DEVELOPING A NOVEL *IN VITRO* PREPARATION TO STUDY THE MECHANISMS UNDERLYING OVIDUCT FLUID FORMATION IN HEALTH AND DISEASE

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Abstract

Studying the detailed operation of the oviduct is essential considering the association between ailments, such as cancer, and those conceived by IVF — a technique which bypasses the oviduct. However studying the oviduct *in vivo* is impractical. This thesis addresses this by developing a preparation enabling, for the first time, the study of an *in vitro* derived oviduct fluid (*iv*DOF). Highlights of this fluid include a similar amino acid composition to previously reported *in vivo* data, presence of the oviduct specific glycoprotein, the capacity to support early embryo development, and responsiveness to hormonal stimuli.

In addition to providing an avenue for optimising *in vitro* embryo culture using *iv*DOF this thesis investigates some potential underlying molecular aetiologies of subfertility. For instance, artificially induced hyperandrogenism results in a *leaky* oviduct phenotype — demonstrated by reduced *zona occludin* gene expression (vital for epithelial monolayer integrity), decreased transepithelial electrochemical resistance, and increased *iv*DOF volume.

This thesis also shows that supplementation with the drug metformin moderately altered *iv*DOF amino acid content whereas drugs clomifene and diclofenac had no effect. The transport properties of the oviduct epithelium are also investigated using the nutrient carnitine and the dietary derived embryo-toxin genistein — both of which transverse the *in vitro* oviduct epithelial barrier.

In addition to presenting data indicating that dimethyl sulfoxide affects *iv*DOF in a manner resembling the action of 17β-oestradiol, bovine *in vivo* derived uterine luminal fluid is analysed to investigate uterine responses to day of pregnancy, conceptus sex, maternal lactation, and ipsilateral *vs* contralateral uterine horn variations. A theoretical model of subfertility is also presented.

This thesis characterises a novel *in vitro* oviduct model for investigating oviduct fluid formation and regulation within a controlled laboratory environment. The data are important because embryos are particularly sensitive to periconceptual environmental perturbation, potentially leading to adverse lifelong effects.

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Thesis outputs

Correct at the date of final submission

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Manuscripts under Review

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Publications In Preparation

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Conference Abstract Presentations

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Simintiras CA, Courts FL, Sturmey RG **(2012)** "Genistein transport across the bovine oviduct epithelium." *International Embryo Transfer Society (**IETS**), Hanover, Germany.* Abstract published in ***Reproduction, Fertility & Development***, Vol. 25, Issue 1, pp. 208-209. **DOI**: 10.1071/RDv25n1Ab123.

Declaration

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Abbreviations

1°	Primary
2°	Secondary
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
βME	Beta-mercaptoethanol / 2-mercaptoethanol
ΔG	Change in Gibbs' free energy
ΔG_T	Transepithelial conductance
ΔV_A	Apical membrane potential
ΔV_T	Transepithelial voltage
λ	Wavelength
μ	Micro — unitary division depicting $x10^{-6}$
Ω	Ohms
A	Amps
AA	Amino acid
Ab	Antibody
ABC	Adenosine triphosphate binding cassette
ADAM3	A-disintegrin and metalloprotease 3
ADP	Adenosine diphosphate
AI	Artificial insemination
AIJ	Ampullary-isthmic junction
Akt	Protein kinase B
Ala (A)	Alanine
AMPK	Adenosine monophosphate-activated protein kinase
AngII	Angiotensin II
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APS	Ammonium persulphate
Arg (R)	Arginine
ART	Assisted reproductive technology
Asn (N)	Asparagine
ATM	Adipose tissue macrophages
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AQP	Aquaporin
BBB	Blood-brain barrier
BHBA	Beta-hydroxybutyrate
BL	Broad ligament
BOEC	Bovine oviduct epithelial cell
BOFC	Bovine oviduct fibroblast cell

bp	Base pair
BSA	Bovine serum albumin
BSP	Bovine seminal plasma
BUEC	Bovine uterine epithelial cell
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium concentration
$[Ca^{2+}]_e$	Extracellular calcium concentration
$CaCl_2$	Calcium chloride
Caco-2	Human epithelial colorectal adenocarcinoma cell line
Calu-3	Human epithelial lung adenocarcinoma cell line
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation glycoprotein
CD1	Cesarian derived 1
cDNA	Copy deoxyribose nucleic acid
CIDR	Controlled intra-vaginal drug-releasing device
CK	Cytokeratin
Cl^-	Chloride
cm	Centimetres
O_2	Carbon dioxide
COC	Cumulus oocyte complex
Ct	Threshold cycle
$CuSO_4$	Copper (II) sulphate
CYC	Cyclic
CYP19	Aromatase
Cys (C)	Cysteine
DEPC	Diethylpyrocarbonate
DES	Diethylstilbestrol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Di-nucleotide triphosphate
DOHaD	Developmental origins of health and disease
dsDNA	Double stranded deoxyribonucleic acid
E	Epithelium or ethanol
E1	Estrone
E2	17β-oestradiol
E3	Estriol
ECM	Extracellular matrix
EDP	Oestrogen dependent protein / Entner-Doudoroff pathway
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid

EGA	Embryonic genome activation
EGF	Epidermal growth factor
ENaC	Epithelial sodium channel
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ERE	Oestrogen response element
F	Fimbriae
F_o	Folds
F12	Nutrient mixture F12 ham
FACS	Flow assisted cell Sorting
FADS	Female androgen deficiency syndrome
FBS	Foetal bovine serum
$FeSO_4$	Iron (II) sulphate
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
Gln (Q)	Glutamine
Glu (E)	Glutamate
Gly (G)	Glycine
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
GnRH	Gonadotropin releasing hormone
GOPEX	Pentavalent *Branko Grünbaum* Subfertility Model
H^+	Hydrogen proton
H_2S	Hydrogen sulfide
HBSS	Hank's balanced salt solution
HCEC	Human corneal epithelial cells
hCG	Human chorionic gonadotrophin
HCO_3^-	Bicarbonate
HDL	High density lipoprotein
HEPES	4-(2-hydroxy-ethyl)-1-piperazine ethane sulphonic acid
HFEA	Human fertilisation and embryology authority
His (H)	Histidine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IEC-18	Rat immortalised small intestinal epithelial
IFNτ	Interferon tau
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
ILβ	Interleukin beta
Ile (I)	Isoleucine
IRS1	Insulin receptor substrate 1

I_{SC}	Short circuit current
in vivo	Latin for 'within the living'
in vitro	Latin for '*in glass*'
in situ	Latin for '*in position*'
***iv*DOF**	*in vitro* derived oviduct fluid
IVF	*in vitro* fertilisation
K^+	Potassium
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
kDa	kiloDaltons
KR	Krebs Ringer medium
L-PGTS	Lipocalin-type prostaglandin D synthase
LDL	Low density lipoprotein
Leu (L)	Leucine
LH	Luteinising hormone
LP	*Lamina propria*
LTF	Lactoferrin
Lys (K)	Lysine
m	Milli — unitary division depicting $x10^{-6}$
M	Molarity unit — $mol \cdot l^{-1}$
MAP4K3	Mitogen activated protein kinase kinase kinase kinase 3
MAPK	Mitogen activated protein kinase
MDR1	Multi-drug resistance protein 1
Met (M)	Methionine
Mes	Mesothelium
Mg^{2+}	Magnesium
$MgSO_4$	Magnesium sulphate
miRNA	Micro ribonucleic acid
MIQE	Minimum info. for publication of qRT-PCR experiments
ml	Millilitres unit
mRNA	Messenger ribonucleic acid
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic yarget of rapamycin
MUC9	Mucin 9
n	Nano — unitary division depicting $x10^{-9}$
Na^+	Sodium
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National centre for biotechnology information

$NaHCO_3$	Sodium bicarbonate
NCS	Newborn calf serum
NEFA	Non-esterified fatty acid
NFkB	Nuclear factor kappa beta
NHS	National health service
nm	Nanometre — unit
NO	Nitric oxide
NOX	NADPH oxidase
NTP	Nucleotide triphosphate
O	Ovary
OEC	Oviduct epithelial cell
OGP	Oviductal glycoprotein
OPA	O-phthaldialdehyde reagent
OVGP	Oviduct specific glycoprotein
P_4	Progesterone (pregn-4-ene-3, 20-dione)
P45011a1	Cholesterol desmolase
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAI1	Plasminogen activator inhibitor 1
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
PCO	Polycystic ovary
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PD	Potential difference
PDGF	Platelet derived growth factor
PDMS	Polydimethylsiloxane
PDK	Phosphatidylinositol dependent kinase
Pe	Peritoneum
PenStrep	Penicillin-streptomycin solution
PEP	Phosphoenolpyruvate
PET	Polyethylene terephthalate
$PGF_{2\alpha}$	Prostaglandin F2α
pH	Power of hydrogen
Phe (F)	Phenylalanine
PI3K	Phosphatidylinositol-3-hydroxyl kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PNS	Parasympathetic nervous system
PPAR	Peroxisome proliferator activated receptors

Pro (P)	Proline
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse transcription PCR
R	Resistance
R_A	Apical membrane resistance
R_{ABS}	Absolute resistance
R_B	Basal membrane resistance
R_{IC}	Intercellular resistance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
R_{PC}	Paracellular resistance
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
R_{TJ}	Tight junction resistance
R_{TRC}	Transcellular resistance
SACN	Scientific advisory committee on nutrition
SD	Standard deviation of the mean
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean / Scanning electron microscopy
Ser (S)	Serine
SKG	Serine-threonine kinase
SLC	Solute carrier protein
SNAT	Sodium coupled neutral amino acid transporter
SNS	Sympathetic nervous system
SOF	Synthetic oviduct fluid
SpM	Sub-peritoneal musculature
SSP1	Osteopontin
T	Testosterone
TBS	Tris buffered saline
TCA	Tricarboxylic acid (Krebs) cycle
TER	Transepithelial electrochemical resistance
TER*f*	Fractional transepithelial electrochemical resistance
TEM	Transmission electron microscopy
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th	Helper T cell
THF	Tetrahydrofuran / Tetrahydrofolate
Thr (T)	Threonine
TIMMP1	Tissue inhibitor of matrix metalloprotease 1
TJ	Tight junction

T_m	Melting temperature
T_{muc}	*Tunica mucosa*
T_{musc}	*Tunica muscularis*
TNFα	Tumour necrosis factor alpha
Trp (W)	Tryptophan
T_{ser}	*Tunica serosa*
T_{sub}	*Tunica submucosa*
Tyr (Y)	Tyrosine
TZD	Thiazolidinedione
U	Uterus
UK	United Kingdom
UCD	University College Dublin
ULF	Uterine luminal fluid
UTJ	Uterotubal junction
V	Voltage
v*/*v	Concentration as *volume* to *volume*
WB	Western blot
WPI	World precision instruments
w*/*v	Concentration as *weight* to *volume*
XX	Female
XY	Male
ZO1	Zona occludin 1
ZP	Zona pellucida

ἓν οἶδα ὅτι οὐδὲν οἶδα

I know one thing, that I know nothing.

Socrates (c. 469 BC - 399 BC)

Chapter 1

General Introduction

Chapter 1 — General Introduction

1.1. The Developmental Origins of Health and Disease

Professor David Barker first hypothesised that a low birth weight was associated with later incidence of type 2 diabetes, heart disease, and high blood pressure (Barker & Osmond 1988; Barker 1997; Godfrey & Barker 2000; Armitage *et al* 2004). These epidemiological observations were the precursor to the Developmental Origins of Health and Disease (DOHaD) concept which describes the capability of the environment surrounding early life (from conception to early childhood) to induce changes that have long term impact on adult health. Owing to the widespread adoption of assisted reproductive technologies, this has become one of the greatest concerns facing the field of reproductive medicine (SACN, 2011).

The most striking reproductive pathology has traditionally been infertility which is thought to affect about 1 in 6 couples globally (Hull *et al* 1985). However landmark discoveries in Assistive Reproductive Technologies (ARTs) offer scope to circumvent this problem, at least in principle. The most commonly used ART is *in vitro* fertilisation (IVF) which has led to the birth of over 5 million children (Kamphuis *et al* 2014) since its inception (Steptoe & Edwards 1978). However some startling patterns of negative health impacts of IVF are emerging (Kamphuis *et al* 2014). For instance evidence hints at links between IVF and the development of afflictions such as insulin resistance (Ceelen *et al* 2008), hypospadias (Silver *et al* 1999), retinoblastoma (Moll *et al* 2003) and even cancer (Klip *et al* 2001). Such reports lead one to enquire into the differences between conception *in vivo* and *in vitro*.

Perhaps the most obvious difference is that IVF bypasses the oviduct. Ironically, one historic consequence of the success of IVF has been to diminish scientific

inquiry into the role of the oviduct (Leese *et al* 2001). However, despite the oviduct being unessential to basic reproduction, the notion that the oviduct plays a key reproductive role nonetheless is gaining traction (Leese *et al* 2008b; Aviles *et al* 2010; Feuer & Rinaudo 2012; Menezo *et al* 2015).

Four crucial reproductive events are facilitated by the oviduct: (***i***) oocyte (egg) capture, (***ii***) sperm capacitation, (***iii***) fertilisation, and (***iv***) early embryo development (Hunter 2012). Insights into the detailed operation of the oviduct are therefore of paramount importance to (***a***) our understanding of essential reproductive processes, (***b***) improving existing *in vitro* embryo culture methods, (***c***) targeting suboptimal fertility *in vivo* and (***d***) augmenting our understanding of the way in which the first environment of the developing embryo can influence development and lifelong health.

1.2. Reproductive Endocrinology

1.2.1. The Oestrous Cycle

The oestrous cycle describes the endocrine activity facilitating mammalian female transition between reproductive non-receptivity to receptivity thereby enabling mating and subsequent pregnancy (Forde *et al* 2011). The stages of the oestrus cycle can be divided into two broad phases (follicular and luteal) and four sub-phases (proestrus, estrus, metestrus and diestrus) as depicted in Figure 1-1. In eutherian mammals the phenomenon of reproductive cycling is governed by the endocrine system which exerts function by positive and negative feedback mechanisms (Roche 1996) described briefly below.

Day 0 of the oestrous cycle is defined by ovulation which is triggered by a rise in LH levels (Rahe *et al* 1980). The LH surge is preceded by 17β-oestradiol (E2) secretions from the ovarian granulosa cells (Gordon *et al* 1994) which circulate to

the brain. In this case (at around day -3 or 18) this E2 surge stimulates rapid bursts of Gonadotropin Releasing Hormone (GnRH) release (Schally *et al* 1971a 1971b).

It is worth noting that GnRH secretion is pulsatile throughout the cycle whereby the frequency of secretion varies (Tsutsumi & Webster 2009). Specifically E2 regulates GnRH secretion frequency by both negative and positive feedback — low circulating E2 inhibits whereas high E2 promotes GnRH secretion (Moenter *et al* 2009; Christian *et al* 2008) (Figure 1-2).

Following ovulation, the ruptured follicle forms the corpus luteum which begins secreting progesterone (P4) (Taya *et al* 1991). P4 in combination with E2 also suppresses GnRH release thereby reducing the amplitude of concomitant LH pulses (Kulick *et al* 1999). In the absence of pregnancy, the corpus luteum regresses over approximately 10 days. P4 levels fall, enabling E2 to begin to rise and thus allowing the cycle to restart (Gordon 2003) (Figures 2-1 and 2-2).

