assumption may not apply to cleavage-stage embryos. On the other hand according to the Goldilocks hypothesis, described earlier, it may be hypothesized that higher rates of glucose consumption observed by the embryos cultured in the '11+' and '11-' environments could be related to lower viability and failure of these embryos to progress to later stages of development and implantation (Leese *et al.*, 2016). Given the extreme modifications of that environment compared to the environment of the control conditions, it can be speculated that high glucose consumption is associated with an increased rate of glycolysis, associated with embryos which are less metabolically healthy (Leese *et al.*, 2016). Given that the only treatment that was associated with glucose consumption significantly different from zero was the '11+', and keeping in mind that there are conflicting theories regarding the impact of glucose consumption rates on embryo viability, further experiments with increased n-numbers are required in order to establish the impact of culture conditions based on *iv*DOF secretions, on embryo viability and metabolism.

Finally, lactate production is known to follow a pattern that mirrors glucose consumption (Guerif *et al.*, 2013). As mentioned earlier, according to the Goldilocks hypothesis, high embryo metabolism, and in particular, high rates of glucose consumption are associated with low viability (Leese *et al.*, 2016). Given that high glucose consumption could be potentially associated with high rates of lactate production, through glycolysis, this could suggest low viability for the embryos cultured

in the ‘11+’ conditions. Due to the high variability in the rates observed in the other treatments, no safe conclusion can be drawn at this stage about them, and consequently, further experiments should be performed.

An interesting observation is the similarity between the ‘8.5’ and the control conditions. Indeed, culture in a medium that was based on the secretions of cells exposed to mild hyperglycaemia (‘8.5’ group) led embryos to display a pattern of substrate consumption which was very similar to the control conditions, however, lactate was marginally consumed when all embryos were collectively assessed and analysis restricted to cleaved embryos, yet it was marginally produced by uncleaved embryos. As mentioned in the previous paragraph, the data on lactate production should be assessed with caution, due to their high variability. The similarity between the modified conditions and control could be possibly attributed to the fact that, as mentioned in the Experimental Design (section 5.3) of this chapter, the ‘8.5’ environment was based on concentrations of nutrients measured in the secretions of BOECs treated under 8.5mM+I conditions, which would potentially mimic more accurately the conditions physiologically.

As mentioned earlier, in the cases of the embryos cultured in conditions mimicking *iv*DOF secretions by BOECs treated under 11+insulin and 11-insulin, glucose consumption was greatly increased. According to the thrifty genotype hypothesis, mentioned first by Neel (Neel, 1962), and further discussed by Leese (Leese, 2002) in the Quiet Embryo hypothesis, there are genes that offer an advantage over other genes, according to the presence of over- or under-nutrition. Consequently, we might hypothesize that a conversion of the main energy substrate from pyruvate to glucose is favoured in the ‘11+’ and ‘11-’ environments, especially since the glucose-to-pyruvate ratio in the culture medium was 2.6-fold and 4-fold respectively. Consequently, it is possible that glucose became an important substrate in cleavage stage embryos under these embryo culture conditions; however, this would require further investigation. The high variability observed in substrate consumption/ production for embryos cultured under the ’11-’ conditions, indicated by high interquartile range, indicates that a higher number of embryos would help us clarify the impact of these conditions on embryo metabolism. Glucose uptake by mouse preimplantation embryos is not known to be affected by the presence of insulin (Gardner and Leese, 1988). In the bovine, the presence of insulin does not affect cleavage rate or the percentage of embryos reaching

morula stage, even though the combined presence of glucose and insulin in the culture system was associated with increased number of blastocyst cells (Matsui *et al.*, 1995). To the best of our knowledge, there is no existing literature investigating glucose uptake in the presence of insulin in the bovine.

Unfortunately, due to limited tissue availability, the impact of normo-and hyperglycaemic *iv*DOF mimicking conditions to the concentrations of glucose, lactate and pyruvate could not be performed for blastocyst stage embryos.

5.5.3. Modified embryo environment altered the rates of amino acid consumption and production in cleavage-stage and blastocyst-stage embryos

Embryo survival and the capacity to give rise to live births in clinical IVF has been previously associated with amino acid turnover (Houghton *et al.*, 2002; Brison *et al.*, 2004). As observed for the rates of glucose, lactate and pyruvate consumption and production under control conditions, the amino acid profile of cleavage-stage embryos under control conditions (Figure 5.9) was similar to previously reported data (Partridge and Leese, 1996; Houghton *et al.*, 2002; Brison *et al.*, 2004; Sturmey, Brison and Leese, 2008; Sturmey *et al.*, 2010). In particular, arginine was consumed and alanine was produced by cleavage-stage embryos cultured under control conditions. This pattern was largely consistent regardless of whether the embryos had cleaved or not. The pattern of amino acid consumption/production for blastocyst-stage embryos cultured in control conditions also broadly followed expected profiles (Figure 5.17). This suggests that the embryos growing under control conditions had similar patterns of consumption/production as previously reported, and were therefore suitable for comparisons.

In terms of the modified embryo environments, glycine consumption in cleavage-stage embryos was significantly higher in the 8.5 and 11+ -modified environments compared to the control group, while arginine was released in the 11+ conditions. The BOEC-modified embryo environments induced significant differences in the rates of glycine consumption/production, with glycine consumption in blastocyst-stage embryos was higher in the '11+' conditions, regardless whether the embryo progressed or arrested. Glycine plays an important role in the maintenance of a stable internal osmotic

environment in early embryos (Dawson, Collins and Baltz, 1998; Baltz and Zhou, 2012) and the increased rate of glycine consumption in the present work might involve a balanced influx and secretion of this amino acid, which, in turn, is a result of glycine transporter activity to maintain internal osmolarity to ensure embryo health and survival (Steeves *et al.*, 2003; Steeves and Baltz, 2005).

Interpretation of findings on arginine should be performed with caution, due to high sample variability, though it is worth noting that in blastocyst-stage embryos, arginine production by embryos in the '11-' culture environment was significantly different from arginine depletion under control conditions (Figure 5.20).

Overall, the variability in the rates of both amino acids' consumption/production was quite high, with the exception of the rates for the embryos under control conditions, where variation was slightly decreased; consequently, repetition of these experiments would be required to clarify the pattern arginine depletion/production.

5.5.4. Limitations of embryo segmentation

The distinction of the embryos into cleaved and uncleaved embryos, when examined at cleavage stage, and between non-degenerate and degenerate, as well as progressed and non-progressed blastocysts introduces a number of potential limitations. First, it reduces the number of embryos examined per group, which will tend to lead to an increase of the sample variance. This is reinforced by the fact that when embryos were separated into cleaved and uncleaved, substrate consumption/production results were changed from significantly different from zero to no difference. In addition, there is no information on whether the embryos which were classified in a given category would remain in the state seen at the time of observation. For example, when an embryo is classified as uncleaved, this does not necessarily mean that it would remain uncleaved, since there is a possibility that it would cleave if left in individual culture for longer. Since there is a time window within which embryo development occurs, there is always a possibility for mis-allocation of the embryos into set categories and data from such experiments should be interpreted with caution.

5.5.5. Conclusion

Modified embryo culture conditions designed to mimic the nutritional environment to which early bovine embryos are exposed had no impact on the developmental progression through cleavage and towards the formation of the blastocyst. The embryos in the modified environments were able to withstand the changes in the environment and survive in rates comparable to control conditions. This is in contrast to work in mice, where varying embryo culture environments impacted on embryo metabolism but not on embryo survival (Morbeck *et al.*, 2014; Morbeck, Baumann and Oglesbee, 2017). However, the results of the present work are in agreement with work performed in the human, where the timing of cleavage divisions as well as implantation and pregnancy rates were not affected by varying embryo culture conditions (Basile *et al.*, 2013).

The above suggest that in the experiments described in this chapter, embryos retained the ability of adapting to the modified embryo environment and surviving in the adverse conditions introduced. However, embryo adaptation and survival does not ensure the subsequent health of the embryo for the remaining pregnancy period or even postnatally. In addition to that, such modified culture conditions suggested some modest effects on embryo metabolism in terms of the consumption/production of nutrients. The impact that these metabolic alterations may have on further foetus development, as well as on the future health of the organism, in terms of the development of metabolic conditions in the future, remains to be investigated.