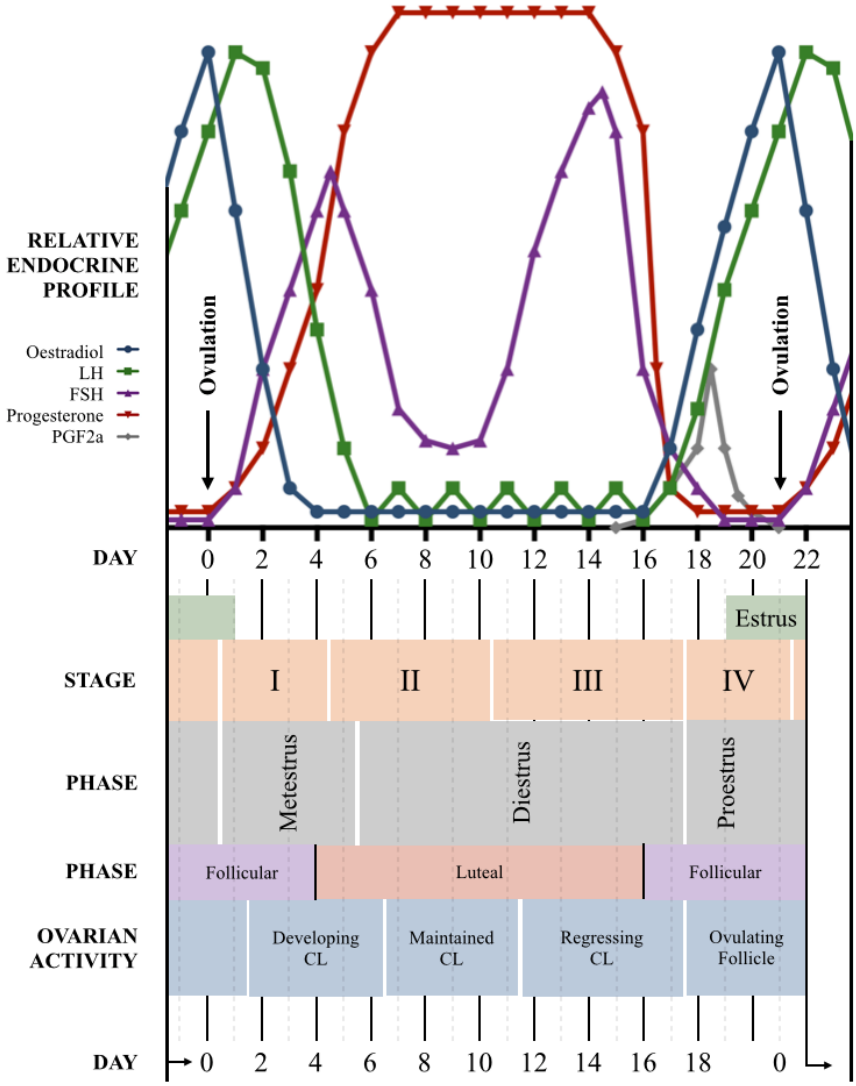

Figure 1-1 — Representative bovine oestrous cycle with relative systemic circulating hormone flux during two waves of follicular growth within 22 days.

1.2.2. The Menstrual Cycle

There are key differences between the human menstrual cycle and the bovine oestrus cycle (Kol & Homburg 2008). For instance the bovine enters oestrus for a few days around ovulation. During this brief window the animal is receptive to the male, and is referred to as *standing heat*. In contrast human females are sexually active throughout their cycle although not typically during their menstrual period (Tanfer & Aral 1996). Moreover, although both menstrual and oestrus cycles have secretory and proliferative phases, the menstrual cycle undergoes a third phase

wherein the endometrium is shed (menstruation) whereas in cattle, uterine tissue is reabsorbed (Devkota *et al* 2012). Furthermore, the oestrus cycle lasts approximately 21 days whereas the menstrual is typically around 28 days (Murphy *et al* 1991).

In spite of these differences, the principle of hormonally-mediated cycling is conserved between the two species. Progesterone, testosterone and estradiol play key roles in regulating the oestrous cycle is highlighted in Figure 1-2.

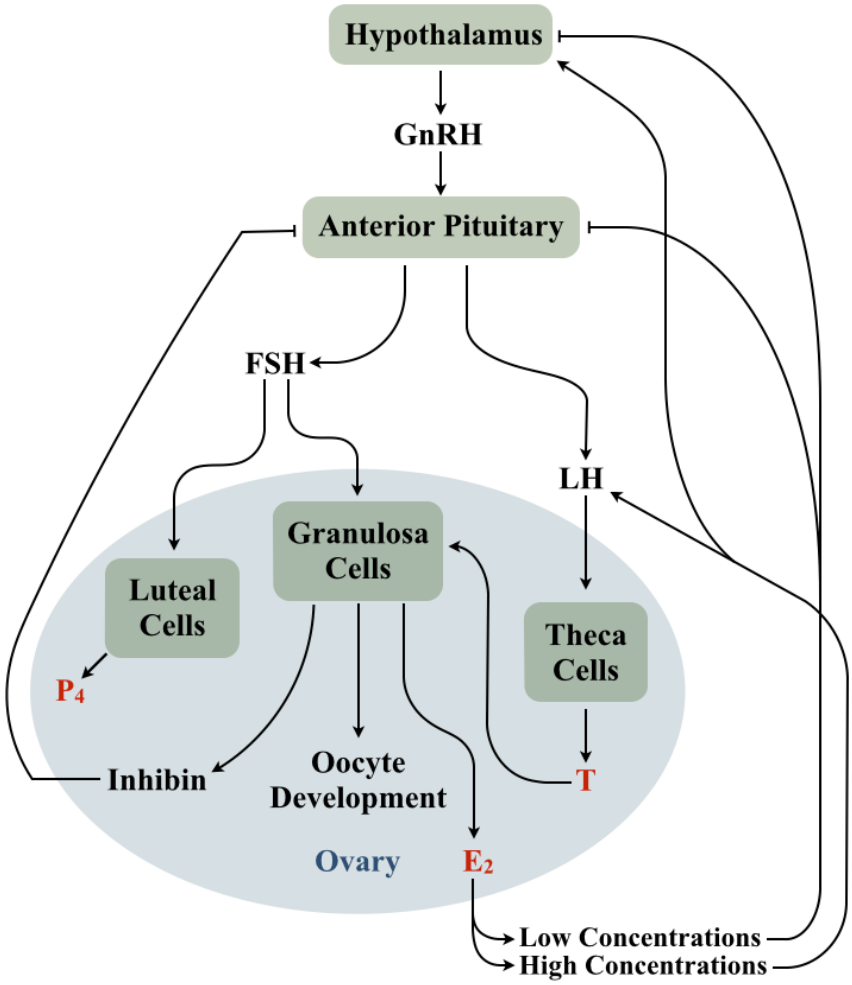

Figure 1-2 — The physiological relationships between ovarian hormones, the hypothalamus and the anterior pituitary gland; referred to as the hypothalamic-ovarian axis. Within this complex feedback loop including 17β-oestradiol (E_2), progesterone (P_4), and testosterone (T), pointed arrows represent stimulation whilst blunted arrows indicate inhibition (adapted from Arrais & Dib 2006 and Diamanti-Kandarakis *et al* 2008).

1.2.3. 17β-Oestradiol

17β-oestradiol (Figure 1-3:A) is one of three primary naturally occurring oestrogens — estrone (E1), 17β-oestradiol (E2), and estriol (E3). E1 is the least abundant oestrogen at its maximal concentration post-menopause (Cauley *et al* 1989). E3 is exclusively produced by the placenta and is the weakest oestrogen in terms of affinity for oestrogen receptors yet becomes the most dominant during late pregnancy (Siler-Khodr *et al* 1986).

The biosynthesis of E2, which is the dominant estrogen in cycling females, requires the synergistic activity of the theca and granulosa ovarian epithelia (Havelock *et al* 2004). It is synthesised by thecal cells which convert pregnenolone to androstenedione (Fowler *et al* 1978). Androstenedione is subsequently converted into testosterone prior conversion of testosterone to the final E2 (Nobel *et al* 2001). This second stage of E2 synthesis occurs in granulosa cells as the key enzymes, CYP19 and 17β-HSD are not present in theca cells (Gulliver 2013) (Figure 1-3:B). E2 is a potent oestrogen and elicits cellular responses by action on oestrogen receptors alpha (ERα) and beta (ERβ) (Katzenellenbogen *et al* 2000).

1.2.4. Progesterone

Progesterone (P4) (Figure 1-3:A) is primarily synthesised in the ovaries by two sequential reactions starting with cholesterol desmolase catalysing the conversion of cholesterol to pregnenolone followed by the synthesis of P4 from pregnenolone by 3β-hydroxysteroid dehydrogenase (3β-HSD) (Pikuleva 2006) (Figure 1-3:B). P4 centrally regulates critical reproductive events including pregnancy establishment, implantation and uterine myometrium contractility (Etgen 1984; Mulac-Jericevic & Conneely 2004).

1.2.5. Testosterone

Testosterone (T) is the androgenic anabolic steroid hormone (Figure 1-3:A). It is synthesised in the female by the ovarian theca cells and to a lesser extent the adrenal glands. Similar to E2 and P4, T is derived from cholesterol via sequential enzymatic reactions (Figure 1-3:B). Unlike E2 and P4 however T is not directly involved in oestrous cycle regulation although an increase in circulating testosterone has been linked with reduced fertility (Speranda & Papic 2004) — specifically hyperandrogenism, defined as a high T to E2 ratio, is a common characteristic of women with disorders such as polycystic ovary syndrome (PCOS) who are subfertile (Cho *et al* 2008). A mechanism underpinning this phenomenon has been proposed by Nelson *et al* (2001) who showed that 3β-HSD in theca cells of women with PCOS convert steroid precursors to testosterone to a greater extent than in those without PCOS. In the female 25% of T comes from the adrenals, 25% is produced by the ovaries, and 50% originates in peripheral tissues from adrenal and ovarian pre-hormones (Bachmann 2002).

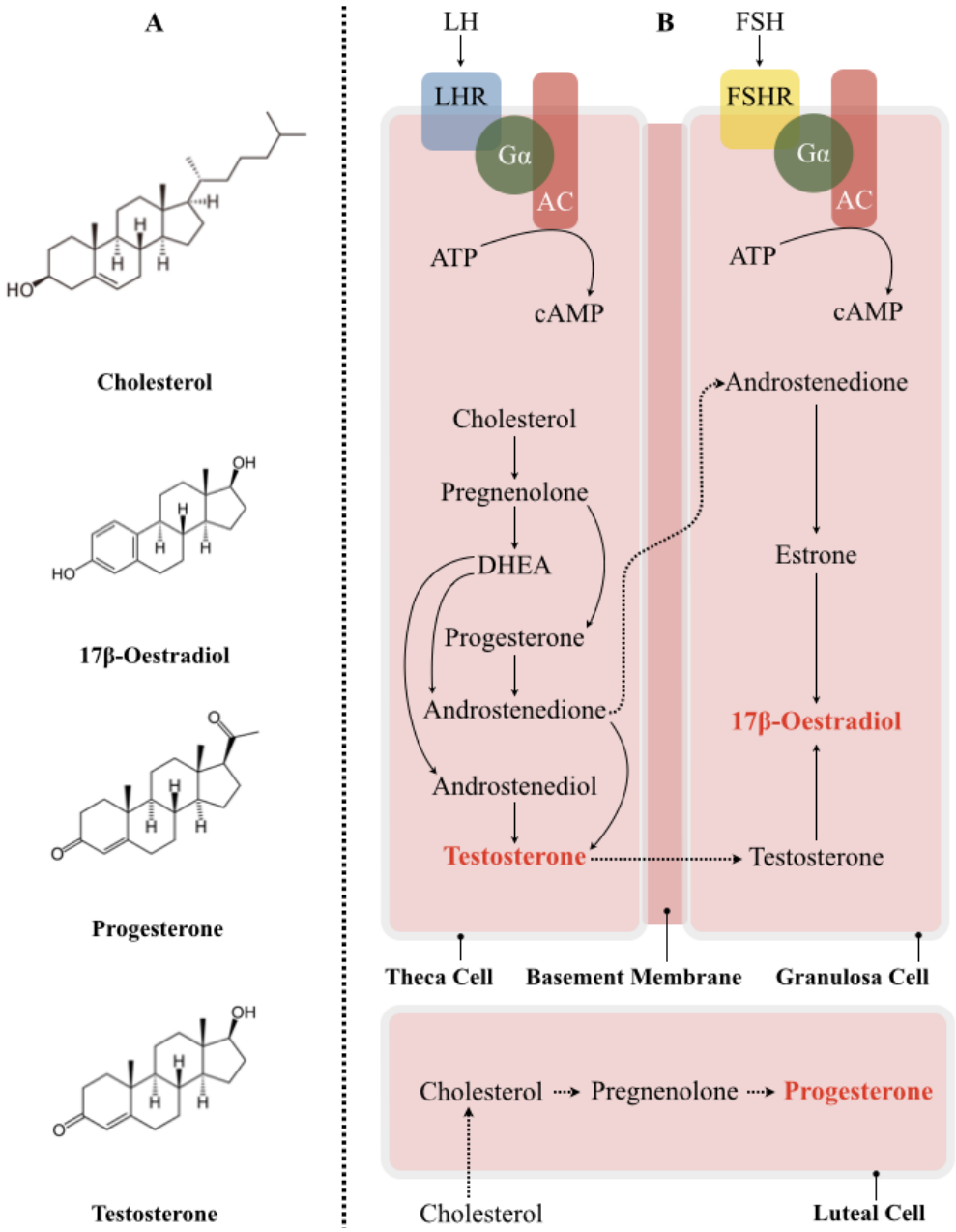

Figure 1-3 — **A**: Chemical structures of ovarian steroids; cholesterol, 17β-oestradiol (E2), progesterone (P4), and testosterone. (T) **B**: Representation of ovarian steroidogenesis in the developing follicle (theca and granulosa) and corpus luteum (luteal cells). LH-stimulated theca sequentially convert cholesterol to androstenedione and T which diffuse through the basement membrane for conversion to E2 in FSH-stimulated granulosa cells. Dashed arrows indicate molecular translocations, solid arrows represent enzymatic conversions. Cyclic adenosine monophosphate (cAMP) produced by protein hormone signalling (LH and FSH binding) activates most of the enzymes involved in the steroidogenesis cascade (adapted from Craig *et al* 2011 and Andersen & Ezcurra 2014).

1.3. Reproduction

1.3.1. Oocyte Retrieval

Ovulation is the release of the female gamete (oocyte/egg/ovum) from the ovary. At ovulation, the cumulus oocyte complex (COC) is released from the ruptured ovarian Graafian follicle. The COC is either lost into the peritoneal (abdominal) cavity, collected by the oviduct for fertilisation, or move unfertilised to the uterus. Although the oviduct has intrinsic muscular contractility (Ruckebusch & Bayard 1975), progesterone released from the ovarian *corpus luteum* (ruptured follicle) post-ovulation leads to increased blood flow into the distal oviduct thereby increasing dilation and motility. The cilia lining the inner oviduct are moreover highly active during this period increasing the likelihood of oocyte capture (Eddy & Pauerstein 1980).

Post-ovulation the oocyte's extracellular *zona pellucida* (ZP) coat undergoes changes to enable the binding of oviduct secreted proteins which further modify ZP carbohydrate and protein composition (Funahashi *et al* 2001). Such oviduct derived proteins include oviduct specific glycoprotein one (OVGP1), osteopontin (SSP1), lipocalin-type prostaglandin D synthase (L-PGTS), lactoferrin (LTF) (Goncalves *et al* 2008), plasminogen activator inhibitor 1 (PAI1) (Kouba *et al* 2000), and tissue inhibitor of matrix metalloproteinase 1 (TIMMP1) (Buhi *et al* 1997); assumed to protect the oocyte from degradation and facilitate fertilisation.

1.3.2. Sperm Capacitation and Fertilisation

In human reproduction the ejaculate is deposited at the anterior vagina; an immunologically hostile and acidic environment (Kumamoto & Iwasaki 2012). To avoid death the sperm 'swim' rapidly towards the cervix where the cervical mucus further filters out sperm with poor morphology and motility (Suarez & Pacey

2006). A minority of ejaculated sperm pass the cervix and into the uterus where muscular contractions facilitate sperm transit through the uterine cavity. From the ~100 million sperm ejaculated only a few thousand will reach the oviduct (utero-tubal) junctions (Suarez & Pacey 2006).

Current data suggest that sperm migration through the oviduct to the oocyte occurs via a chemotactic mechanism (Hunter 2012) — such as a progesterone-mediated chemical gradient originating from the ovary (Eisenbach & Giojalas 2006; Guidobaldi *et al* 2012; Coy *et al* 2012). However this does not appear to be the only mechanism by which sperm reach the oocyte in the oviduct. Muscular contractions of the uterus and oviduct are thought to aid the process, as are oviductal ciliary movements. It has also been shown that sperm cannot enter the oviduct in the absence of certain sperm derived proteins such as A-Disintegrin and Metalloprotease 3 (ADAM3) (Okabe 2013). The mechanisms underlying this process remain unclear but could provide insights into male factor infertility.

Sperm released at ejaculation are incapable of oocyte fertilisation since they must first remain in the oviduct to capacitate (mature) – a process independently discovered by Austin (1951) and Chang (1951) in the rat and rabbit respectively. In these studies the authors reported that sperm did not penetrate the oocyte unless first deposited into the oviduct for at least 4 hours in both species. However optimal fertilisation rates were observed when the duration of sperm-oviduct incubation was 8 hours in the rabbit and 24 hours in the rat.

It is now known that sperm adhere to the oviduct epithelium to form a so-called ‘sperm reservoir’ until ovulation. This has been shown to occur biochemically via carbohydrate-protein interactions (Fazzeli *et al* 1999; Koelle *et al* 2009; Topfer-Petersen *et al* 2002; Suarez 2001) and by physical entrapment within oviduct *sperm crypts* (Bedford *et al* 1997).

A number of mechanisms have been shown to induce release of sperm from the reservoir including signals from the COC (Koelle *et al* 2009), oviduct epithelial reorganisation and secretion of oviduct specific glycoprotein 1 (OVGP1) (Killian 2011; Kan *et al* 2006), progesterone (P4) and 17β-oestraiol (E2) (Talevi & Gualtieri 2010; Coy *et al* 2012), sperm hyper-motility (Suarez 2008), and sialidase release by the sperm (Ma *et al* 2012). Al-Dossary *et al* (2013) have furthermore discovered exosomes in the oviduct during sperm transit which may play a role in oviduct-sperm interactions. Whichever the case, capacitation is a crucial reproductive event since its inhibition or even premature occurrence can compromise fertilisation (Colledge 2013).

1.3.3. Early Embryo Development

The preimplantation embryo spends approximately 3-4 days in the oviduct, though there are species differences (Wang & Dey 2006; Suarez 2006). During this time critical embryonic developmental events occur — notably, cleavage (cell division) and embryonic genome activation (EGA) (Lonergan *et al* 2003). This is reflected in the observation that moving an embryo from the *in vivo* to *in vitro* environment or *vice versa* during any stage of pre-implantation development significantly disrupts the gene expression profile of the resulting blastocyst (Gad *et al* 2012). It is also known that embryo cleavage is blocked. For example at the 8-cell stage in bovine and 2-cell in mouse, when the *in vitro* culture is suboptimal (Lequarre *et al* 2003).

The morphology, gene expression, cryotolerance and post-transfer pregnancy rates of *in vivo* embryos are superior to those for embryos developed *in vitro* (Rizos *et al* 2010; Mondejar *et al* 2012). The reasons for this phenomenon are unclear but it is possible to list five features of oviduct physiology that provide an appropriate environment for the embryo during its most critical stage of development.

It *firstly* acts as physical barrier sterically hindering the transit of potentially hostile influences from the maternal vasculature into the oviductal lumen. *Secondly*, the secretory pockets of the oviduct epithelial landscape secrete a multitude of biochemical components (Section 1.6.) to create a metabolically and nutritionally favourable environment for the developing offspring. *Thirdly*, oviductal cilia play a biophysical role in establishing fluid flow towards the uterine body in the presence of the embryo (Belve & McDonald 1968) whilst simultaneously mixing the luminal milieu. *Fourthly*, the oviduct communicates with the embryo via paracrine and possibly juxtacrine signalling; and *fifthly*, the oviduct spatiotemporally synchronises itself as the embryo passes towards the uterus for implantation, to establish a physicochemical (temperature and pH) gradient — to maximise the likelihood of pregnancy maintenance (Coy *et al* 2012).

Oviducts from different species exhibit similar transcriptomic and proteomic profiles, reflecting conserved biological function – to the extent that embryos cultured from one species can survive physiologically within the oviduct of a different species (Mondejar *et al* 2012; Rizos *et al* 2002; Lazzari *et al* 2010). Figure 1-4 schematically depicts the spatiotemporally dynamic processes of oocyte maturation, fertilisation and early embryo development.

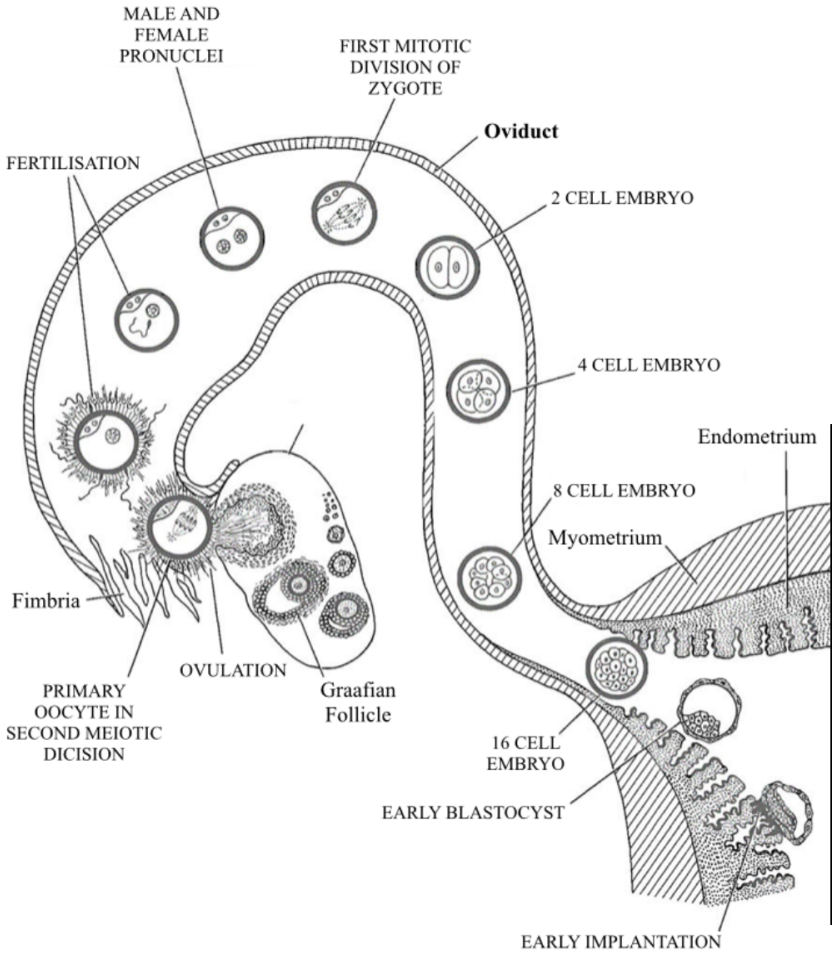

Figure 1-4 — Schematic diagram of early pre-implantation reproductive events in the human reproductive tract, specifically showing egg maturation in the ovary prior to ovulation and COC retrieval by the oviduct. Assuming peri-ovulatory intercourse, fertilisation will occur 12-24 hours post-ovulation and form a zygote. The zygote will then divide to form a 2-cell embryo and division continues until the 16-32 cell stage at which point the embryo compacts to form a morula (~ day 4). After around Day 5 the early blastocyst will develop further in the uterus. Image adapted from discovery.lifemapsc.com.

1.4. Oviduct Gross Morphology

The oviduct (Fallopian tube or uterine tube or salpinx) is a seromuscular tubular organ whose distal portion surrounds the ovary whilst the proximal oviduct adjoins the uterus. This is why the oviducts were first described by the Italian anatomist Gabriele Fallopius (1523-1562) as *tuba uteri* or the *trumpets of the uterus* – as they extend bilaterally from the uterine body towards the ovaries and

are shaped like a musical instrument (Cappello *et al* 2010). The human oviduct is ~12 cm in length and 0.5-1.0 cm in diameter. The longitudinal anatomy comprises 4 principal sections: the fimbriae, infundibulum, ampulla, and isthmus (Coy *et al* 2012) (Figure 1-5).

1.4.1. Fimbriae

The fimbriae are the fine protrusions of the *ostium* — the peripheral opening of the oviduct into the peritoneum, specifically extending proximally from the infundibular fringe towards the ovary. These protrusions are not connected to the ovary although of the many fimbriae one is long enough to reach the ovary — the *fimbria ovarica* (Ahmad-Thabet 2000). Fimbriae are internally lined with ciliated epithelial cells and are cumulatively responsible for COC retrieval at ovulation (Gordts *et al* 1998). Adjoining the fimbriae is the oviductal infundibulum.

1.4.2. Infundibulum

The human infundibulum is ~ 3 cm long and is the widest section of the oviduct at ~1.0 cm in diameter. It resembles a 'funnel' opening into the peritoneum. The infundibulum is internally lined with a ciliated simple columnar epithelium indicative of its function in gamete transport facilitation (Hunter 2012). The infundibulum medially adjoins the ampulla.

1.4.3. Ampulla

The ampulla is the oviduct mid-region, and the longest section at approximately 6 cm with a diameter of ~1.0 cm in the human. The ampulla curves over the ovary and is the site of fertilisation in addition to being the most active secretory region of the oviduct . It is also the site of fertilisation — specifically around the

ampullary-isthmic junction (AIJ) (Aguilar & Reyley 2005; Buhi *et al* 1990; Erickson-Lawrence *et al* 1989; Hyde & Black 1986; Leese 1983, 1988; Wegner & Killian 1992).

1.4.4. Isthmus

The isthmus is the most proximal region of the oviduct, medially adjoining the ampulla (AIJ) and terminating at the uterotubal junction (UTJ). The human isthmus is 2-3 cm in length and 0.1-1.0 mm in diameter (Pauerstein & Eddy 1979) making it the region of lowest 'vacant' diameter, and thus the area of greatest shear stress, as reflected in its considerably thick wall (Figure 1-5) (Underwood *et al* 2010).

1.5. The Oviduct in Cross Section

The oviduct is a dynamic organ with several variations from the *fimbriae* to the *isthmus* which is reflective of its various functions. This is also true when it is observed transversely. Anatomically and histologically the oviduct comprises four principal sections: the inner *tunica mucosa* ($\mathbf{T}_{muc}$), the *tunica submucosa* ($\mathbf{T}_{sub}$), the *tunica muscularis* ($\mathbf{T}_{musc}$), and the outermost *tunica serosa* ($\mathbf{T}_{ser}$) (Figure 1-5). These regions of the oviduct are responsible for the establishment and maintenance of the oviductal luminal environment (Figure 1-5).

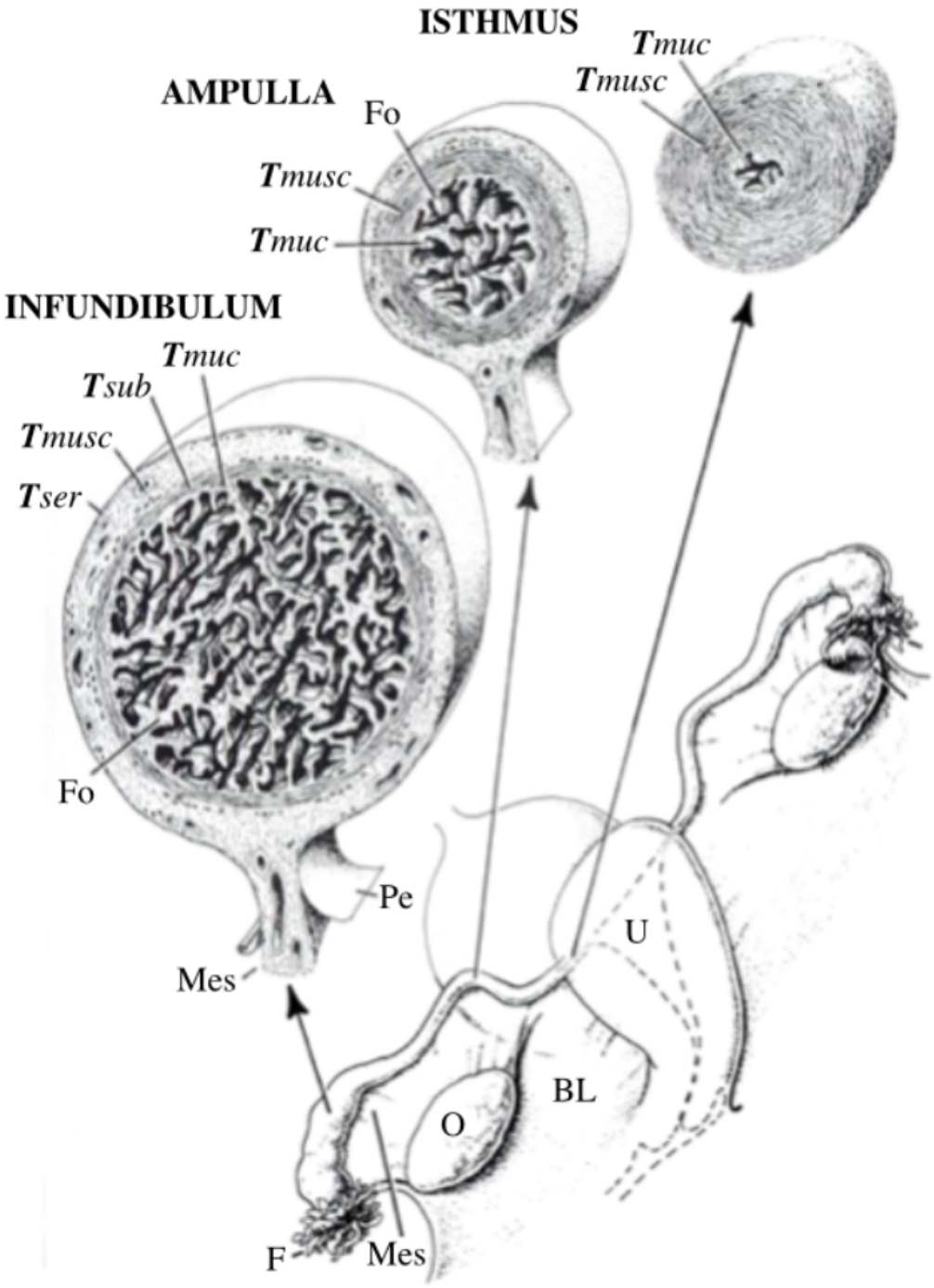

Figure 1-5 — The four main longitudinal sections of the oviduct: *fimbriae*, *infundibulum*, *ampulla*, and *isthmus*, including cross sections of the showing variation in *tunica mucosa* ($\mathbf{T}_{muc}$), *tunica submucosa* ($\mathbf{T}_{sub}$), *tunica muscularis* ($\mathbf{T}_{musc}$), and *tunica serosa* ($\mathbf{T}_{ser}$) in addition to highlighting epithelial folds, (Fo), mesothelium (M), peritoneum (Pe), fimbriae (F), ovary (O), broad ligament (BL), and uterus (U) — adapted from discovery.lifemapsc.com.

1.5.1. *Tunica muscularis*

The tunica muscularis comprises the circular and longitudinal smooth muscle fibres of the oviduct — responsible for its peristaltic contractions in response to ovarian hormones (Hafez & Hafez 2000). Contractions are greatest around the time of ovulation to facilitate oocyte capture. Specifically, smooth muscle bundles radiate from the longitudinal muscle into the fimbriae [termed *sub-peritoneal musculature* (SpM)] enabling the distal oviduct to position itself around the protruding tertiary follicle to minimise the risk of oocyte loss. It is believed that *tunica muscularis* contractions, in synchronisation with the pulsatile movement of cilia, facilitate sperm transport towards the egg and subsequent embryo conveyance towards the uterus (Gordts *et al* 1998). Total *tunica muscularis* increases from infundibulum to isthmus despite the parallel longitudinal narrowing of the whole oviduct (Coy & Aviles 2010). Directly beneath the *tunica mucosa* lies the *tunica serosa* (**T**$_{ser}$).