Chapter 6

General Discussion and Conclusions

6.1. Summary of findings

The broad aim of the thesis was to study the effect of a hyperglycaemic challenge, in the presence or absence of physiologically relevant levels of insulin, on the secretions of bovine oviduct epithelial cells and on preimplantation embryo development and metabolism. This was done with the use of a previously established *in vitro* model of the oviduct, whereby the oviduct epithelium was cultured in a monolayer of polarised cells and then a hyperglycaemic challenge was introduced at the basal side of the cells, which represented blood. The measured glucose, lactate, pyruvate, and amino acid concentrations secreted by the oviduct epithelium were used for the development of modified embryo culture conditions, and the cleavage and blastocyst rates, as well as nutrient consumption/production by the embryos was investigated.

The work in this thesis has demonstrated that the air-liquid interface model of bovine epithelial cell culture *in vitro* provides a valid approach for the investigation of the composition of oviduct secretions. It has been established that the method used for epithelial cell extraction results in the successful isolation of an oviduct epithelial cell population, which can then be cultured to form a single-cell monolayer, with selective barrier properties. It has been possible to further explore this model in a long-term culture experiment, which has shown successful cell growth in an air-liquid interface for as long as 36 days. After this time, the amino acid composition of the oviduct fluid was modified, with nine amino acids having increased concentrations compared to earlier days of culture in air-liquid interface.

Importantly, the data in this thesis have confirmed that the content of the media in which the cells were incubated and the gene expression profile of the cells cultured in Transwells, produce a similar pattern to the much more widely used T25 flask. Cell culture in Transwells allows them to form a confluent monolayer of polarised cells, which enables access to both the apical and the basal compartment of the cells. This, in turn, facilitates transport of molecules across the monolayer. Overall, the above suggest that this model is successful, reproducible and can be used for experiments on the oviduct epithelium, as an intact, polarised monolayer.

Having confirmed the validity of the air-liquid interface model of the oviduct, it has been used to measure the impact of physiologically relevant interventions on the gene expression and substrate composition of the oviduct cells and their secretory fluid. In this work, it was possible to re-create in the cell preparation the effect of one of the major consequences of metabolic syndrome, which is elevated glucose. A series of experiments were performed, described in Chapter 4, to investigate the impact of pathophysiological levels of elevated glucose on the secretory properties of the oviduct epithelial cells. The data revealed that exposing oviduct epithelial cells to elevated glucose for a prolonged period, in the presence or absence of insulin, resulted in altered composition of oviduct secretions. Notably, chronic exposure to hyperglycaemic conditions in the absence of insulin resulted in significantly modified concentrations of glutamine, glycine, arginine and alanine in the luminal secretions of the oviduct epithelial cells. By contrast, the cells of the oviduct appeared to buffer the luminal secretions in response to a short-duration treatment of hyperglycaemic conditions. Overall, the experiments did not find any impact of the above conditions on the gene expression profile of the cells.

In order to understand the impact of the hyperglycaemic-modified luminal environment in more detail, experiments to explore the effect on embryos were undertaken. Embryo culture in media modified according to the substrate and amino acid concentrations detected in the oviduct-cell derived fluid produced under pathophysiological conditions did not affect embryo cleavage or blastocyst rates. However, the metabolic profile of embryos was altered in response to culture in these conditions. Consumption of pyruvate was significantly modified under extremely hyperglycaemic conditions, both in the presence and in the absence of insulin, where it appeared to be marginally produced. In cleavage-stage embryos, lactate production was also significantly modified; however high variability did not allow for safe conclusions to be drawn with regards to the results. In addition, embryo culture in modified embryo environment was associated with significantly altered glycine and arginine consumption in both cleavage-stage and blastocyst-stage embryos. These findings are summarised in Figure 6.1.

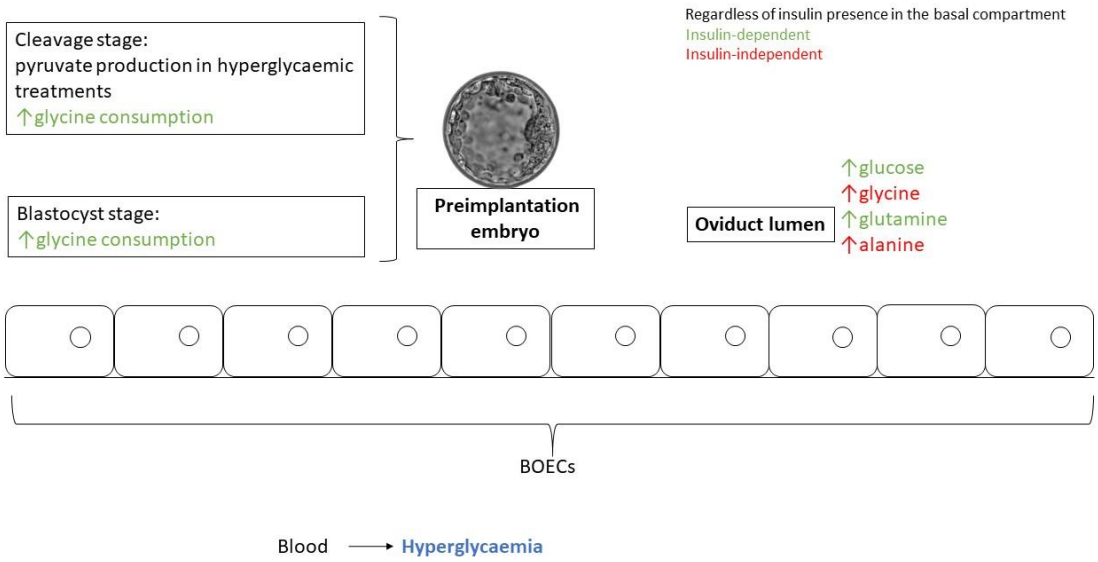


Figure 6.1: Summary of findings on the impact of hyperglycamia on early embryo environment and development. Chronic hyperglycaemia results are summarised, as these conditions appeared to have a more pronounced impact on secretion into the oviduct lumen. In green are highlighted the insulin-dependent effects of hyperglycaemia whereas the insulin-independent effects of hyperglycaemia are shown in red colour. Severe hyperglycaemia in the presence of insulin induced significantly higher glycine and alanine concentrations in the apical compartment, compared to all other treatments regardless of the presence or absence of basal insulin, whereas hyperglycaemia in the absence of insulin was associated with increased glucose and glutamine concentrations, and this was insulin-dependent. In terms of the preimplantation embryo, cleavage-stage embryos produced pyruvate regardless of whether the initial basal treatment included insulin. Glycine uptake by both cleavage-stage and blastocyst stage embryos increased in all treatments, in particular in embryo culture medium modified according to the '11+insulin' conditions.

The importance of these findings is linked to the key role of these nutrients in embryo survival and viability. For example, in early mouse embryos, glycine can act as an osmolyte (Dawson, Collins and Baltz, 1998; Baltz, 2001), glutamine consumption has been associated with high DNA damage (Sturmey *et al.*, 2009) and alanine (Donnay, Partridge and Leese, 1999) and arginine (Orsi and Leese, 2004) release may be associated with ammonium ion recycling. In particular, increased glycine secretion by the epithelial cells and subsequent increased glycine consumption by both cleavage-stage and blastocyst-stage embryos, may be associated with cell and embryo response to adverse osmolarity and the importance of maintaining optimal osmolarity in the embryo in response to adverse conditions.

6.2. Strengths and limitations

As with any piece of scientific study, this work has a number of strengths and limitations. Previous work performed in the laboratory has demonstrated the validity of the method of cell collection used and the robustness of the method of oviduct fluid production (Simintiras *et al.*, 2016), as well as of those for oocyte isolation and maturation, fertilisation and embryo culture (Guerif *et al.*, 2013). In addition, the metabolic content analyses of biological fluids are well-established and have been used in a variety of systems (Guerif *et al.*, 2013; Leary, Leese and Sturmey, 2015; Simintiras *et al.*, 2016). An experimentally tractable system was used throughout, in order to perform this study in a tightly defined, controlled manner. Exclusion of experiments that did not follow the appropriate standards was systematically performed; for example, experiments where the standard curves of glucose, lactate and pyruvate had a linearity below 98% were excluded, and the method of amino acid detection was validated prior to each chromatography experiment to confirm low levels of error. Finally, gene expression experiments were confirmed to be specific and efficient before the target samples were assessed.

However, no piece of research or method comes without limitations. One limitation of the present work was the number of replicates for some of the experiments performed, due to time and tissue constraints, limiting the numbers of observations that could be made. This may have contributed to the high variability in some of the experiments. In addition, and due to the same restrictions, spent media from blastocyst-stage embryos was analysed only in terms of its amino acid composition, a known marker of embryo quality (Sturmey *et al.*, 2010), but not for glucose, lactate and pyruvate consumption/ production. Undertaking a full metabolic profile would have allowed a comparison with the respective cleavage-stage embryos as well as the determination of the impact of the modified embryo conditions. This limitation was further increased by the low blastocyst rates encountered, attributable to the extreme ambient heat at the time the embryo experiments were done (MetOffice, 2018), which could have affected the oocytes

aspirated from the ovaries (Ahmed *et al.*, 2017) as well as progression of zygotes to the blastocyst stage (Alves *et al.*, 2013).

Another limitation of the current work was the use of the bovine as a model species. To the best of our knowledge, there are no studies on the extent to which hyperglycaemia in the cow replicates the situation in the human. The only exception to this is the presence of insulin resistance during late pregnancy and early gestation, to ensure nutrient supply to the bovine mammary gland (Mair *et al.*, 2016). It is possible that modified mouse strains may have provided a more useful in the representation of these conditions, however, the similarities between the human and the bovine in oocyte and embryo development (Ménézo and Hérubel, 2002; Elsik, Tellam and Worley, 2009) reinforce the usefulness of the use of the bovine in the study of mammalian reproductive processes (Ellington *et al.*, 1998; Ménézo and Hérubel, 2002). In addition, there are a number of practical advantages to using bovine tissue; the approaches used in this thesis make use of material that would otherwise be discarded. Moreover, the use of bovine slaughterhouse-derived material meant that experimental animals were not required, thus fitting in with the 3Rs agenda of reducing the use of animals in experimental research.

6.3. Future work

6.3.1. *In vitro* model of the oviduct

This research has raised a number of questions regarding the future research possibilities of using the *in vitro* model of the oviduct. Further to BOEC visualisation with Transmission Electron Microscopy described in Chapter 3, which demonstrated the presence of a single cell epithelium growing on the Transwell membrane, it would be valuable to repeat the observation on BOECs grown under conditions of the air-liquid interface. This would be of particular interest, as it would provide some indication of how well the *in vivo* appearance of the oviduct epithelium is retained *in vitro*; specifically the proportion of ciliated to secretory cells, previously observed in a related preparation in the porcine (Miessen *et al.*, 2011) and in the human oviduct following oestradiol addition to the basal compartment (Comer, Leese and Southgate, 1998).

It is well-established that the composition of the oviduct-derived fluid is modified according to the stage of the oestrous cycle (Aviles, Gutierrez-Adan and Coy, 2010), a change well-known for glycosidases (Carrasco *et al.*, 2008) and steroid hormones (Aguilar and Reyley, 2005). It would therefore be of interest to exploit the air-liquid interface model to determine whether the different stages of the cycle impact on the gene expression profile and the energy substrate composition of BOECs, and compare to the data previously observed *in vivo* (Hugentobler *et al.*, 2008).

The interaction between the oviduct epithelium and sperm is a topic of great interest, both in the maintenance of the fertilisation capacity of sperm (Suarez, 2002) and sperm capacitation and hyperactivation (Coy *et al.*, 2012). It would be of particular interest to investigate the potential impact of sperm on the maintenance of the integrity of the monolayer, as well as possible differences in monolayer potential, current and transepithelial resistance. The data might suggest a potential association of sperm binding to the oviduct sperm reservoir with modification of oviduct fluid secretion. This would align with observations that the presence of embryos and gametes can affect the composition of oviduct fluid (Aviles, Gutierrez-Adan and Coy, 2010).

The present and previous studies (Simintiras *et al.*, 2016) in the laboratory have established the robustness of the *in vitro* model of the oviduct in producing an epithelial cell monolayer similar to the one observed *in vivo*. It would therefore be of interest to discover if this model could be translated to other organisms for the description of oviduct epithelia and secretions. Given the small size of a mouse oviduct, experiments on this species would be very difficult. However, with appropriate ethical approval, the human oviduct could be studied extensively using this model. Isolation and culture of Human Oviduct Epithelial Cells in an air-liquid interface and analysis of their secretions into the apical medium would be of special interest, since it would enable the composition to be compared with human embryo culture media used in IVF. This could be done under control conditions and following modification of the basal environment to mimic pathophysiological conditions, such as the ones examined in this thesis.

6.3.2. Hyperglycaemia in the oviduct

Experiments could also be carried out to discover how the secretion of other molecules into the oviduct fluid is affected by the modified basal conditions. Oviduct-specific glycoprotein has been previously detected in *in vitro* Derived Oviduct Fluid produced using the air-liquid interface system as described in this thesis (Simintiras *et al.*, 2016). In addition, growth factors, proteases and their inhibitors, and cytokines have also been detected in the oviduct fluid and they are known to promote development and maturation of gametes and embryos (Pillai *et al.*, 2017). Furthermore, glycosidases in the oviduct fluid are potentially associated with sperm release into the oviduct lumen (Coy *et al.*, 2012). Similarly, steroid hormones are secreted and are differentially expressed according to the stage of the oestrous cycle (Aguilar and Reyley, 2005). Protein detection by ELISA assays, would be useful in the determination of whether the treatments modify the amount of protein secreted by the cells.

In addition, it would be of considerable interest to determine whether the hyperglycaemic treatments, in the presence or absence of insulin, influence the secretion of extracellular vesicles, which have previously been found to be secreted by the oviduct epithelial cells (Lopera-Vasquez *et al.*, 2016). The composition of these vesicles and whether this is modified under these conditions could also be explored. Finally, fatty acids have been detected in the oviduct lumen (Drews *et al.*, 2018) and

NEFA composition of the oviduct lumen have previously been reported to reflect non-esterified fatty acid (NEFA) concentrations in blood (Jordaens, Van Hoeck, De Bie, *et al.*, 2017). Whether the treatments described in this thesis have the potential to affect luminal NEFA concentrations remains to be determined.

Related experiments could be carried out to discover if the levels of amino acids in oviduct fluid are attributable to differences in the gene and/or protein expression of the various amino acid transporters. Similar experiments could examine transporters for glucose, lactate, pyruvate and amino acids in the blastomeres of cells of the blastocyst, trophoectoderm or inner cell mass cells (Purcell and Moley, 2009, reviewed by Gardner, 2015, reviewed by Van Winkle *et al.*, 2006) and whether their gene/protein expression is changed in the modified environments.

Further to the work described in this thesis, insulin resistance combined with hyperinsulinaemia would be of interest to be studied. The use of an insulin antagonist followed by incubation in the presence of high levels of insulin would be able to mimic these conditions, and would potentially be the next step in mimicking the symptoms of obesity and diabetes. In addition, it would be fascinating to examine the impact of anti-diabetic drugs, such as metformin, and whether these can restore the adverse effects of hyperglycaemia and insulin resistance.

Also, further work should be performed on deciphering what the composition of oviduct secretions would be under a combination of symptoms which constitute obesity and diabetes, such as the combined introduction of high levels of glucose, insulin resistance, and inflammation-related factors, at levels at which these would be encountered in metabolic syndrome. This would enable safer predictions regarding the impact of these conditions on oviduct secretions.

6.3.3. Embryo in the hyperglycaemic oviduct

The work described in this thesis regarding the impact of the modified embryo environment on embryo cleavage rates, blastocyst rates and metabolism raises the question of whether there is an impact on sperm. Since sperm are present in the oviduct until fertilisation, within a sperm reservoir (Coy *et al.*, 2012), and given the known

interaction between sperm and the oviduct epithelium (Fazeli, 2011) it would be of particular interest to determine whether the modified environment, which in this thesis has been found to affect oviduct secretions and embryo metabolism, has an impact on sperm physiology, motility and fertilisation capacity.

Although the impact on the modified embryo environment was not shown to have an overtly detrimental effect on embryo survival and development, the results of this thesis cannot lead to conclusions about the long-term health of the embryos produced. Indeed, according to the Developmental Origins of Health and Disease Hypothesis (DOHAD), described in the Introduction, embryo exposure to adverse conditions at or around conception are associated with the development of chronic metabolic conditions (Martin-Gronert and Ozanne, 2006; Feuer and Rinaudo, 2016). Consequently, the adverse environment created under the modified conditions described in this thesis, may be associated with the development of chronic conditions that may not be obvious until later in life.