1.5.2. *Tunica serosa*

The *tunica serosa* is the outermost cylindrical portion surrounding the oviduct and defining it as a distinct intraperitoneal organ (Otsuki *et al* 1989). The *tunica serosa* is composed of dense connective tissue, primarily comprising collagen, forming a continuous sheath with the uterine *tunica serosa*. The role of the *tunica serosa* is structural with minimal histological variation between different species (Johnson & Foley 1974). Beneath the *tunica serosa* is the **T**$_{sub}$.

1.5.3. *Tunica submucosa*

The **T**$_{sub}$ (also known as the mesothelium or extracellular matrix (ECM) or *lamina propria* (LP) or stroma) is a biologically complex thin lattice of loose irregular

connective tissue – created and maintained by fibroblast cells. This network of connective scaffolding primarily comprises laminins, integrins and collagens embedded in proteoglycans (Fazleabas *et al* 1997) that are responsible for its mechanical properties – a highly elastic and compressible plexus providing the organ with tensile strength (Frantz *et al* 2010). For example the bladder exhibits a high contractile and expansive capacity owing to its extensive $\mathbf{T}_{sub}$ (Young *et al* 2013). In addition to the important biophysically stress resistant properties of the $\mathbf{T}_{sub}$, a number of cell types — including lymphocytes, macrophages, plasma cells and leukocytes (Cardenas *et al* 1998) — are embedded within this mesh.

The $\mathbf{T}_{sub}$ is highly vascularised via which endocrine, metabolic, and lymphatic signals are transferred to the oviduct. The rapid diffusion of compounds between the vasculature and epithelium is facilitated by the *tunica submucosa.* Further dynamism of the *tunica submucosa* is reflected in the differential expression of its components during the oestrous cycle (Gabler *et al* 2001). The epithelium or $\mathbf{T}_{muc}$ is situated beneath the $\mathbf{T}_{sub}$. The tubal *mucosa* and *submucosa* are also known as the endosalpinx and myosalpinx respectively.

1.5.4. *Tunica mucosa*

The substructure largely responsible for the creation and regulation of oviduct fluid is the epithelium (E) located in the $\mathbf{T}_{muc}$ a monolayer of simple columnar epithelial cells resting on a thin basement membrane mainly consisting of collagens and laminin, under which resides the *lamina propria mucosae* (histology.leeds.ac.uk). The $\mathbf{T}_{muc}$ is arranged in numerous, branched, latitudinal invaginations giving a folded structure which increases the ratio of total surface area to volume (Figure 1-6:A).

The total ‘vacant’ diameter of the oviduct reduces from the lateral fimbriae to medial *isthmus* in parallel with a mild increase in total invaginations of the *tunica mucosa* – resulting in a considerable increase in the surface area to volume ratio as the oviduct approaches the uterotubal junction (Figure 1-5). Aside from the directionality of the oviduct on a macroscopic scale, each epithelial cell comprising the inner *tunica mucosa* is itself specifically orientated. In other words, oviduct epithelia are directionally polar about their apical (luminal side) to basal (*lamina propria* side) axis; they are biochemically and electrochemically asymmetrical (Figure 1-6:D). The significance of polarity is reflected in the observation that uterine epithelia cultured *in vitro* without defined polarity lose their ability to respond to steroid hormones (Glasser & Mulholland 1993).

In general terms, epithelial cells may be thought of as ‘gatekeepers’ separating the body from the external environment. They have structural and functional roles including nutrient trafficking, gas exchange, secretion, transportation, and external stimulus transduction (Garnett *et al* 2014). The oviductal *tunica mucosa* epithelium consists of two cell types – secretory and ciliated (non-secretory). Cilia are motile narrow projections of the apical surface of the plasma cell membrane. In the oviduct cilia modulate lateral intra-oviductal fluid flow and facilitate embryo transit. Ciliated epithelial cells are terminally differentiated and do not regulate the composition of the luminal fluid although they do determine the flow. By contrast, secretory cells are responsible for the formation and secretion of the oviduct fluid. For this reason, although there are no oviductal glands, the oviduct is loosely considered a secretory organ (Leese 1988).

Ciliated and secretory cell activity in the oviduct is regulated locally by steroid hormones oestrogens and progestogens. On oestrogen stimulation secretory cells will differentiate into ciliated cells (Comer *et al* 1998). Despite this the landscape of the *tunica mucosa* is never entirely dominated by a single cell type. Exceptions

to this are the *fimbriae* which are always exclusively comprised of a ciliated epithelium and the *isthmus* which is consistently dominated by secretory cells (Reeder & Shirley 1999; Lyons *et al* 2006; Comer *et al* 1998). The ratio of ciliated to secretory cells therefore dramatically decreases longitudinally (laterally to medially or from *fimbriae* to *isthmus*).

This is consistent across species (Pedrero-Badillo *et al* 2013) and reflects the numerous roles of the oviduct. For instance lateral oviduct ciliation aids successful oocyte capture and propulsion to the *ampulla* for fertilisation (Kölle *et al* 2010) whilst medial oviduct secretory cell dominance reflects the need for sperm capacitation and the later nourishing responsibility of the oviduct to the embryo.

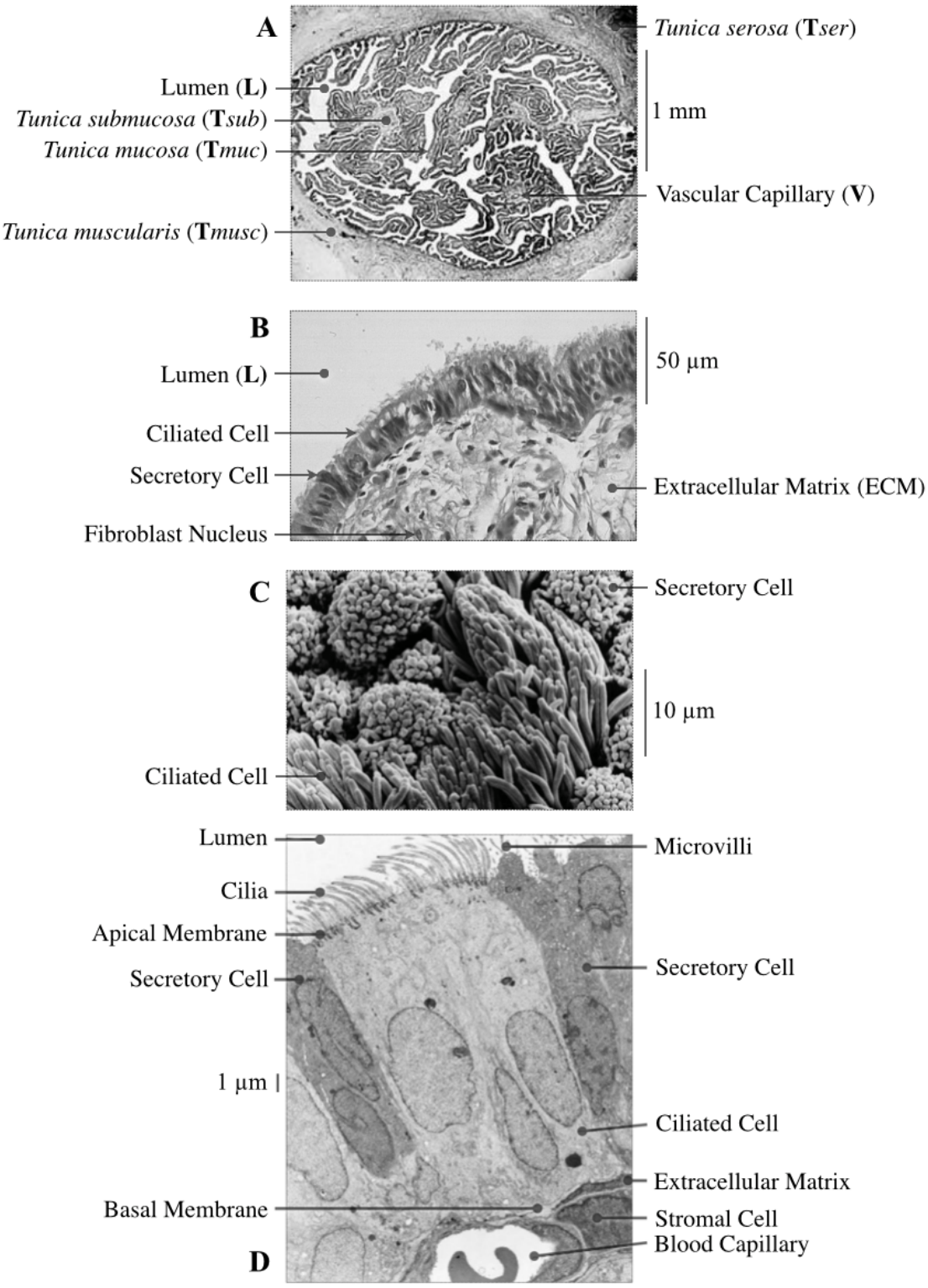

Figure 1-6 — Oviduct images at increasing magnification. **A**: Photomicrograph of a human ampulla in cross section (adapted from glowm.com), **B**: the $\mathbf{T}_{muc}$ and $\mathbf{T}_{sub}$ of an infundibular section (adapted from pathguy.com), **C**: a scanning electron micrograph (SEM) image of a human oviduct epithelium from a luminal view (Lyons *et al* 2006), and **D**: a high resolution SEM of oviduct epithelia in cross section (adapted from med.umich.edu/histology).

Despite this generalisation, both the height and proportion of ciliated to secretory cells are dynamic and correlate with the oestrous cycle. The epithelium is taller in the first half of the follicular phase than during the second half. Moreover, the proportion and activity of secretory epithelia increases during the luteal (secretory) phase of the cycle, indicative of the spatiotemporally active *tunica mucosa* (Verhage *et al* 1973; Steinhauer *et al* 2004).

Besides the fluid created by the *tunica mucosa*, the oviductal lumen also contains some follicular fluid (entering during the peri-ovulation period) in addition to compounds which passively and actively arise from the vasculature of the *tunica subserosa*. This can be mechanistically attributed to either intracellular or paracellular transport (Leese 1988).

As the oviduct epithelium is by definition *open* to the external environment it is unsurprising that it is lymphatically catered for, specifically by the inguinal nodes. The role of the immune system in reproduction has been studied to an extent (Kowsar *et al* 2013; Cronin *et al* 2016) however the lymphatic contribution to oviduct fluid formation is less well understood. Oviduct lymphatics were first described in the sow by Andersen (1927) who observed that the lymphatics expand around oestrus. Leese (1988) later suggested that the local lymphatic system might play a role in the transfer of ovarian steroids, which would conceivably impact fluid formation. Moreover the identification of lymphocytes, macrophages and neutrophils amidst the sow oviduct epithelium itself (Jiwakanon *et al* 2005) coupled with the presence of cytokines in the *Xenopus laevis* oviduct lumen (Jantra *et al* 2011) furthermore suggests that the contribution of the lymphatic system in oviduct fluid formation should not be overlooked.

Biological fluids including that of the oviduct would not exist without junctional complexes. The epithelial monolayer of the oviduct is a fairly tight barrier,

regulating the formation and composition of the luminal environment. Tight lateral cell-cell barriers or junctions are responsible for the apical:basal cellular polarity by facilitating the formation and maintenance of distinct luminal and basal environments (Figures 1-7 and 1-8) (Garnett *et al* 2014).

Cell junction proteins are a characteristic of all epithelia (Garnett *et al* 2014). There are two broad categories of cell junctions — those linking cells to one another (cell-cell) and those linking to the *lamina propria* (cell-matrix). Cell-cell junctions include: occludin (tight) junctions, adherens junctions, desmosomes, and gap junctions (Garnett *et al* 2014). Cell-matrix junctions include anchoring junctions, hemidesmosomes and focal adhesions — discussed below.

1.5.4.1. Tight Junctions

Tight junctions (TJs) are primarily responsible for creating an electrochemically tight seal between adjacent cells (Furuse *et al* 1993). TJs therefore limit the diffusion of compounds across the epithelium, thereby separating apical and basal fluids. This physical compartmentalisation allows for the selective transport of compounds basally to apically and *vice versa* (Tsukita & Furuse 1999; Tsukita *et al* 1999).

Three main proteins link adjacent cells to form TJs: occludins, claudins, and tetraspanins. Common to these proteins are four transmembrane repeats with two extracellular domains that homodimerise with their respective proteins at adjacent plasma membranes (Gonzalez-Mariscal *et al* 2003). Intracellularly linked to all these TJ complexes is zona occludin 1 (ZO1) — a large protein (210–225 kDa) that is essential for TJ function (Tornavaca *et al* 2015). In epithelial cells ZO1 is concentrated at the immediate sub-membranous domain of TJs whereas in cells that do not form TJs, such as fibroblast cells, ZO1 is dispersed in the cytoplasm

(Gonzalez-Mariscal *et al* 2003). The importance of ZO1 for TJ formation and maintenance is highlighted by observations that the aberration of ZO1 abolishes transepithelial resistance (Zehendner *et al* 2011; Stuart & Nigam 1995; Underwood *et al* 1999).

The Resistance (R) (tightness) of epithelial monolayers is measured in units of Ohms (Ω) where any resistance is defined as the ratio between Potential Difference (PD) in Volts (V) to short circuit current (I_{SC}) in Amps (A) in accordance to Ohm's law: R (Ω) = PD (V) / I_{SC} (A). However because resistance is influenced by the surface area (cm^2) of a monolayer the unit of choice for epithelial integrity measurement is Transepithelial Electrochemical Resistance (TER) measured in $\Omega \cdot cm^{-2}$ (Ferrell *et al* 2010).

TER is strictly a measure of many resistances. The absolute resistance ($\mathbf{R}_{ABS}$) of a confluent epithelium is defined as the ratio of transcellular resistance ($\mathbf{R}_{TRC}$) to paracellular resistance ($\mathbf{R}_{PC}$), both of which are an amalgamation of resistances in accordance to equation 1-1 below and schematically depicted by Figure 1-7.

Eq. 1-1: $\mathbf{R}_{ABS} = \mathbf{R}_{TRC} / \mathbf{R}_{PC}$

It is worth noting however that an absolute resistance of a cellular monolayer differentially factors four individual resistances – apical membrane resistance ($\mathbf{R}_A$), basal membrane resistance ($\mathbf{R}_B$), tight junction resistance ($\mathbf{R}_{TJ}$) and intercellular resistance ($\mathbf{R}_{IC}$) (for more detail please refer to Odijk *et al* 2014). TJs are sensitive to calcium (Ca^{2+}) and its removal from culture using calcium chelating agents such as ethylenediaminetetraacetic acid (EDTA) reversibly opens the junctions. This is measurable as a decrease in TER of the epithelial monolayer (Gonzalez-Mariscal *et al* 1990; Bleich *et al* 2012).

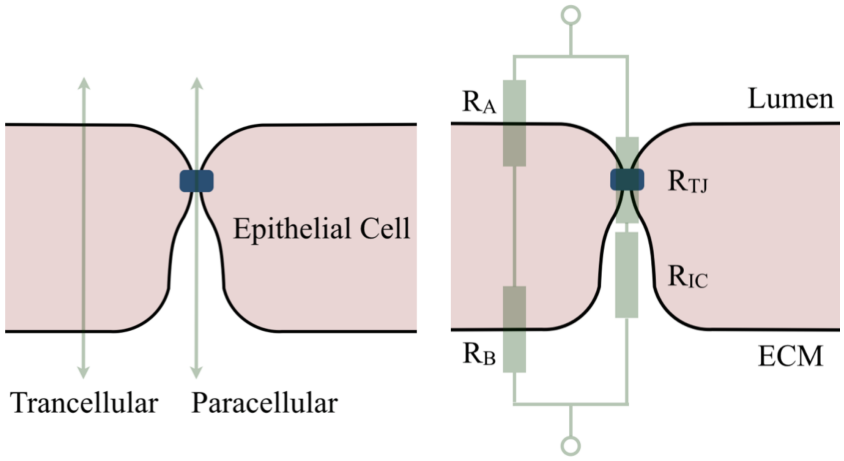

Figure 1-7 — Schematic depiction of transcellular and paracellular flux across epithelia with corresponding resistances cumulatively creating an absolute resistance (R_{ABS}) — adapted from Odijk *et al* (2014).