In addition, modified embryo culture conditions are associated with a differentiated pattern of expression of imprinted genes (Doherty *et al.*, 2000). Therefore, it is not unlikely that the modified environment may be associated with differences in imprinting in the embryo. Additionally, work in diabetic rats has shown an increased rate of cell death compared to control embryos and nuclear fragmentation, particularly in the inner cell mass cells (Pampfer *et al.*, 1997), and high glucose has also been shown to be associated with cell allocation in the equine blastocyst (Choi *et al.*, 2015). Whether these findings apply to the bovine embryos grown in the modified environment described in this thesis, remains a possibility for future research. Embryo transfer experiments, where embryos would be grown in culture under control or modified conditions to the blastocyst stage and then implanted into healthy cows, would allow the determination of whether the adverse environments described in this thesis have a long-term impact on the offspring health.

An important finding of this work was that exposure to culture conditions informed by the effects of hyperglycaemia did not affect embryo development to the blastocyst stage. The ability of mouse blastocyst-stage embryos to exhibit developmental plasticity

to ensure further conceptus development and postnatal fitness in adverse conditions has been previously presented (Watkins *et al.*, 2008). Embryonic developmental plasticity has been reviewed, and a variety of mechanisms are known to be used by the embryo to ensure developmental plasticity (Fleming *et al.*, 2004). The embryo uses epigenetic, proliferative and metabolic adaptations to ensure its survival, which may be reflected in disturbances during pregnancy and foetal growth, as well as in the development of metabolic conditions later in life (Fleming *et al.*, 2004). Therefore, future work might focus on the determination of whether the conditions modified to mimic different adverse environments of early embryo development affect patterns of epigenetic modifications. This could be performed with the use of single-cell RNA sequencing, which is a marker of blastomere heterogeneity and has previously been used for experiments of genome activation in the first days of development (Lavagi *et al.*, 2018) or with the use of bisulphite sequencing, which has been previously used in preimplantation embryos for observation of DNA demethylation patterns (Wu *et al.*, 2017). Based on the existing literature, comparison of the epigenome of embryos cultured in the modified environments would also be of great interest.

Knowledge of the anticipated composition of the secretions of the oviduct epithelium in women with metabolic disturbances would be of crucial importance in the determination of the appropriate assisted reproduction treatment for them. The work described in this thesis is a first step towards this direction, as replication of the above experiments in humans would enable the prediction of the composition of embryo culture media used when obese or diabetic women wish to undergo IVF. This would introduce a whole new approach in personalisation of IVF culture media and would be very useful in further research in the field, although many steps are required until this stage is reached.

6.4. Importance of work described in this thesis

The oviduct has been described as a "neglected epithelium" (Leese *et al.*, 2001), in that, historically, it has tended to be ignored since IVF was first developed to overcome the blockage of the Fallopian tube and works successfully without the need for the oviduct (Leese *et al.*, 2001). On the contrary, extensive research has focused on the impact of a variety of factors on the ovary and the uterus. However, with the realisation that the oviduct plays a pivotal role in the events surrounding conception and has extensive interactions with the gametes and early embryo, there is renewed interest in this organ.

This study contributes to the limited existing literature on the physiology of the oviduct. Of particular interest are the findings of glucose transporter expression in the oviduct tissue. Previous studies have determined the expression of GLUT1 and GLUT3 transporters in the oviduct epithelium of rat and the bat (Tadokoro *et al.*, 1995; Kuchiiwa *et al.*, 2011; Roy and Krishna, 2013). Additionally, GLUT1 expression has been detected in fallopian tube adenocarcinomas (Kalir *et al.*, 2005). The work described herein has confirmed the expression of these transporters in the bovine oviduct epithelium for the first time. In addition, to the best of our knowledge, this study is the first one to report the expression of the insulin receptor gene in the oviduct epithelial cells. These findings provide critical data in understanding the role of the insulin-glucose pathway in the oviduct epithelium and how this is affected by the maternal environment.

This study provides another piece in the puzzle of the impact of maternal health and metabolic conditions on the pre-implantation embryo. Our knowledge this far has focused on the ovarian follicle, since this milieu can be sampled relatively simply. By contrast, accessing the fallopian tubal environment is technically challenging. Using the air-liquid interface model of the oviduct the data in this thesis has illuminated some understanding of the Fallopian tube and the effect of an abnormal basal environment on oviduct secretions and integrity, indicating the complexity of the impact of metabolic conditions on gametes and the preimplantation embryo. For example, non-esterified fatty acids detected in oviduct fluid may affect the barrier properties of the oviduct monolayer (Jordaens, Van Hoeck, De Bie, *et al.*, 2017; Jordaens, Van Hoeck, Maillo, *et al.*, 2017). Additionally, previous work performed in the laboratory has shown the

impact of disturbed hormonal environment on the composition of oviduct-derived fluid (Simintiras *et al.*, 2016), as well as the permeability of the epithelium to genistein (Simintiras and Sturmey, 2017). All these data illustrate the importance of investigating the impact of various pathophysiological conditions on the oviduct epithelium, its selective barrier properties and its secretions.

Overall, the work described in this thesis has confirmed the usefulness of the air-liquid interface model in the generation and composition of oviduct secretions. In addition, the experiments focusing on the impact of hyperglycaemia, in the presence or absence of insulin, are important in helping us to understand the impact of maternal pathophysiological conditions on the secretions of the oviduct and preimplantation embryo.

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Appendices

- Chapter 2

List of chemicals used

Chemical substance	Supplier, Product number
Adenosine 5'-triphosphate disodium salt hydrate, 99% (ATP)	Sigma, A26209
Adenosine triphosphate (ATP)	Sigma, A6419
Alanine	Sigma, A-7627
Amino acid standard solution	Sigma, AAS18
Amphotericin B	Invitrogen 15290026
Antibiotic antimycotic (Ab-Am)	Fisher, 11580486
Antimycin A	Sigma, A8674
Aprotinin from bovine lung	Sigma-Aldrich, A6279
Arginine	Sigma, A-5131
Asparagine	Sigma, A-4284
Aspartate	Sigma, A-4534
Bovine Serum Albumin (BSA)	Sigma-Aldrich A7030
$CaCl_2 \cdot 2H_2O$	Sigma, C7902
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Sigma, C7902
Chloroform, for HPLC, stabilized with amylene	Fisher Scientific, C/4966/17
Cysteine	Sigma, C-1276
Cytokeratin-18 mouse primary antibody	Applied Biosystems, ab82254
D-ABA	Acros Organics, 142140050
D-Glucose	Sigma, G6152
Dithiothreitol	D0632, Sigma-Aldrich
DMEM	Sigma-Aldrich D5546
DNA ladder	ThermoFischer Scientific, SM0373
EDTA	Sigma ED4SS
Embryo-tested water	Fresenius Kabi
Epidermal Growth Factor from murine submaxillary gland	Sigma, E4127
EPPS	Sigma, E9502
Essential amino acids	Sigma, M7145
Fetal Bovine Serum (FBS)	ThermoFischer Scientific, 10270106
Fibroblast Growth Factor from bovine pituitary	Sigma, F3133
Fluorescein	Sigma Aldrich, F6377
Glacial Acetic Acid	Sigma, CHE1018
Glucose standard, 5.0mmol/L, 30ml	Analox instruments LTD, GMRD-010
GlutaMAX, 100x	Thermo-Fisher Scientific, 35050061
Glycine	Sigma, G6388
Glycine, ≥99%	Sigma, G7126
goat anti-mouse, secondary, FITC-conjugated antibody	Applied Biosystems, ab6785
Hank's Balanced Salt Solution (-phenol red, $-CaCl_2$, $-MgCl_2$)	Sigma-Aldrich, H6648
Hanks Balanced Salt Solution (+phenol red, $-CaCl_2$, $-MgCl_2$)	Lonza LZBE10-543F