TJ permeability (resistance) varies amongst different epithelia and between species. Bovine oviduct epithelia typically exhibit an *in vitro* TER of ~ 700 $\Omega \cdot cm^{-2}$ - 1 $k\Omega \cdot cm^{-2}$ (Chen *et al* 2015; Keating & Quinlan 2008; 2012) whereas human oviduct epithelial monolayers *in vitro* are roughly ten times less 'tight' at ~ 150 $\Omega \cdot cm^{-2}$ (Downing *et al* 1997). This suggests that human oviduct epithelia are inherently *leakier* than the bovine although not necessarily less selective (Cereijido *et al* 2008).

1.5.4.2. Adherens Junctions

Adherens junctions (or *zona adherens*) are the cell connections responsible for the formation of an uninterrupted adhesion belt immediately basal to occludin junctions (Fanning *et al* 1998). Adherens junctions are comprised of two main proteins: cadherins – which are the transmembrane adhesion proteins anchoring neighbouring plasma membranes, and catenins – that intracellularly link the adherens junction to cytoskeletal actin bundles (Perez-Moreno *et al* 2003). This arrangement forms a transcellular network regulating folding (invaginations) and mediating the remodelling of epithelial monolayers (Okabe *et al* 2004). When

individual epithelia are seeded *in vitro* the formation of adherens junctions is essential for subsequent alignment and establishment of all other junctions (Alberts *et al* 2002).

1.5.4.3. Desmosome Junctions

Desmosomes also hold adjacent cells together and form a junctional complex basal to the adherens belt (Gumbiner *et al* 1988; He *et al* 2003; Jamora & Fuchs 2002). Like adherens junctions, desmosome extracellular domains belong to the cadherin protein family, but their intracellular domains are intermediary filaments – specifically cytokeratins (CK) in epithelia. Cytokeratin isoforms are highly variable amongst different epithelia and are thus used to distinguish between cell types. For instance CK18 is specific to single monolayer surface columnar epithelia such as those in the trachea and the oviduct (Comer *et al* 1998; van der Velden *et al* 1997). Similar to desmosomes are hemidesmosomes which are found at the basal cell membrane to anchor the epithelium to the extracellular matrix.

1.5.4.4. Gap Junctions

The junctions described thus far play a predominantly physical barrier role — essential to establishing and maintaining cell polarity. By contrast, gap junctions are pores linking two adjacent cells. A gap junction is composed of six connexin proteins which assemble to form a connexon. The hexagonal connexon bridges a distance of ~ 2 nm with an internal diameter of ~ 1.5 nm and allows the intercellular movement of small (1 kDa maximum) molecules and ions only (Figure 1-8) (Veenstra 1996; Brison *et al* 2014).

Gap junction facilitation of the movement of small signalling compounds [such as cyclic adenosine monophosphate (cAMP), inositol triphosphate and calcium

(Ca^{2+})] drives phenomena such as cell synchrony — the indirect response of a cell to a stimulus (Schultz 1985). Gap junctions function in a regulatory capacity; low cytosolic pH and/or high intracellular calcium ($[Ca^{2+}]_i$) leads to connexin hexamer reconfiguration which reversibly closes the junction (Peracchia 2004). An important reproductive role of gap junctions is in the regulation of myometrial contractions during parturition (Garfield *et al* 1988).

Epithelial cells typically lose most of their gap junction coupling *in vitro* (Stein *et al* 1991). However improved cell culture methods such as cell proliferation on an extracellular matrix (ECM) increase gap junction coupling (Amsterdam *et al* 1989). For example *in vitro* equine oviduct epithelia have been shown to display connexin-mediated communication (Ellington *et al* 1993).

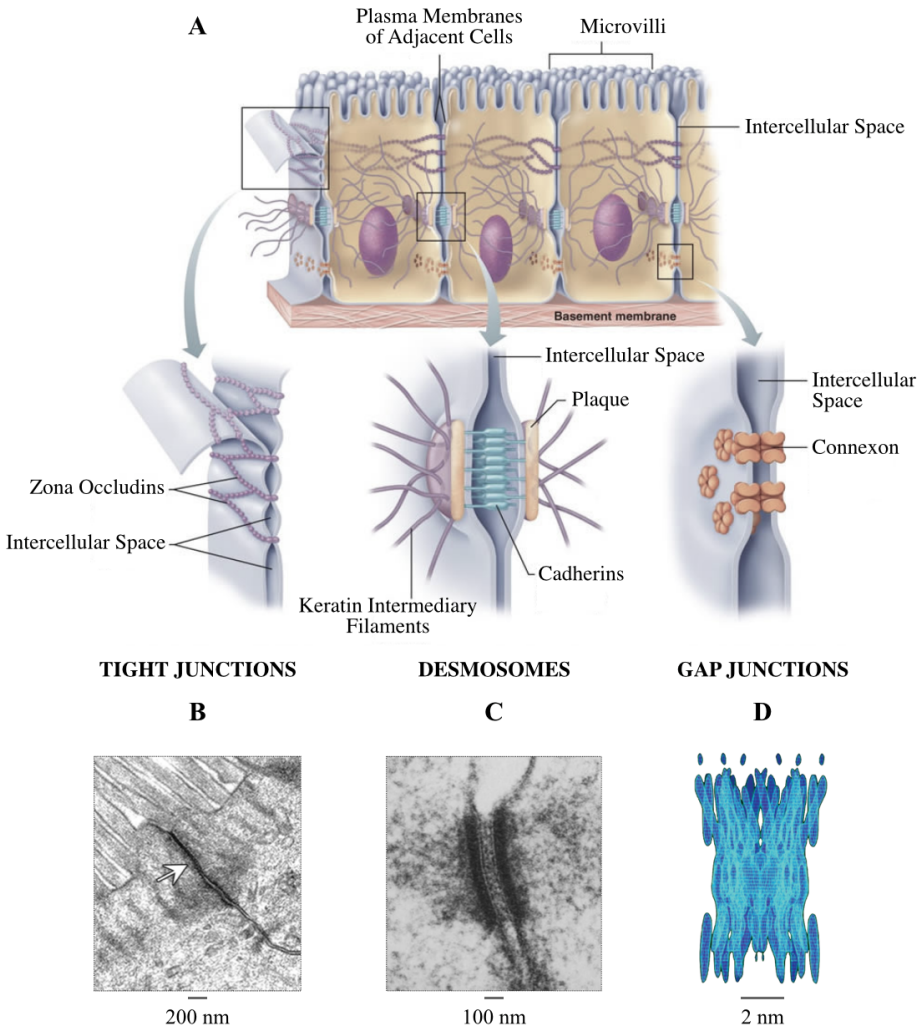

Figure 1-8 — **A**: Schematic diagrams of epithelial junctional complexes (adapted from proprofs.com) coupled with biological images of **B**: tight junctions (Nighot & Bilkslager 2010), **C**: desmosomes (Dembitzer *et al* 1980) and **D**: a protein crystallographic structure of a connexon pore (Unger *et al* 1999).

1.5.5. The Oviduct Lumen

The junctional complexes discussed play a key role in the separation of apical and basal oviduct fluid thereby allowing a unique and spatiotemporally dynamic luminal fluid to be established and maintained. The lumen is a *milieu* whose composition is regulated by the cells of the surrounding epithelium. Analyses of the luminal fluid have identified several biochemically significant compounds including amino acids, proteins, lipids, ions, energy substrates such as glucose and

pyruvate, hormones and growth factors – all known to be present in oviduct fluid (Leese *et al* 2008; Aguilar & Reyley 2005; Menezo & Guerin 1997; Menezo *et al* 2015) and discussed in greater detail in Chapter 3.

Although previously stated that the secretory cells are responsible for oviduct fluid formation (Verhage *et al* 1979) it is worth acknowledging that ciliated cells, which do not directly contribute to the creation of oviduct fluid are involved in mixing the milieu (Lyons *et al* 2006a, 2006b; Chen *et al* 2004) and are part of the overall oviduct mucosa (known as endosalpinx in the human), holistically considered a transporting epithelial tissue (Leese 1988). It is also noteworthy that cilia have recently been shown to secrete ectosomes (Wood & Rosenbaum 2015) but such ciliary ectosomes have yet to be investigated in the reproductive tract.

Nonetheless our knowledge of this environment is not as extensive as related biological fluids such as the blood. Part of the challenge in examining this environment rests in the sampling methods currently available (Section 1.7.). Another difficulty lies in the high inherent variability of fluid composition.

1.6. Oviduct Fluid

The first experimental demonstration that the oviduct is an actively secreting epithelium was conducted by Bishop (1956) who ligated the oviducts of anaesthetised rabbits *in vivo* at the uterotubal junction. A cannula was inserted vertically at the open infundibular end and the pressure in the fluid created was measured. The data demonstrated that at oestrous, rabbit oviducts produced 0.79 ml of fluid over 24 hours whilst ovariectomised rabbits secreted 0.14 ml over 24 hours. Importantly, the secretion rate could be restored in ovariectomised rabbits following exogenous injections of oestradiol. Bishop (1956) also showed that secretion volume and pressure declined during pregnancy.

Since then our knowledge of oviduct fluid formation has progressed with data from the rabbit (Engle *et al* 1968; David *et al* 1969; Iritani *et al* 1971), sheep (Hill *et al* 1997), ewe (Iritani *et al* 1969), bovine (cow) (Roberts *et al* 1975; Hugentobler *et al* 2007a; Hugentobler *et al* 2007b; Hugentobler *et al* 2008), pig (Iritani *et al* 1974; Nichol *et al* 1998), mouse (Gardner & Leese 1990; Harris *et al* 2005), monkey (Stambaugh & Mastroianni 1980) and human (Tay *et al* 1997; Gardner & Leese 1990).

1.6.1. Formation and Composition

Oviduct fluid formation is a highly spatiotemporally dynamic process. Spatially, as discussed, the proportion of secretory cells increases longitudinally from infundibulum to isthmus (Leese 1983) (Section 1.4.) which also affects the spatial composition of oviduct fluid. For instance Leese (1988) suggested that the appearance of primary metabolites (glucose, pyruvate and lactate) in the ampulla at 1.8 times their concentration in the isthmus may not be due to any differential secretion by the ampulla *vs* isthmus but more likely corresponds to the relative secretory mucosal surface being approximately 1.8 times greater in the ampulla.

Because the isthmic luminal landscape is dominated (~70%) by secretory cells in contrast to ~50% in the adjacent ampulla (Crow *et al* 1994) the isthmus is considered by some (*e.g.* Nutu 2009) the most pronounced secretory portion of the oviduct. However this is subject to debate because factoring in both surface area and secretory cell population, the ampulla has a secretory index of (0.5x1.8) 0.9 compared to the isthmic (0.7x1.0) 0.7 (adapted from Abe 1996). This is unsurprising since the oocyte, sperm, and therefore embryo spend the majority of their time in the oviduct at the ampullary-isthmic junction and isthmus (Nichol *et al* 1992) where arguably there should be greatest output of metabolites essential

for sustaining the early embryo until it migrates to the uterus (Aguilar & Reyley 2005).

Aside from spatial variation, oviduct fluid composition and volume furthermore vary temporally as a function of the oestrous cycle mediated by steroid hormones which act via direct and indirect signalling mechanisms (Aguilar & Reyley 2005). Specifically, oviduct epithelia undergo cycles of (*i*) hypertrophy (redevelopment) – cell height and activity increases during oestrogen dominance (Figure 1-1), and (*ii*) atrophy (physiological reabsorption or shedding); cells stop releasing secretory granules during progesterone dominance (Abe 1996; Greenwald 1969; Beck & Boots 1974; Russe & Liebich 1979; Bareither & Verhage 1981; Odor *et al* 1983). Isthmic contractions are more frequent and pronounced under oestrogen whilst progesterone induces the relaxation of the *tunica muscularis* (Jansen 1980).

Fischbarg (2010) stated that "the mechanism of epithelial fluid transport constitutes arguably the last major problem of epithelial function still unsolved". Owing to subject complexity and lack of definitive proof surrounding the detailed mechanisms of epithelial fluid transport, unless otherwise stated this section adopts the predominant view that 'fluid' (*i.e.* water) movement follows an initial (passive or active) flux of ions [such as sodium (Na^+), potassium (K^+), chloride (Cl^-), and bicarbonate (HCO_3^-)] – *i.e.* osmotic water transfer is secondary to solute transport.

The oviduct epithelial monolayer is directionally polar about their apical (luminal) to basal (*lamina propria*) axis; a biochemical and electrochemical asymmetry maintained by the tight junctions and well demonstrated *in vitro* by the presence of a TER. This phenomenon of polarity underpins the basis of epithelial fluid formation (Figure 1-9).

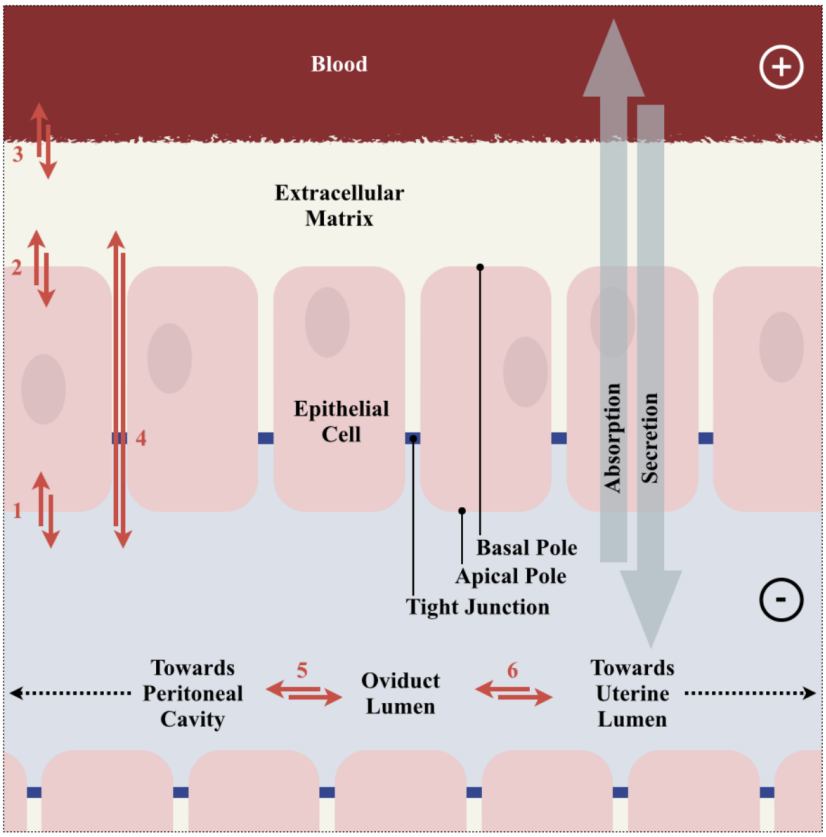

Figure 1-9 — Diagram highlighting the potential routes by which fluid may enter and leave the lumen (adapted from Leese 1988). Routes **3-2-1** and **1-2-3** respectively depict transcellular secretion and absorption, whilst arrows 4 represent paracellular movement. Peritoneal and follicular fluid may also enter and leave the oviduct via the abdominal cavity **(5)**. Similarly uterine fluid, and menstrual blood under pathological retrograde menstruation), may enter the oviduct from the uterus **(6)**. Moreover the potential difference across the epithelial cells is negative in the lumen with respect to the vasculature (Leese 1988).