Heparin sodium salt	Sigma, H3393
HEPES	Sigma, H3375
HEPES sodium salt	Sigma, H3784
HEPES solution, 1M, pH 7.0-7.6, sterile-filtered, BioReagent, suitable for cell culture	Sigma-Aldrich, H0887
Hexokinase/Glucose-6-phosphatase (H3K/G6P)	Roche,10127825001
High Capacity cDNA Reverse Transcription Kit	ThermoFisher Scientific 4368814
Hi-Res Standard Agarose	Molecular Biology Products (formerly Bioproducts Ltd), A4-0700
Histidine	H-8125
Hydrazine sulphate	Sigma, H3376
Hydromount	National Diagnostics, HS-106
Insulin	Sigma-Aldrich, I0516
Isoleucine	Acros Organics, 166170250
Isopropanol, for HPLC	Fisher Scientific, P/7507/17
Lactate Standard, 5.0mmol/L, 25ml	Analox instruments LTD, GMRD-079
Leucine	Acros Organics, 125121000
L-Glutamine	Sigma-Aldrich G7513
L-Lactate Dehydrogenase (L-LDH), from rabbit muscle	Roche 10127884001
Loading dye	ThermoFischer Scientific, R0611
Lysine	Sigma, L-5626
M199	Sigma Aldrich, M0650
Magnesium sulphate	Sigma, M2643
Magnesium sulphate	Fisher, M/1050
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	Sigma, M7774
Menopur/Merional (75IU)	Ferring Pharmaceuticals Ltd
Methanol	Methanol, M/4056/17
Methionine	Sigma, M-9625
Mineral oil	Sigma Aldrich, M8410
Monobasic potassium phosphate (KH_2PO_4)	Sigma, P5655
NAD	Roche 127 981
NAD, Grade II, free acid	Roche 10127981001
NADH Di sodium salt	Roche, 10 128 023 001
NADP	Roche, 128 040
Newborn Calf Serum (NCS)	Sigma-Aldrich N4762
Nutrient Mix F12	Sigma-Aldrich N8641
O-phthaldialdehyde	Sigma-Aldrich, P0532
Pen/Strep	Invitrogen 15140122
Penicillin G	Sigma, P4697
Penicillin/Streptomycin	Sigma, P0781
Percoll	Sigma, P1644
Phenylalanine	Acros Organics, 130310250
Phtaldialdehyde Reagant (OPA)	Sigma, P0532
Platinum Taq DNA polymerase	Invitrogen, 10966018
Poly-L-lysine	Sigma, P1524
Potassium chloride (KCl)	Sigma, P5405
PrecisionPLUS MasterMix with ROX premixed with SYBRgreen mini kit	PrimerDesign, Mini-PrecisionPLUS-R-SY
Proline	Sigma, P-0380
Pyruvate standard 0.45mM/L	Analox, GMRD-140-E
saline solution, 3-7 TIU/mg protein	
Serine	Sigma, S-4500
Sodium bicarbonate ($NaHCO_3$)	Sigma, S5761
Sodium chloride (NaCl)	Sigma, S5886
Sodium DL-lactate solution	Sigma, L1375
Sodium hydroxide	Fisher Scientific, S/4920/60

Sodium hydroxide	Fisher, S4920/60
Sodium pyruvate	Sigma, P5280
Sodium pyruvate	Sigma, P2256
Streptomycin	Sigma, S1277
SYBR Green PCR Master Mix	ThermoFischer Scientific, 4309155
SYBR™ Safe™ DNA	Invitrogen, 10328162
Tetrahydrofuran (THF)	Sigma, 34865
Threonine	Sigma, T-8625
Trizol	Thermo-Fischer Scientific, 15596026
Trypan Blue solution, 0.4%, liquid, sterile-filtered, suitable for cell culture	Sigma T8154
Trypsin	Sigma-Aldrich, 59427C
Tryptophan	Sigma, T-0254
Tyrosine	Sigma, T-1020
Valine	Sigma, V-0500
VECTASHIELD anti-fade mounting medium with DAPI	Vector Laboratories, H-1200
β-mercaptoethanol	Sigma M6250
(L-)Glutamate	Sigma, G-1251
(L-)Glutamine	Sigma, G8540
1% eosin, aqueous solution	BDH
100% Hematoxylin Solution, Harris Modified	Sigma-Aldrich, HHS128
2-deoxy-glucose	Sigma, D8375

List of equipment used

Equipment Used	**Used For**
Nanodrop 2000c, Thermo Scientific	RNA extraction
EVOM voltohmmetre, World Precision Instruments	TEER measurement
STX2 Electrodes of EVOM voltohmmetre, World Precision Instruments	TEER measurement
Voltage/Current Clamp (World Precision Instruments)	Voltage clamp experiment
Applied Biosystems 2720 Thermal Cycler	cDNA synthesis
BioRad C1000 Touch Thermal Cycler	PCR Thermocycler
StepOne Plus Real-Time PCR system, Applied Biosciences	qRT-PCR
Molecular Imager VersaDoc™ MP 4000 System	DNA gel imaging
BMG plate readerFLUOstar Omega Microplate Reader - BMG LABTECH	Glucose, Lactate, Pyruvate assays
Agilent 1100/1200	High Performance Liquid Chromatography
Class II cabinet	Cell culture and maintenance
Zeiss LSM 710 confocal microscope	BOEC staining

List of consumables

Consumables	
96-Well PCR Plates, Skirted, Low Profile	Starlabs, E1403-5200
MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 mL	Applied Biosystems, 4346907
Corning TranswellTM 0.4µm PolyEthylene Tetraphtalate cell culture inserts	Corning 3450
Corning 12mm Snapwell Insert with 0.4 µm pore, pore density 1 x 10^8 pores/cm^2, polycarbonate membrane	Corning Costar 3407
Falcon® 60 mm x 15 mm Not TC-treated Treated Bacteriological Petri Dish, 20/Pack, 500/Case	Corning, 351007
Nunc™ 4-Well Dishes for IVF, Nunclon Delta Surface	ThermoScientific, 176740
Tissue Culture Flask, 25cm, Adherent Surface, Canted Neck, Non-Vented Cap, Sterile, Non-Pyrogenic, Non-Cytotoxic	Sarstedt, 83.3910
Tissue Culture Flask, 75cm, Adherent Surface, Canted Neck, Non-Vented Cap, Sterile, Non-Pyrogenic, Non-Cytotoxic	Sarstedt, 83.3911
Tube Conical Skirted Base 50ml 115x28mm Polypropylene Screen Printed	Sarstedt, 62.559.001
Serological Pipette 5ml Individually Wrapped Sterile Non Pyrogenic	Sarstedt, 86.1253.001
Serological Pipette 10ml Individually Wrapped Sterile Non Pyrogenic	Sarstedt, 86.1254.001
Serological Pipette 25ml Individually Wrapped Sterile Non Pyrogenic	Sarstedt, 86.1685.001
Micro Tube Conical Base Push Cap 0.5ml Standard Neutral	Sarstedt, 72.699
Micro Tube Conical Base Push Cap 1.5ml Standard Neutral	Sarstedt, 72.690.001
Tip Standard 10µl Bagged	Sarstedt, 70.1130
Tip Standard 200µl Bagged	Sarstedt, 70.760.002
Tip Standard 1000µl Bagged	Sarstedt, 70.762

- Chapter 3

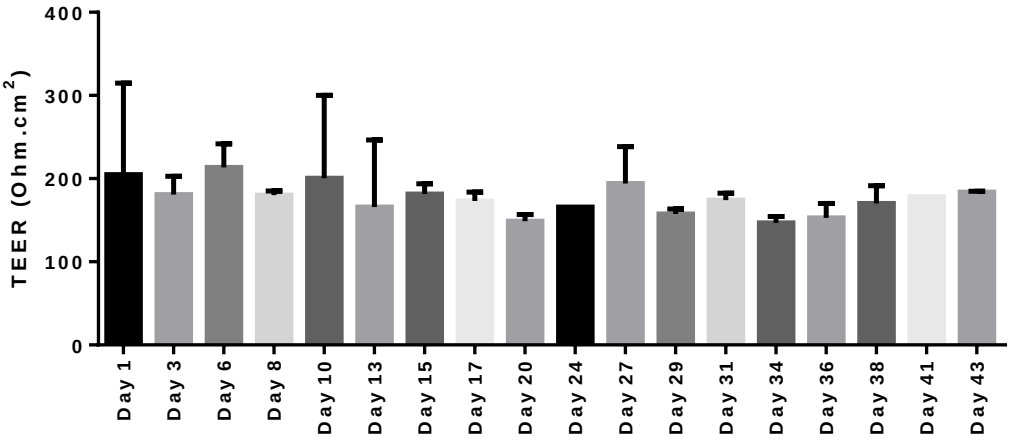


Figure 3.12_Appendix: Impact of long-term *in vitro* culture on the TransEpithelial Electrical Resistance over time. n=1-7 technical replicates, depending on snapwell availability and ability to measure TEER, median+ interquartile range