It is established that a primary mechanism of oviduct fluid formation and regulation is by Cl^- secretion (Brunton & Brinster 1971; Quinton 1990; Gott *et al* 1988; Dickens *et al* 1993; Keating & Quinlan 2008) with K^+ flux also a likely mechanism for moving water apically (Dickens & Leese 1994). This occurs on the plasma membrane level of epithelial cells – shuttling Cl^- and K^+ inside and out of the cell to drive water flux. Paracellular fluid formation and transport may make a contribution owing to oviduct epithelial cells exhibiting an inherently relatively low TER (Leese & Gray 1985).

Osmotic transfer of water is secondary to the transit of the solute (*e.g.* Cl^- and K^+) and may be demonstrated by a temporary disruption of TER when the voltage is artificially clamped to 0 V. A classic *in vitro* example of epithelial fluid formation and TER disruption is the basal effect of purinergic agents [adenosine triphosphate (ATP) and adenosine diphosphate (ADP)] on confluent oviduct epithelial monolayers – resulting in a near-immediate (~ 1 second response time) and transient (~ 3 second duration) substantial decrease in TER (Cox & Leese 1995).

Introducing ATP basally temporarily raises the short circuit current (I_{SC}) across the monolayer thereby indirectly lowering the resistance in accordance with Ohm's law and Equation 1-2 below (Keating & Quinlan 2008).

Eq. 1-2: $\mathbf{R}(\Omega) = \mathbf{PD}\ (V) \div \mathbf{I_{SC}}\ (A)$

This short dip in TER is however not a reflection of any loosening of tight junctions previously discussed. Rather ATP signals induce Cl^- release into the lumen (Mendelssohn *et al* 1981). Examples of an ATP induced purinergic response are provided in Figure 3-12.

A consequence of this apical Cl^- flux is the establishment of an electrochemical gradient; the importance of which is twofold. Firstly the active creation of a chemical concentration gradient drives the passive secretion of water to the apical compartment, leading to a reestablishment of an osmotic equilibrium. Secondly, the creation of a greater electrical gradient (more negatively charged lumen than previously) raises $\mathbf{I_{SC}}$ thereby indirectly reducing the *fractional TER* which is defined in Equation 1-3 below.

Eq. 1-3: $\mathbf{TER_F} = \mathbf{R_A} \div (\mathbf{R_A} + \mathbf{R_B}) = \mathbf{\Delta V_A} \div \mathbf{\Delta V_T}$

where $\mathbf{R}_A$ is the apical resistance, $\mathbf{R}_B$ is the basal resistance, $\mathbf{\Delta V}_A$ is the apical membrane potential, and $\mathbf{\Delta V}_T$ is transepithelial voltage. This formula can be further rearranged as equation 1-4 given equation 1-5.

Eq. 1-4: $\mathbf{TER}_F = (\mathbf{G}_T)^{-1} = (\mathbf{\Delta V}_T) \div \mathbf{I}_{SC}$

Eq. 1-5: $\mathbf{I}_{SC} = (\mathbf{V}_T) \cdot (\mathbf{G}_T)$

where $\mathbf{G}_T$ is transepithelial conductance. As $\mathbf{V}_T$ is clamped near enough to 0 V, any increase in electrons apically will raise $\mathbf{G}_T$ thereby dropping $\mathbf{TER}_F$ (Stutts *et al* 1992). As surface area is constant it can be negated, therefore having no impact on $\mathbf{TER}_F$.

Regulation of fluid secretion by extracellular ATP is one means of controlling fluid formation by the oviduct epithelium — consistent with the airway epithelium (Stutts *et al* 1992). Extracellular ATP furthermore induces increased ciliary beat frequency in non-secretory oviduct epithelia (Barrera *et al* 2004). However other agents also drive Cl^- secretion. For example, Downing *et al* (2002) showed that platelet activating factor (PAF) – secreted by sperm (Toledo *et al* 2003) and early embryos (Hansel *et al* 1989) can modulate Cl^- flux in human oviduct epithelia.

1.6.1.1. Ions

In general the ionic composition of oviduct fluid is highly conserved across mammalian species studied thus far. For instance the concentration of potassium (K^+) is consistently higher in oviduct fluid than present in plasma across mammalian species (Aguilar & Reyley 2005). Human oviduct fluid is notably

higher in Cl^- than plasma (Lippes *et al* 1972; David *et al* 1973; Borland *et al* 1980) whilst in the bovine K^+ content is highest in the lumen at oestrous (Olds & VanDenmark 1957). Consistent with this *in vivo* information, more pregnancies were established in mice by IVF when using an embryo culture medium with high K^+ (Quinn *et al* 1985) and this formed the basis of the medium known as synthetic oviduct fluid (SOF), used for cattle embryos (Tervit et al 1972; Gandhi et al 2000).

Calcium (Ca^{2+}) in the bovine oviduct is significantly higher in the isthmus than in the ampulla (Grippo *et al* 1992). Ca^{2+} is moreover present in the bovine oviduct in concentrations higher than that in plasma at ovulation (Grippo *et al* 1992), in contrast to the human where *ex situ* apical Ca^{2+} concentrations rarely exceed basal levels (Borland *et al* 1980). Whereas Ca^{2+} concentrations are longitudinally (from infundibulum to isthmus) dependent, magnesium (Mg^{2+}) appears to be oestrous cycle dependent and consistently lower apically than basally (Grippo *et al* 1992), with the exception of the horse (Campbell *et al* 1979). In the sheep, Na^+, K^+, Cl^- and HCO_3^- are all lowest at met-oestrus (Restall & Wales 1996). Hugentobler *et al* (2007a) showed that the ionic composition and rate of secretion of bovine oviduct fluid differed considerably to that for uterine fluid. Figure 1-10 shows the broad range of channels pertinent to ion and macromolecule movement about biological membranes.

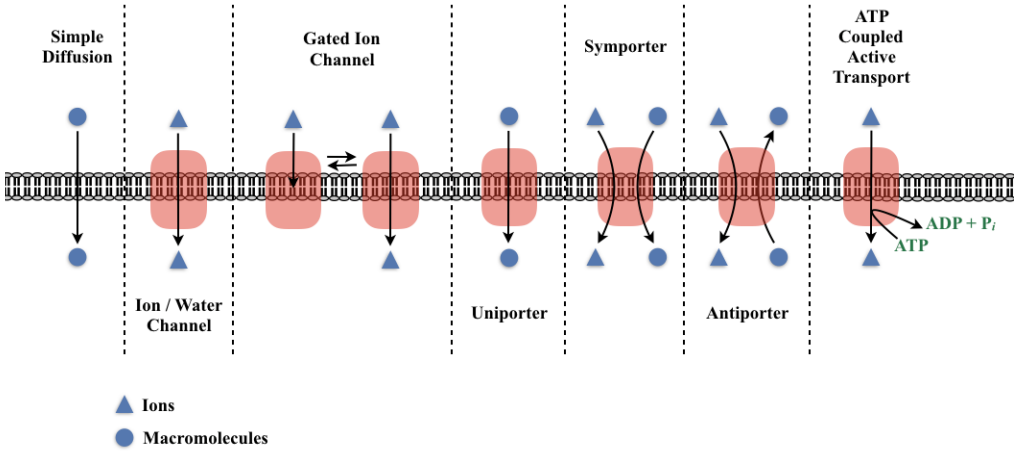

Figure 1-10 — Schematic depiction of routes of ion and macromolecule movement about biological membranes, adapted from (themedicalbiochemistrypage.org).

1.6.1.2. Carbohydrates

The primary energy substrate in the oviduct lumen is glucose (Brewis *et al* 1992) which is present in the oviduct fluid of every species examined to date (Nichol *et al* 1992) at a concentration of approximately 20% of that found in blood plasma in most species (*i.e.* ~ 1 mM *vs* ~ 5 mM) (Gardner & Leese 1990; Hamner 1973; Nichol *et al* 1992; Dickens *et al* 1995; Carlson *et al* 1970; Campbell *et al* 1979; Aguilar & Reyley 2005). Glucose has a fundamental role in sustaining the oviduct epithelium in addition to sperm and preimplantation embryo metabolism (Leese & Gray 1985; Leese 2012; Smith & Sturmey 2013) with a lesser, but important, role in the oocyte (Sutton-McDowall *et al* 2010). Glucose is transported into the rabbit oviduct lumen predominantly by facilitated diffusion (Leese & Jeffries 1977).

Glucose Transporter 1 (GLUT1) is expressed and localised on the apical and basal surfaces of rat oviduct epithelia (Tadokoro *et al* 1995) and serves to shuttle glucose into the lumen. The concentration of glucose in the human oviduct varies spatiotemporally. For instance, glucose is present at 1.8 times higher levels in the ampulla *vs* isthmus (Leese 1983). Apical glucose concentrations are furthermore oestrous cycle dependent (Gardner *et al* 1996).

Glucose is present at its lowest concentration in the oviduct lumen mid-cycle (in contrast to lactate and pyruvate) (Kaye 1986; Gardner *et al* 1996). Interestingly the glucose concentration of rabbit oviduct fluid was found to decrease three days after mating – around the time the embryo begins to enter the uterus (Edwards & Leese 1993). In ovariectomised pigs, oviduct glucose concentrations were unaffected by embryo presence (Nichol *et al* 1998). It seems likely that the glucose content is therefore endocrine dependent as opposed to being driven by maternal-embryo communication in the pig. In species such as the human the provision of specific energy substrates follows a pattern mirroring embryo demand; that is pyruvate during the cleavage stages (Nichol *et al* 1992) before a glucose increase coinciding with blastocyst formation (Gardner *et al* 1996). The precise mechanisms underpinning these phenomena remain to be clarified.

1.6.1.3. Protein

Protein can enter the oviduct lumen by one of two primary routes – (*i*) 'filtration' from the basal vasculature and (*ii*) synthesis and secretion by the epithelium (Aguilar & Reyley 2005). The role of vesicular *bulk* transport of macromolecules across the oviduct epithelium has not been examined in any great detail. Protein concentration of oviduct luminal fluid is approximately 10% to 15% of that observed in basal plasma (Leese 1988) with serum albumin and serum immunoglobulin G (IgG) representing approximately 95% of the total (Oliphant *et al* 1978). Other proteins observed in the oviduct include high-density lipoproteins (HDLs) which are secreted during the follicular (non-secretory) phase of the oestrous cycle and are thought adhere to cholesterol secreted by the sperm membrane to capacitate them until ovulation (Ehrenwald *et al* 1990).

Further to temporally regulated secretions, there is a spatiotemporal pattern of protein secretion from ampulla to isthmus. Although the function of many of these proteins remains unknown (Abe 1996; Nieder & Macon 1987; Buhi *et al* 1990), extensive work has been done to characterise one protein in particular – the oviduct specific glycoprotein one [(OVGP1) or 'oviductal glycoprotein' (OGP) also known as 'oestrogen dependent protein' (EDP), 'mucin 9' (MUC9) or 'oviductin'], first observed in the sheep by Sutton *et al* (1984) as a 'an oestrus-associated glycoprotein in oviductal fluid'.

1.6.1.4. Oviduct Specific Glycoprotein

Across species, OVGP1 is consistently observed in the ampulla and is known to enter the lumen via epithelial secretory granule exocytosis. The prevailing view is that OVGP1 mediates sperm binding and release to and from the oviduct epithelium (McNutt *et al* 1992) following ejaculation and ovulation in order to maximise the likelihood of fertilisation (Gandolfi 1995). OVGP1 has been identified in the bovine (Sendai *et al* 1994; Malayer *et al* 1988; Boice *et al* 1990; Gerena and Killian 1990), mouse (Kapur and Johnson 1986), hamster (Suzuki *et al* 1995; Leveille *et al* 1987; Oikawa *et al* 1988; Abe *et al* 1992), rat (Abe & Abe 1993), sheep (DeSouza & Murray 1995), pig (Buhi *et al* 1996), goat (Abe *et al* 1995), baboon (Jaffe *et al* 1996; Fazleabas & Verhage 1986), human (Arias *et al* 1994; Verhage *et al* 1988; Wagh & Lippes, 1989), and monkey (Verhage *et al* 1997).

Luminal secretion of OVGP1 correlates with the stage of the oestrous cycle, or more specifically, with oviduct cellular differentiation states (Verhage *et al* 1998) although patterns of OVGP1 production and secretion differ between species. For instance OVGP1 expression in the goat is limited to the fimbriae and ampulla during the follicular phase (Abe *et al* 1995) – *i.e.* OVGP1 is secreted around the

time of fertilisation not at the site of fertilisation. In contrast, rat OVGP1 is predominantly secreted by the isthmus which is where the sperm reservoir forms (Abe 1996). The strategy employed by the bovine differs with OVGP1 located at both the isthmus and ampulla – the sites of sperm capacitation and fertilisation respectively (Lefebre *et al* 1997) during the follicular phase of the oestrus cycle (Figure 1-1) (Sendai *et al* 1994).

In the bovine, Sendai *et al* (1994) purified and sequenced both OVGP1 cDNA and protein to reveal a 95 kDa [97 kDa (Boice *et al* 1990)] structure with an integral transmembrane repeat and numerous post-translational modification sites. Sendai *et al* (1994) also raised a monoclonal antibody against OVGP1 and observed cross-reaction with a 55 kDa protein. As the core molecular weight of the protein is 59.62 kDa the cross-reacted protein observed in blots is most likely the de-glycosylated OVGP1 structure (Sendai *et al* 1994). In the sheep OVGP1 is a 92 kDa molecular weight protein exclusively produced by the ampullary region (Gandolfi *et al* 1991) at highest concentration at oestrous or on day 1 of pregnancy (ovulation) (Murray 1993) – consistent with mRNA (gene expression) data (DeSouza & Murray 1995).

As a direct consequence of the presence of OVGP1, sperm capacitation and binding to the bovine isthmic tunica mucosa *in vitro* results in a drastic shift in the volume and content of the oviduct secretome (Ellington *et al* 1993) – indicative of a wider signalling role for OVGP1. This notion is highlighted by the observation that oocytes and spermatozoa exposed to OVGP1 *in vitro* exhibited decreased polyspermy (Coy *et al* 2008) and a higher rate of fertilisation (Ambruosi *et al* 2013). Also porcine embryos reached the blastocyst stage when cultured with OVGP1 present in the media (McCauley *et al* 2003). OVGP1 physically binds to oocytes and developing embryos (Wegner & Killian 1991; Boice *et al* 1990) which may result in an increased resistance of the *zona pellucida* (ZP) to

enzymatic digestion (Mondejar *et al* 2012).

Human oviduct epithelial cells cultured *in vitro* do not readily express OVGP1 unless supplemented with oestrogen (Briton-Jones *et al* 2003). Moreover, human chorionic gonadotropin (hCG), a protein similar to Luteinising hormone (LH), increases the half life of OVGP1 (Sun *et al* 1997). Hamster embryo internalisation of OVGP1 by virtue of an endosomal mechanism has also been reported (Kan *et al* 1993).

Another important component of oviduct fluid is amino acids. This is discussed in Chapter 3. The following section outlines the technical challenges associated with studying the dynamic composition of oviduct fluid *in vivo* and *ex situ*.

1.7. Technical Challenges

Aside from inter-species and intra-individual variation — an individual oviduct lumen is a highly variable environment influenced by pregnancy (Forde *et al* 2014), spatial determinants (Bahat *et al* 2012), temporal elements (Bauersachs *et al* 2004), pathology (Dixon *et al* 2009), nutrition (Newbold *et al* 2001), age (Evers 2002) and genetics (Matzuk & Lamb 2008). Technical sampling challenges associated with oviduct fluid composition determination (Leese *at al* 2008b), are discussed below.

1.7.1. Direct Retrieval

A range of approaches to sample the oviduct luminal environment have been described. Direct retrieval is one of the most common methods and has been utilised to sample fluid from humans (Gardner *et al* 1996), mice (Borland *et al* 1977), and pigs (Nichol *et al* 1992). Direct retrieval involves collecting fluid from

oviducts *in situ* by micro puncture or the insertion of a catheter. Fluid obtained by direct retrieval probably best resembles the true environment. However disadvantages include extracting very low volumes which limit the scope for analysis as well as the need to conduct the sampling under anaesthesia – effects of which can include the disruption of carbohydrate and fat metabolism (Allison *et al* 1969), immune system interference (Kumar *et al* 2002), respiratory depression (Swerdlow 1957), reduced blood pressure and smooth muscle relaxation (Epstein and Smith 1973).

1.7.2. Perfusion

A second approach to studying the oviduct lumen is combined vascular and luminal perfusion. This involves excising the oviduct prior to cannulating the blood supply to enable the physiological provision of nutrients for a short duration over which greater volumes of luminal fluid may be collected. Combined vascular and luminal perfusion is particularly useful *in vitro* method for sampling human oviducts since *in situ* cannulation of the human is deemed unethical (Leese *at al* 2008b). Raess & Vincenzi (1980), Leese *et al* (1981) and Leese & Gray(1985) were among the first to employ perfusion to investigate the appearance and metabolism of nutrients in the oviduct lumen. Data obtained correlated well with *in vivo* observations but this method is limited by time since the tissue loses physiological characteristics approximately 3 hours after cannulation. Removing the oviduct from the body also makes perfusion more prone to artefacts than direct sampling (Leese *et al* 2008b).

1.7.3. Chronic and Acute Cannulation

Chronic cannulation has been one of the most widely used methods and involves cannulating the oviduct *in situ* under anaesthesia allowing the collection of

oviduct fluid over a period of days or weeks (Gerena & Killian 1990; Kavanaugh & Killian 1988). Although chronic cannulation enables the generation of insightful data, introducing a catheter into the oviduct can disturb fluid flow and formation in addition to causing localised inflammation of the tunica. Moreover sample collection without refrigeration beyond a few hours will cause significant degradation of fluid constituents such as pyruvate and glutamine (Leese *et al* 2008b). Acute cannulation of anaesthetised animals overcomes this limitation as sampling occurs within minutes and can be continued for approximately 3 hours (Kenny *et al* 2002; Hugentobler *et al* 2007). The oviduct tunica is still breached, but unlike chronic cannulation the fluid collected is uncontaminated by cellular debris (Leese *et al* 2008b).

1.7.4. Excision and Rapid Sampling

Excision and rapid sampling involves oviduct removal and sampling *post mortem*. Fluid retrieval *post mortem* introduces an inevitable degree of cell death and lysis (Leese *et al* 2008b) although this can be minimised by a high speed (within minutes) of sampling. To highlight the importance of *rapid* sampling Elhassen *et al* (2001) reported an average bovine oviduct total amino acid concentration of 41 mM. whereas Hugentobler *et al* (2007b) who used acute cannulation reported a value of 3.37 mM. In the case of Elhassen *et al* (2001), the time interval between the death of the animal and obtaining the sample was 90 minutes. Such a prolonged time interval will have induced cellular lysis attributable to *post mortem* effects, protein degradation and greater shearing during collection, and distorted the fluid composition.

This introduction has described the strengths and weaknesses of the various methods for collecting oviduct fluid. Whilst acute cannulation generally yields the best results it is unfeasible to employ routinely, particularly using animals such as

anaesthetised heifers (Leese *et al* 2008b). Moreover none of these methods is useful for studying underlying secretory mechanisms. There is therefore a need for a method to generate oviduct fluid *in vitro* in as physiological manner as possible.

1.8. Studying the Oviduct *in vitro*

Following the first studies into whole oviduct fluid formation by Bishop (1956) (Section 1.6.), oviduct epithelial cells began being harvested and cultured *in vitro* with the aim of improving blastocyst rates (Whittingham 1968), a concept later termed co-culture (Gandolfi & Moor 1987). Six years later the first oviduct epithelial cell line (porcine) was isolated and characterised (Boullant & Greg 1973). Since then oviduct cell lines have been established from ciliated (Ando *et al* 2000) and secretory human (Lee *et al* 2001; Umezu *et al* 2003; Ling *et al* 2005) and bovine (Schoen *et al* 2008) oviduct epithelia.

Publications such as *establishment of human ampullary cell cultures* (Bongso *et al* 1989) and *culture of epithelial cells derived from the oviduct of different species* (Ouhibi *et al* 1989), led to the most rapid rise in research output concerning the *in vitro* oviduct epithelium, specifically between 1990 and 2000. This decade saw numerous contributions to our knowledge of the biology of oviduct-sperm interactions (Suarez *et al* 1991; McNutt & Killian 1991; Raychoudhury & Suarez 1991; Parish *et al* 1994; Lefebvre *et al* 1995; Ellington *et al* 1998), oviduct-oocyte interactions (Carolan *et al* 1994; Martinez *et al* 2000) in addition to the role of the oviduct in fertilisation (Walker et al 1997; Confino et al 1997; Martus *et al* 1998) and early embryo development (Pavasuthipaisit *et al* 1994; Carolan *et al* 1994; van Winkle & Dickinson 1995; Edwards *et al* 1997; van der Auwera 1999).

These insights into the reproductive biology of the oviduct were accompanied by understanding of the fundamental biochemical mechanisms underlying oviduct fluid formation (Dickens *et al* 1993, 1995, 1996; Dickens & Leese 1994; Cox & Leese 1995, 1997; Downing *et al* 1997, 1999) as described in Section 1.6.1.

Similarly, this decade saw significant advances in oviduct epithelial culture techniques (Thomas *et al* 1995; Van Langendonckt *et al* 1995; Cox & Leese 1997; Reinhart *et al* 1998; Wijayagunawardane *et al* 1998; Comer *et al* 1999; Reischl *et al* 1999) including the first *in vitro* oviduct cell culture on semi-permeable supports (Dickens *et al* 1993). Dickens *et al* 1993 cultured cells on impregnated collagen filters, a system which better mimicked the *in vivo* environment as epithelia were cultured between two fluid filled chambers. This was followed by oviduct epithelial cell culture on the commercially available semi-permeable Transwell™ membranes (Walter 1995).

Such progress in cell culture techniques enabled electrophysiological aspects of the oviduct epithelium to be examined *in vitro* (Downing *et al* 2002; Keating & Quinlan 2008, 2012) whilst technological advances in nucleic acid amplification by polymerase chain reaction (PCR) further enabled extensive studies into the gene expression (Rizos *et al* 2002, 2003; Ulbrich *et al* 2003; Briton-Jones *et al* 2004; Lam *et al* 2005; Rottmayer *et al* 2006; Gaytan *et al* 2007; Kubota *et al* 2009; Gauvreau *et al* 2010) of oviduct epithelial cells *in vitro* as well as *in vivo.*

Progress in cell culture therefore enabled better *in vitro* experimentation, prompted the issue of co-culture to be revisited and even spurred suggestions of reconstructing a bioartificial oviduct (Orsi & Reischl 2007), although this has not been achieved to date. Recent advances have been made in oviduct epithelial purification (Cronin *et al* 2012) and culture. Specifically, Levanon *et al* (2010) advanced the Transwell™ model to culture oviduct epithelia in an air-liquid

interface whilst oviduct epithelial have also been cultured in 3D clusters (Gualtieri *et al* 2012; Lawrenson *et al* 2013) and under hormonal supplementation (Chen *et al* 2013), all in an attempt to better mimic the *in vivo* environment.

Although beyond the scope of this project, it is worth noting that oviduct epithelia *in vitro* have even also been used as a cellular model to study ciliary activity in a context unrelated to reproduction (Niwa *et al* 2012) and oviduct has moreover been modelled computationally (Vasieva *et al* 2013; Aranda *et al* 2015).

1.8.1. The Oviduct *in vivo vs in vitro*

As discussed, limitations in *in vivo* and *ex situ* sampling created the need for robust and reproducible *in vitro* methods for studying the oviduct luminal environment. However there are some important similarities and differences between these cells *in vivo* and *in vitro*. Oviduct epithelial cells have a relatively low proliferation rate (Steinhauer *et al* 2004) attributable to the fact that the oviduct epithelium is mitotically relatively inert; an observation that may explain the low incidence of primary oviductal tumours (Perlman *et al* 2005). However it should be noted that recent studies show several ovarian cancers (the most lethal gynaecological malignancy) originating from neighbouring organs with most serous ovarian tumours actually arising from the oviduct epithelium (Kurman & Shih 2010).

In an *in vitro* setting, this low proliferative rate results in monolayers taking over a week to reach confluence, a significant implication of which is the loss of cilia and the well-defined columnar shape of the epithelial cells. Potential explanations for deciliation include (*a*) inherently less ciliated cells harvested, (*b*) ciliated cells may not divide as rapidly as secretory cells, and/or (*c*) ciliation is the final stage of oviduct epithelial cell differentiation – a process not directly induced *in vitro*. The

latter explanation is the most likely given that oviduct epithelial regain cilia *in vitro* following basal oestradiol supplementation (Comer *et al* 1998).

These findings of oviduct epithelial physiology serve as a further example of how our understanding of the detailed operative of the oviduct *in vivo* is dependent on better *in vitro* models — all of which have a major limitation to date: the inability to monitor secretions directly.

1.9. Aims and Objectives

This thesis revolves around an observation that bovine oviduct epithelia cultured on Transwell™ permeable supports in an apical:basal air:liquid interface (Levanon *et al* 2010) secrete a thin film of clear fluid following 24 hour incubation. Stemming from the discovery of this *in vitro* Derived Oviduct Fluid (*iv*DOF), the broad primary research questions became:

1. Does *iv*DOF composition differ to the culture medium provided basally?
2. Do hormones influence *iv*DOF composition?
3. Does hypoandrogenism and hyperandrogenism influence *iv*DOF content?
4. Does hypoandrogenism and hyperandrogenism impact cell physiology?
5. Does hypoandrogenism and hyperandrogenism impact gene expression?
6. How responsive is *iv*DOF to epithelial supplementation with drugs?
7. Can the model be used to investigate the transport properties of the oviduct?
8. How might dietary derived compounds impact *iv*DOF composition?
9. Does *iv*DOF composition resemble *in vivo* derived luminal fluid?
10. Can insights into the biochemistry of subfertility be gained using this model?

Chapter 2

Materials and Methods

Chapter 2 — Materials and Methods

This section outlines the materials, apparatus and methodologies employed to create a preparation for examining oviduct fluid formation *in vitro*. Methods exclusive to specific experiments are discussed in their respective chapters. Throughout this thesis experimentation was performed with bovine tissue, unless otherwise stated.

2.1. Comprehensive List of Materials

17β-Oestradiol (Sigma Aldrich E8875)
2-MercaptoEthanol (βME) (Sigma Aldrich P0532)
4-[2-Hydroxy Ethyl]-1-Piperazine Ethane Sulfonic Acid (Sigma Aldrich H0887)
4-hydroxy-diclofenac (4OH-DFC) (Cambridge Biosciences 10008518)
ã-cytokeratin-18 Primary (1º) Antibody (AbCam AB82254)
ã-mouse IgG1 Primary (1º) Antibody (ABD-Serotec MCA928)
ã-vimentin Primary (1º) Antibody (AbCam AB8069)
Alexafluor-488_{nm} Secondary (2º) Antibody (Invitrogen A11001)
Ammonium Persulfate (APS) (Sigma Aldrich A3678)
Amphotericin B (Fungizone) (Invitrogen 15290026)
Anti-biotin (not streptavidin) HRP linked 2º antibody (CST 7075)
Aprotonin from Bovine Lung (Sigma Aldrich A6279)
Bis-Acrylamide Solution (Sigma Aldrich A3547)
Bovine Serum Albumin (BSA) essentially fatty acid free (Sigma Aldrich A6003)
Caco-2 human colon carcinoma cell line (American Type Culture Collection)
Chloroform (Fisher Scientific 10615492)
Clomid™ clomifene citrate (Sanofi)
Corning® Transwell™ Cell Culture Inserts (Sigma Aldrich CLS3450)
DEPC treated water (VWR E476)
Dimethyl Sulphoxide (DMSO) (VWR E476)
Dulbecco's Modified Eagle Medium (DMEM) low glucose (Sigma D5546)
Dulbecco's Modified Eagle Medium (DMEM) high glucose (Sigma D5671)
Eosin (Sigma Aldrich E4009)
Ethylene Diamine Tetraacetic Acid (EDTA) (Sigma Aldrich 93302)
Ethylene Glycol Tetraacetic Acid (EGTA) (Sigma Aldrich E3889)

Foetal Bovine Serum (FBS) (Invitrogen 10270098)
Formic Acid (Sigma Aldrich F0507)
Genistein (Sigma Aldrich G6649)
Glycine (Sigma Aldrich G8898)
Haematoxylin (Sigma Aldrich H3136)
Hank's Balanced Salt Solution (HBSS) - $CaCl_2$ and - $MgCl_2$ (Invitrogen 14170)
Hank's Balanced Salt Solution (HBSS) - phenol red (Sigma Aldrich 55037C)
High Capacity cDNA Reverse Transcription Kit (Fisher Scientific 10400745)
HRP-Linked Biotinylated Protein Ladder (CST 7727)
Hydromount (Natural Diagnostics HS106)
Igepal (Sigma Aldrich I7771)
Insulin *from Bovine Pancreas* (Sigma Aldrich I6634)
Kodak® Autoradiography Solution (Sigma Aldrich P7042)
Kodak® Fixer Solution (Sigma Aldrich P6557)
Laemmli Buffer (Sigma Aldrich S3401)
Laminin (Sigma Aldrich L2020)
L-Carnitine (Sigma Aldrich 11242008001)
L-Glutamine (Sigma Aldrich G7513)
Luminol (Sigma Aldrich 123072)
MEM Non-essential Amino Acid Solution (100x) (Sigma Aldrich M7145)
Metformin hydrochloride 500 mg (RelonChem)
Methanol (Fisher Scientific 10675112)
Newborn Calf Serum (NCS) (Sigma Aldrich N4762)
N,N,N′,N′-TetraMethylEthyleneDiamine (TEMED) (Sigma Aldrich T9281)
Nutrient Mixture F12 Ham (F12) (Sigma Aldrich N8641)
Nystatin (Sigma Aldrich N6261)
O-PhthaldiAldehyde (OPA) Reagent (Sigma Aldrich P0532)
OVGP1 Primary (1º) Antibody (Prof S Ulbrich and Dr T Fröhlich)
P-Coumaric Acid (Sigma Aldrich C9008)
Penicillin-Streptomycin Solution (PenStrep) (Invitrogen 15140122)
Phenylmethanesulfonylfluoride (PMSF) (Sigma Aldrich P7626)
Phosphate Buffered Saline (PBS) (Sigma Aldrich P4417)
Polyvinylidene Fluoride (PVDF) 0.2 µm Membranes (Sigma Aldrich P2813)
Progesterone (Sigma Aldrich P0130)
Sheep Anti-Mouse HRP Secondary (2º) Linked Antibody (CST 7076)
Sodium Acetate Tri-hydrate (Fisher Scientific 10030350)
Sodium Dodecyl Sulphate (SDS) (Sigma Aldrich L3771)
Sodium Chloride (NaCl) (Sigma Aldrich S7653)

T_{75} Vented Cell Culture Flasks (Starstedt 83.3911.002)
Testosterone (Sigma Aldrich T1500)
TetraHydraFuran (THF) (Sigma Aldrich 401757)
Tris Base (Fisher Scientific BP 154-1)
Trizma base (Fisher Scientific BP 154-1)
Trypan Blue (Sigma Aldrich T1503)
Trypsin 2.5% (10x) (Fisher Scientific 10217723)
Tris Buffered Saline (TBS) Tween 20 (Sigma Aldrich T9039)
TRIzol Reagent (Fisher Scientific 15596026)

2.2. Comprehensive List of Apparatus

96 well plates v-bottomed and clear (Griener 651101)
Aspirator (Vacusafe Comfort IBS Integra Biosciences)
Biological Safety Cabinet (ESCO Sentinel Gold Class II)
Centrifuge (Fisher Scientific accuspin™ 400)
CO_2 Incubator (Thermo Scientific HeraCell 150i)
FACS-Calibur running CellQuest software (Becton Dickinson)
FLUOstar Omega micro-plate reader (BMG LabTech)
Haemocytometer (Sigma Aldrich Z359629)
Handheld Evometer with chopstick electrodes (World Precision Instruments)
HyperClone® 5mm C-18 ODS 250 mm x 4.6 mm column (Phenomenex)
Hyperfilm™ (Sigma Aldrich GE28-9068-38)
High Performance Liquid Chromatography (HPLC) (Agilent 1100 Series)
Inverse Microscope (Leica Leitz Laborlux)
Inverted Fluorescence Microscope (Olympus IX51)
L-Carnitine Assay Kit (AbCam AB83392)
MacLab (AD Instruments)
Mini Plate Spinner (Labnet MPS-1000)
Minicentrifuge (Eppendorf 5414R)
Modified Ussing Chambers (World Precision Instruments Inc)
NanoDrop Lite Spectrophotometer (Thermo Scientific)
Osmomat 030 Osmometer (Gonotec GmbH, Berlin, Germany)
pH Meter (Mettler Toledo Seven Multi HB502)
PCR Plates (Starlab E1403-0200)
StepOne Quantitative Real Time (q-RT) PCR Machine (Applied Biosystems)
Trans-Blot® Turbo™ Transfer System (Bio-Rad)
Transmission Electron Microscope (Jeol UK Ltd)

Upright Microscope (Optika 0311859)
Voltage Clamp Amplifier DVC-100 (World Precision Instruments)
Zorbax™ C-18 Silica Chromatography Column (Agilent)

2.3. Practical Methods

2.3.1. Tissue Transport

Primarily stage II (mid-luteal phase) bovine reproductive tracts (Ireland *et al* 1980) were collected from the ABP abattoir in Murton, York, UK, although tracts were not staged for experimentation. Reproductive tracts were transported at room temperature in a **transport medium** comprised of HBSS (Hank's Balanced Salt Solution) excluding $CaCl_2$ and $MgCl_2$ supplemented with 10 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) and 1 µM Aprotonin. The tracts reached the laboratory within 90 minutes of slaughter.