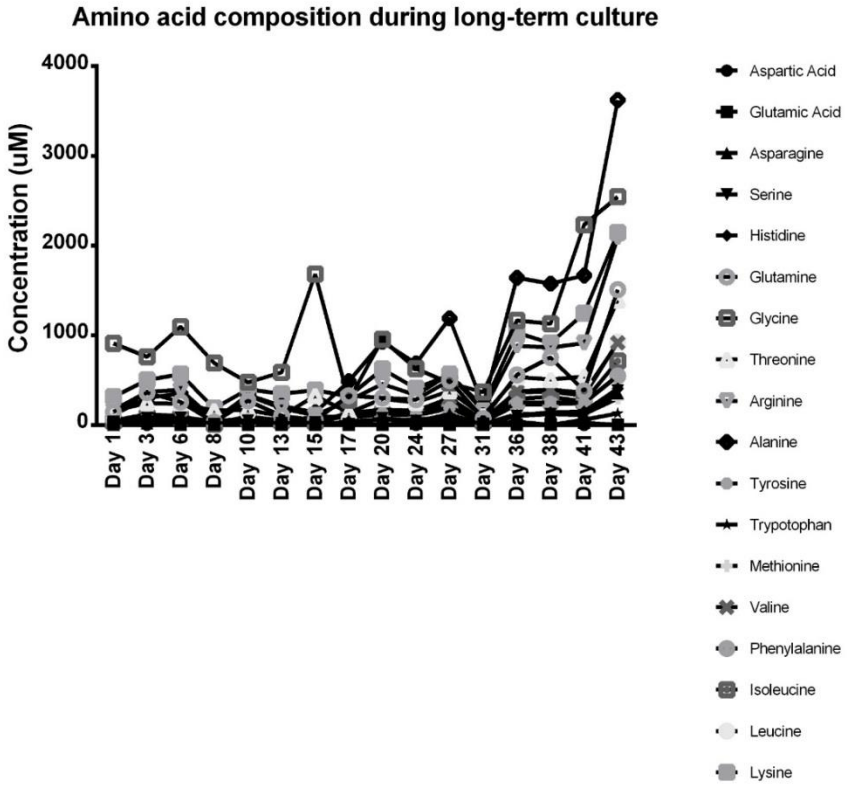


Figure 3.13_Appendix.: Summary of amino acid composition after long-term BOEC culture. As it was described previously, the levels of most amino acids increase significantly during the final days of culture.

Table 3.1_Appendix: Significance in amino acid composition in different days on long-term culture

(a)

Glutamine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	Ns	ns	ns	ns	ns	*	ns	****
Day 3			ns	ns	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	****
Day 6				ns	ns	ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	****
Day 8					ns	ns	ns	ns	Ns	ns	ns	ns	ns	**	ns	****
Day 10						ns	ns	ns	Ns	ns	ns	ns	ns	ns	ns	****
Day 13							ns	ns	Ns	ns	ns	ns	ns	*	ns	****
Day 15								ns	ns	ns	ns	ns	ns	*	ns	****
Day 17									ns	ns	ns	ns	ns	ns	ns	****
Day 20										ns	ns	ns	ns	ns	ns	****
Day 24											ns	ns	ns	ns	ns	****
Day 27												ns	ns	ns	ns	****
Day 31													ns	*	ns	****
Day 36														ns	ns	***
Day 38															ns	**
Day 41																****
Day 43																

(b)

Glycine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	***	**	ns	ns	ns	*	ns	ns	***	***
Day 3			ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	***	***
Day 6				ns	*	ns	ns	***	ns	ns	*	**	ns	ns	***	***
Day 8					ns	ns	***	ns	ns	ns	ns	ns	ns	ns	***	***
Day 10						ns	***	ns	ns	ns	ns	ns	*	*	***	***
Day 13							***	ns	ns	ns	ns	ns	ns	ns	***	***
Day 15								***	ns	***	***	***	ns	ns		**
Day 17									ns	ns	ns	ns	***	**	***	***
Day 20										ns	ns	ns	ns	ns	***	***
Day 24											ns	ns	ns	ns	***	***
Day 27												ns	*	ns	***	***
Day 31													**	**	***	***
Day 36														ns	***	***
Day 38															***	***
Day 41																ns
Day 43																

(c)

Threonine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 6				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 8					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 10						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 13							ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 15								ns	ns	ns	ns	ns	ns	ns	ns	***
Day 17									ns	ns	ns	ns	ns	ns	ns	***
Day 20										ns	ns	ns	ns	ns	ns	***
Day 24											ns	ns	ns	ns	ns	***
Day 27												ns	ns	ns	ns	***
Day 31													ns	ns	ns	***
Day 36														ns	ns	**
Day 38															ns	***
Day 41																**
Day 43																

(d)

Alanine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	**	*	***	ns	***	***	***	***
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***	***	***
Day 6				ns	ns	ns	ns	ns	ns	ns	***	ns	***	***	***	***
Day 8					ns	ns	ns	ns	***	*	***	ns	***	***	***	***
Day 10						ns	ns	ns	ns	ns	***	ns	***	***	***	***
Day 13							ns	ns	*	ns	***	ns	***	***	***	***
Day 15								ns	*	ns	***	ns	***	***	***	***
Day 17									ns	ns	*	ns	***	***	***	***
Day 20										ns	ns	ns	ns	ns	ns	***
Day 24											ns	ns	***	***	***	***
Day 27												***	ns	ns	ns	***
Day 31													***	***	***	***
Day 36														ns	ns	***
Day 38															ns	***
Day 41																***
Day 43																

(e)

Arginine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	***	***	****
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 6				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 8					ns	ns	ns	ns	ns	ns	ns	ns	***	***	****	****
Day 10						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 13							ns	ns	ns	ns	ns	ns	**	**	**	****
Day 15								ns	ns	ns	ns	ns	**	*	**	****
Day 17									ns	ns	ns	ns	ns	ns	ns	****
Day 20										ns	ns	ns	ns	ns	ns	****
Day 24											ns	ns	ns	ns	ns	****
Day 27												ns	ns	ns	ns	****
Day 31													*	*	*	****
Day 36														ns	ns	****
Day 38															ns	****
Day 41																****
Day 43																

h

(f)

Valine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 6				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 8					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 10						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 13							ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 15								ns	ns	ns	ns	ns	ns	ns	ns	***
Day 17									ns	ns	ns	ns	ns	ns	ns	**
Day 20										ns	ns	ns	ns	ns	ns	ns
Day 24											ns	ns	ns	ns	ns	**
Day 27												ns	ns	ns	ns	ns
Day 31													ns	ns	ns	**
Day 36														ns	ns	ns
Day 38															ns	ns
Day 41																ns
Day 43																

(g)

Isoleucine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
Day 6				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
Day 8					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
Day 10						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
Day 13							ns	ns	ns	ns	ns	ns	ns	ns	ns	*
Day 15								ns	ns	ns	ns	ns	ns	ns	ns	*
Day 17									ns	ns	ns	ns	ns	ns	ns	ns
Day 20										ns	ns	ns	ns	ns	ns	ns
Day 24											ns	ns	ns	ns	ns	ns
Day 27												ns	ns	ns	ns	ns
Day 31													ns	ns	ns	*
Day 36														ns	ns	ns
Day 38															ns	ns
Day 41																ns
Day 43																

(h)

Leucine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 6				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 8					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 10						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Day 13							ns	ns	ns	ns	ns	ns	ns	ns	ns	****
Day 15								ns	ns	ns	ns	ns	ns	ns	ns	***
Day 17									ns	ns	ns	ns	ns	ns	ns	**
Day 20										ns	ns	ns	ns	ns	ns	*
Day 24											ns	ns	ns	ns	ns	**
Day 27												ns	ns	ns	ns	ns
Day 31													ns	ns	ns	***
Day 36														ns	ns	ns
Day 38															ns	ns
Day 41																ns
Day 43																

(i)

Lysine	Day 1	Day 3	Day 6	Day 8	Day 10	Day 13	Day 15	Day 17	Day 20	Day 24	Day 27	Day 31	Day 36	Day 38	Day 41	Day 43
Day 1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	*	****	****
Day 3			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	****
Day 6				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	****
Day 8					ns	ns	ns	ns	ns	ns	ns	ns	****	**	****	****
Day 10						ns	ns	ns	ns	ns	ns	ns	*	ns	***	****
Day 13							ns	ns	ns	ns	ns	ns	**	ns	****	****
Day 15								ns	ns	ns	ns	ns	ns	ns	**	****
Day 17									ns	ns	ns	ns	*	ns	***	****
Day 20										ns	ns	ns	ns	ns	ns	****
Day 24											ns	ns	ns	ns	**	****
Day 27												ns	ns	ns	*	****
Day 31													**	*	****	****
Day 36														ns	ns	****
Day 38															ns	****
Day 41																***
Day 43																

- Chapter 4

Ion flux across the BOEC monolayer is relevant to the basal glucose concentrations

In order to determine whether the differences observed in the volume of fluid secreted by the cells (Figure 4.8) were attributed to differences in the ion flux across the monolayer, attempts to determine the impact of the treatments on the ion flux from the basal to the apical compartment of the cells were made. For this purpose, BOECs were grown on Snapwells and the voltage clamp experiment was performed as described in section 2.12 of Materials and Methods. ATP was used as a positive control, as it has been previously used to induce an increase in potential difference and current (Downing *et al.*, 1997)(Dickens *et al.*, 1996). This experiment was successful for the acute treatment, as shown in Figure 4.15_Appendix, where it is shown that the impact of all treatments is comparable to cell treatment with 2-deoxy-glucose, which is transported but not metabolised. However, the experiment could not be replicated for the chronic conditions, as the cells were unresponsive to the positive control (ATP).

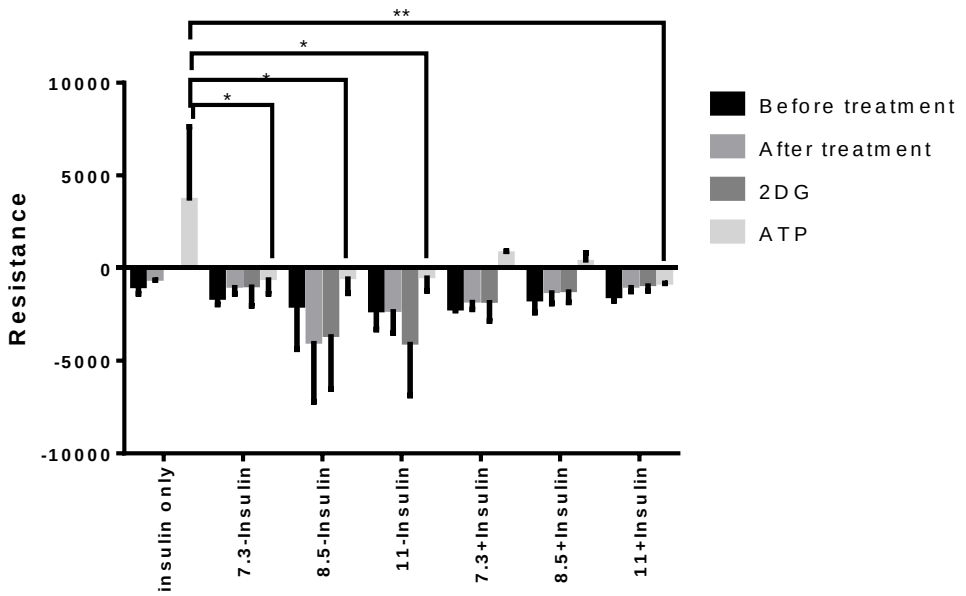
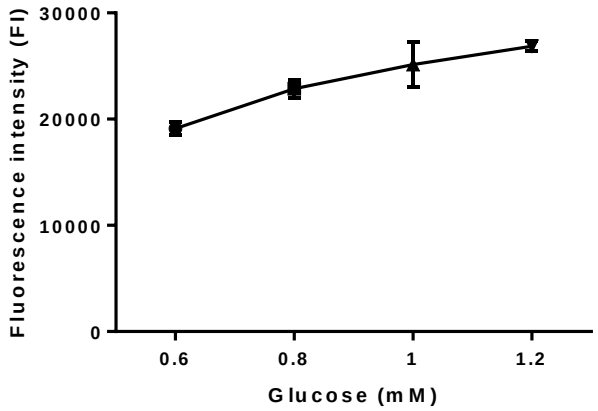


Figure 4.15_Appendix: Ion flux experiment for acute treatment under normo- and hyperglycaemic conditions, in the presence or absence of insulin. Comparison among treatments. Kruskal-Wallis test with Dunn's comparison

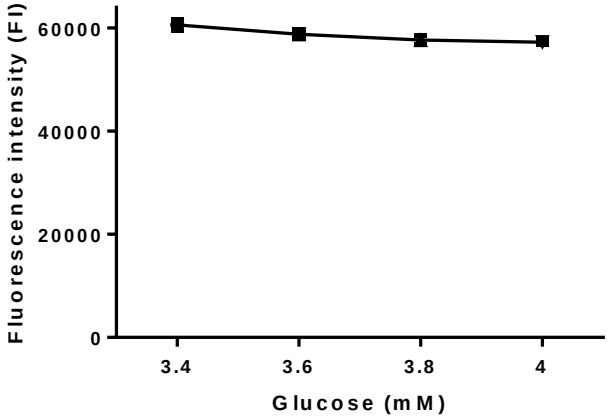
Discussion of this experiment

This experiment suggests that hyperglycaemic treatments, regardless of the presence of insulin, modify the resistance of the monolayer to a very similar pattern to the one of 2-deoxyglucose, which is not metabolised by the cells. The fact that glucose binding to the glucose transporters induces the same response to epithelium polarisation as 2-deoxyglucose, suggests that this induction is not associated with glucose metabolism. The only treatment where this was not observed was the 11- treatment, however, the difference between treatment with glucose and treatment 2-deoxy glucose did not induce a significant difference in resistance. This suggests that the membrane potential is affected by treatment addition, even though high variability did not allow for significance to be determined. Repetition of this experiment and optimisation for chronic conditions will help clarify the impact of the treatments studied on oviduct fluid secretion.

- Chapter 5



a)



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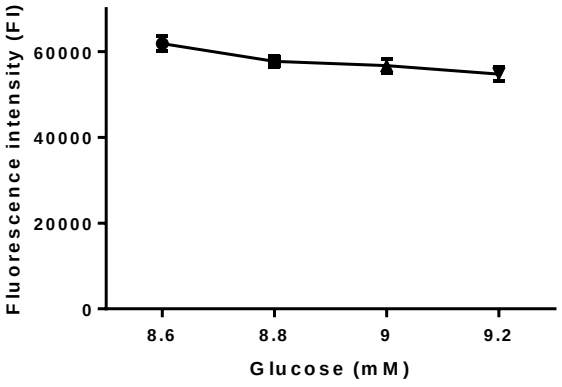


c)

Figure 5.25_Appendix: Examples of standard curves beyond the established linearity. Glucose is not linear within that range.

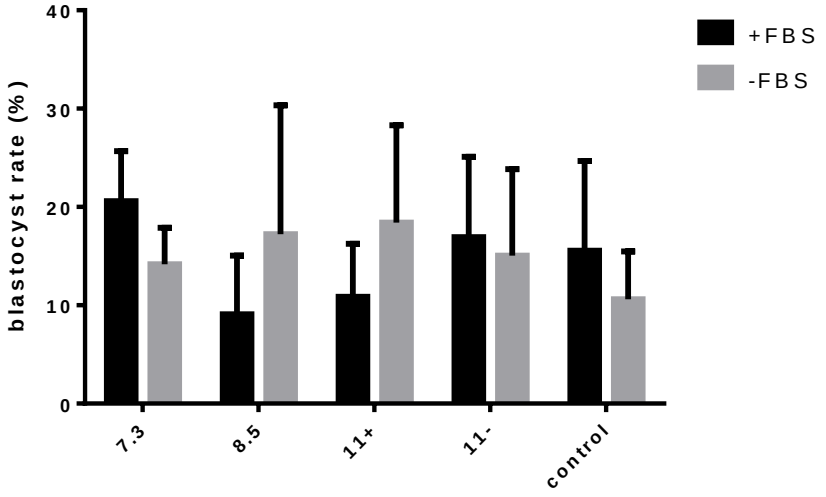
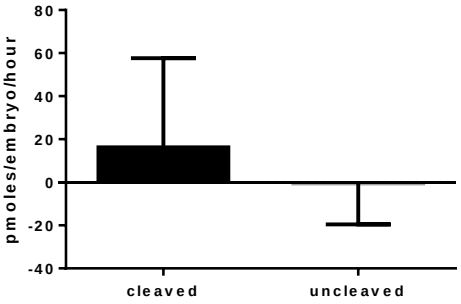
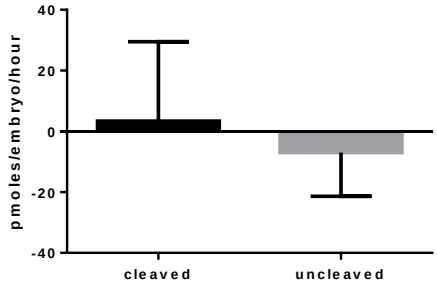


Figure 5.26_Appendix: Comparison of embryo culture conditions, in the presence and absence of FBS.

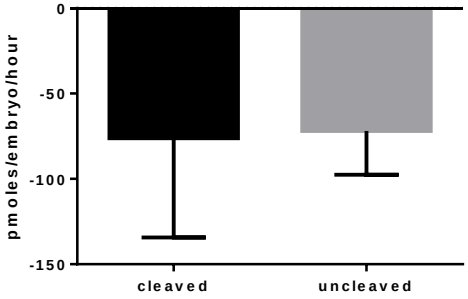


a)

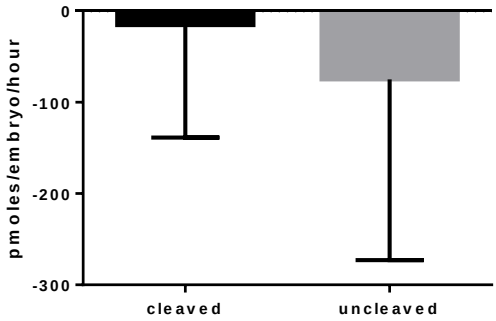


b)

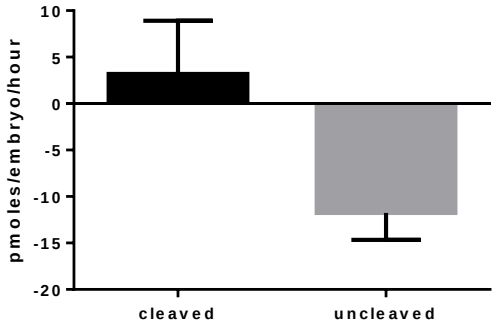
n



c)

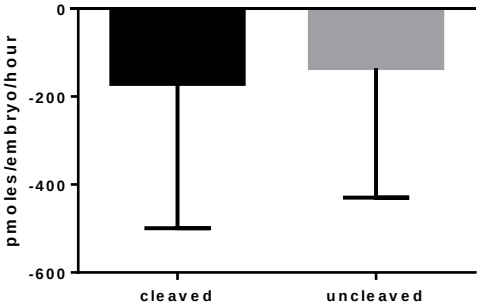


d)

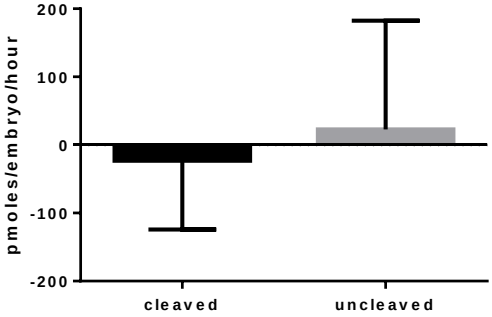


e)

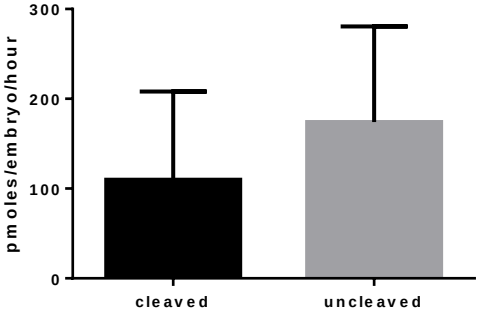
Figure 5.27_Appendix: Comparison of glucose consumption/production in cleaved and uncleaved embryos, under different embryo environments a) '7.3' b) '8.5', c) '11+', d) '11-',e) control


0
-200
-400
-600
pmoles/embryo/hour
cleaved
uncleaved

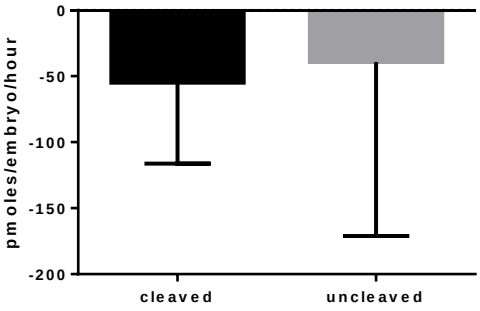
a)


200
100
0
-100
-200
pmoles/embryo/hour
cleaved
uncleaved

b)

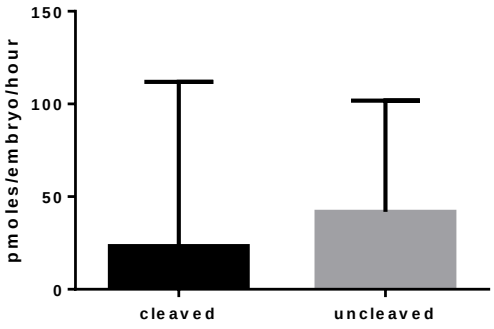

300
200
100
0
pmoles/embryo/hour
cleaved
uncleaved

c)


0
-50
-100
-150
-200
pmoles/embryo/hour
cleaved
uncleaved

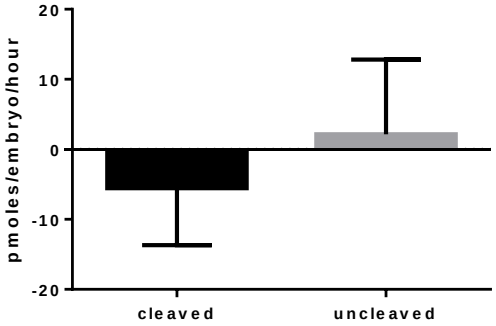
d)

p

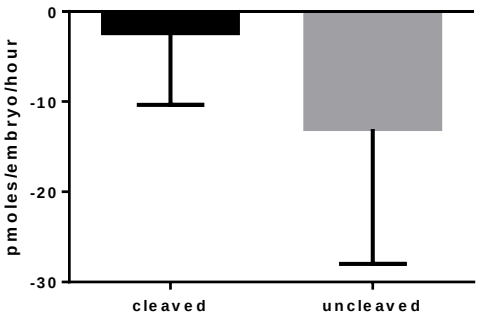


e)

Figure 5.28_Appendix: Comparison of lactate consumption/production in cleaved and uncleaved embryos, under different embryo environments a) '7.3' b) '8.5', c) '11+', d) '11-',e) control

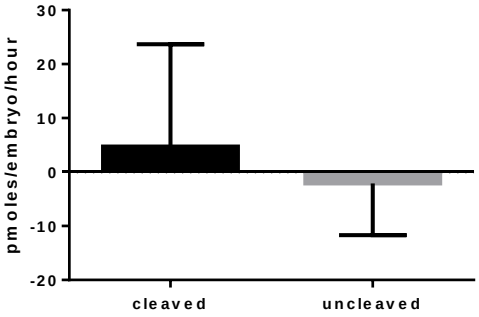


a)

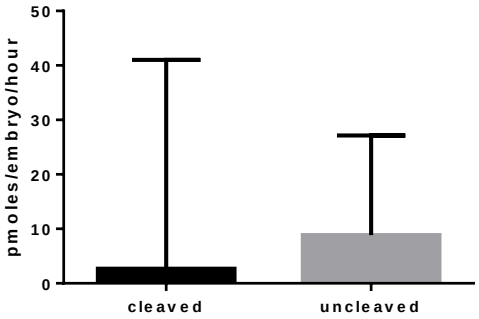


b)

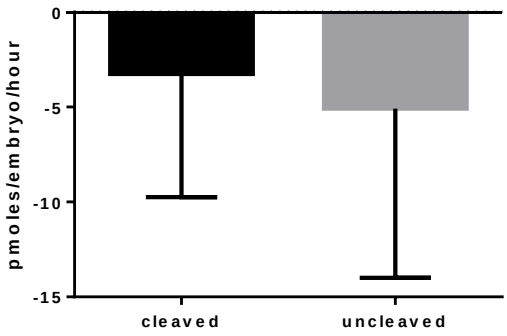
q



c)



d)



e)

Figure 5.29_Appendix: Comparison of pyruvate consumption/production in cleaved and uncleaved embryos, under different embryo environments a) '7.3' b) '8.5', c) '11+', d) '11-',e) control

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