2.3.2. Tissue Harvest

Following manual dissection of whole oviducts, and cellular extraction by hand "by squeezing the oviduct from isthmus to infundibulum" (Ghavideldarestani 2012), into petri dishes containing HBSS without $CaCl_2$ and $MgCl_2$, the Bovine Oviduct Epithelial Cell (BOECs) and Fibroblast Cell (BOFCs) suspension was centrifuged for 5 mins at 400 x *g* at room temperature. The supernatant and upper layer of erythrocytes were removed and discarded. The pellet was resuspended in 10 ml HBSS excluding $CaCl_2$ and $MgCl_2$ and further centrifuged for 5 minutes at 350 x *g*. The cellular pellet was resuspended in 1 ml pre-equilibrated (38.5°C, 5% CO_2, 95% air) of **culture medium** — consisting of 1:1 DMEM and F12; supplemented with 265 $U \cdot ml^{-1}$ PenStrep, 20 $\mu g \cdot ml^{-1}$ Amphotericin B, 2 mM L-Glutamine, 2.5% *v/v* NCS, 2.5% *v/v* FBS, and 0.75% *w/v* BSA – similarly to Dickens *et al* (1993). DMEM and F12 composition is provided in the Appendix.

BOECs and BOFCs were subsequently isolated based on their differential adhesion times - as described by Cronin *et al* (2012). Specifically, BOECs and BOFCs were initially seeded together in T75 flasks. Following 18 hours of culture, BOECs (un-adhered; in suspension) were removed. The purity the epithelial isolates was in excess of 99% as confirmed using Flow Assisted Cell Sorting (FACS) against cells expressing cytokeratin 18 (CK18) (BOECs) *vs* those expressing Vimentin (BOFCs). FACS methodology and data is described in Chapter 3.

2.3.3. Cell Count and Seeding

To evaluate the number and proportion of viable cells recovered, a 50 µl aliquot of the cell harvest was mixed thoroughly with 0.1% Trypan Blue. This suspension was further mixed in 50 µl PBS prior to 10 µl of this composite solution being pipetted between a glass coverslip and a Neubauer improved haemocytometer (Figure 2.1) for viability and total cell count assessment under an upright microscope at x100 magnification as used by Reischl *et al* (1999).

Over 50% of cells usually remained viable for seeding and culture as confirmed by Trypan blue exclusion (Figure 2-1). Trypan blue is a diazo dye derived from toluidine which permeates the plasma membranes of necrotic and apoptotic cells thereby giving them a visually blue appearance. In contrast, viable cells do not take up Trypan blue and therefore appear colourless.

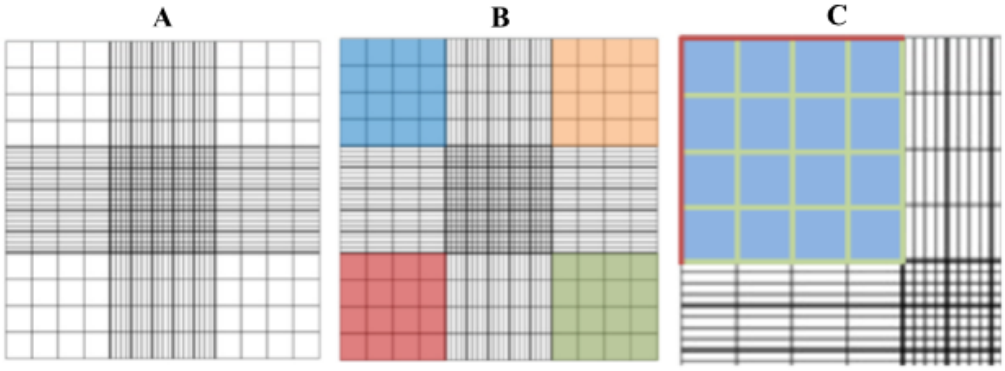

Figure 2-1 — **A**: Image of of the complete haemocytometer at x20 magnification. **B**: Cells were counted within the four corner sections of the haemocytometer highlighted in blue, orange, green and red. **C**: Any cells present on the outer red lines were excluded from the count whereas cells within or verging on the boundaries highlighted in green were suitable for inclusion. To perform a viability assay the following formula was used:

Eq. 2-1: % Viability = [Σ *white cells* ÷ (Σ *white cells* + Σ *blue cells*)] x 100

To determine cellular density, the viable cell mean count established from counting all four chambers was multiplied by $4x10^4$ to convert it into units of cells·ml^{-1}. A typical yield was ~ $75x10^4$ cells·ml^{-1} or $0.75x10^6$ cells·ml^{-1}. Cells were subsequently seeded at a population of $1x10^6$ cells per Transwell™ membrane or T_{25} flask. The corresponding volume at which to $1x10^6$ cells seed was calculated by dividing the actual density of the cellular suspension (*e.g.* $0.75x10^6$ cells·ml^{-1}) by the desired cell number ($1x10^6$ cells). In this particular case, the volume of cells to seed would be 750 µl. For cells seeded to Transwell™ membranes the volume was brought to 2 ml using culture medium whereas for T_{25} flasks the volume was similarly brought to 5 ml.

2.3.4. Cell Culture

Following isolation from fibroblasts, BOECs were directly seeded onto the apical fascia of 24 mm Corning® Transwell™ 0.4 µM pore PolyEthylene Terephthalate

(PET) cell culture inserts coated with 10 μg/ml laminin at a density of 10^6 cells·ml^{-1} per insert. BOECs were subsequently maintained between two culture media-filled chambers (Figure 2-2); apical and basal, with 2 ml culture medium in each compartment, at 39°C in 5% CO_2, 95% air. Apical and basal media were replaced every 48 hours and a polarised confluent monolayer was achieved after 8 days. Cell passaging through flasks was intentionally avoided to minimise the loss of morphological and functional properties of cells described by Rottmayer *et al* (2006).

2.3.5. *in vitro* Derived Oviduct Fluid (*iv*DOF)

Upon reaching confluence, confirmed visually and by a TER greater than 700 Ω·cm^{-2} BOECs were cultured in an 'apical to basal' 'air to liquid' interface as described by Levanon *et al* (2010). Specifically, the basal medium comprised 2 ml of culture medium while the apical compartment comprised moist air in 5% CO_2. However after 24 hours of post-confluence 'air to liquid' interface incubation, a thin film of clear fluid formed in the apical chamber — termed *in vitro* Derived Oviduct Fluid (*iv*DOF) (Figure 2-2). *iv*DOF could subsequently be isolated for analysis. The term 'native' is used to describe *iv*DOF resulting from untreated epithelia.

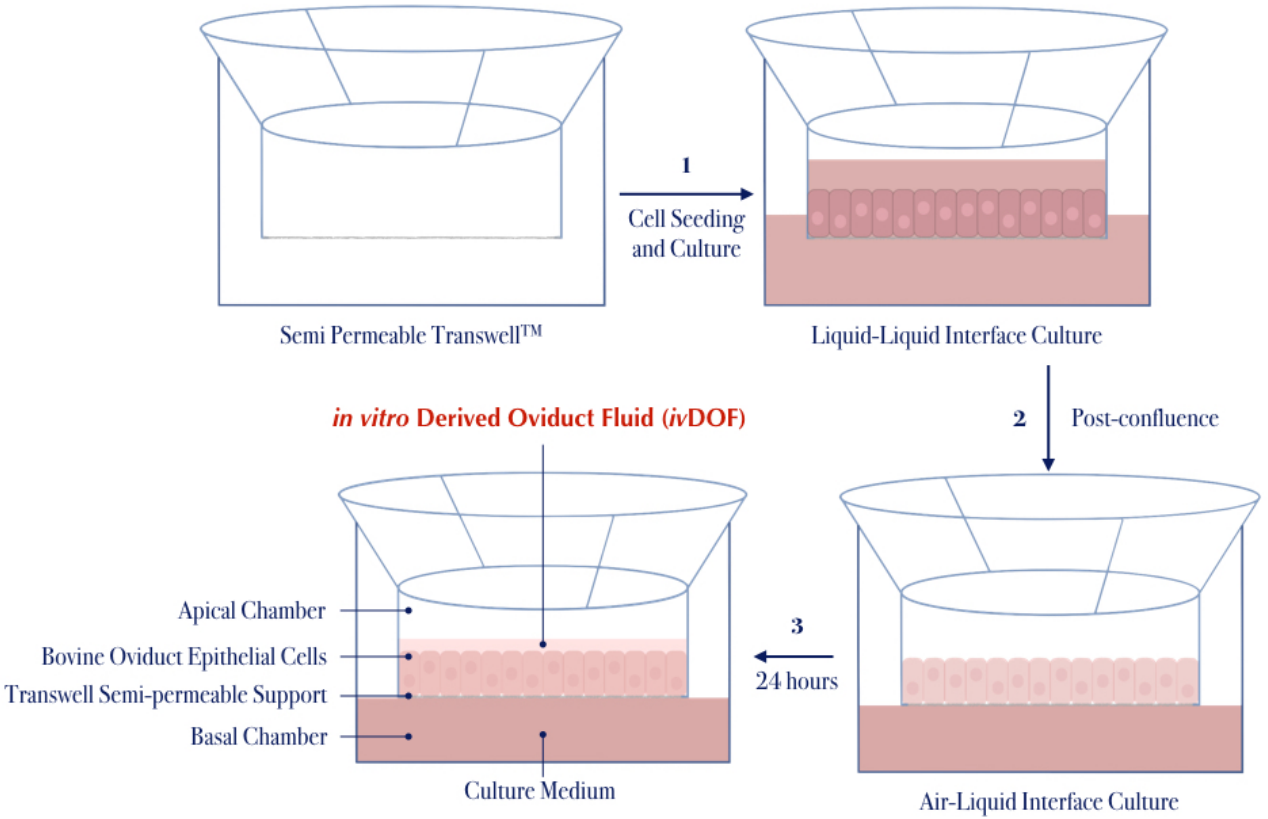

Figure 2-2 — A schematic representation of the process for obtaining *iv*DOF wherein primary BOECs are cultured on Transwell™ semipermeable supports to confluence prior incubation in an air-liquid interface. The basal chamber represents the bloodstream whilst the apical represents the oviduct lumen. The lower side depicts *iv*DOF formation on the apical fascia following 24 hours of air-liquid culture.

2.4. Technical Methods

2.4.1. Transepithelial Electrochemical Resistance (TER)

As alluded to in Section 1.5.4.1. epithelial cell confluency is an indication of the number of adherent in a given culture. Once cells adequately cover a culture surface they are said to have reached confluence. This is not technically the same as a polarised epithelium (Section 1.5.4.) which describes biochemical and electrochemical asymmetry about a cell or monolayer. In the case of the oviduct epithelium however these two phenomena are linked since oviduct epithelial cell polarity is achieved at high confluence. As discussed in Section 1.5.4.1 Transepithelial Electrochemical Resistance (TER) is a measure of both cell confluency and polarity since it is a measure of the ability of a cell culture to establish and maintain an electrochemical gradient (Ferrell *et al* 2010; Cereijido *et al* 1978; Martinez-Palomo *et al* 1980).

TER was measured using an Evom voltmeter fitted with handheld chopstick electrodes. The inherent resistance of a blank Transwell™ (without any cells) immersed in medium was approximately 100 $\Omega \cdot cm^{-2}$. The TER of a tight bovine oviduct epithelial cell monolayer was between 700 $\Omega \cdot cm^{-2}$ - 1000 $\Omega \cdot cm^{-2}$. From cell seeding to reaching full confluence, TER rose from 250 $\Omega \cdot cm^{-2}$ - 800 $\Omega \cdot cm^{-2}$ in the course of ~ 8 days. In addition to assessing monolayer integrity prior to experimentation, TER was also utilised as a measure of post-treatment cellular integrity.

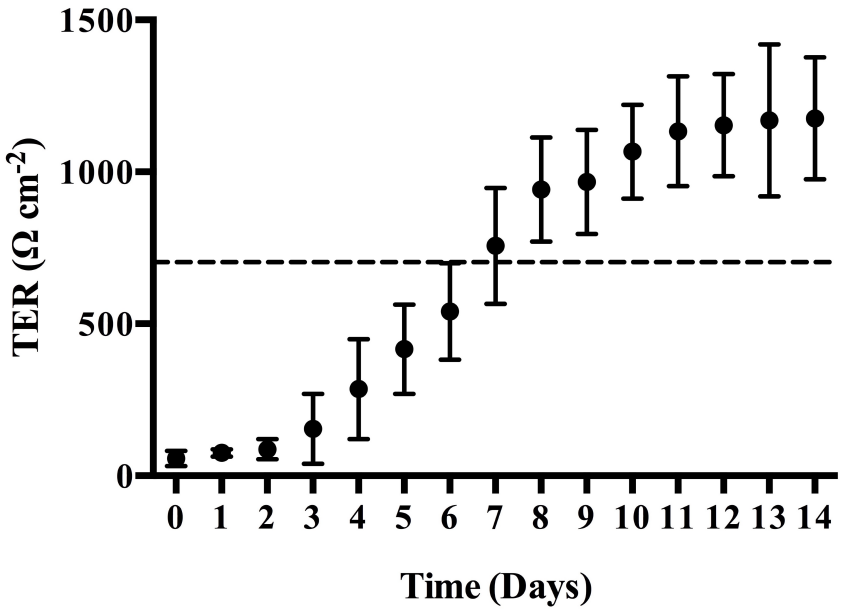

Figure 2-3 — Transepithelial electrochemical resistance (TER) *vs* Time (days) of BOEC cultures on Transwell™ supports (n=8 ± SD).

The value of 700 Ω·cm^{-2} was determined (Simintiras *et al* 2012) based on the previous literature surrounding the subject. At the lower end Tahir *et al* (2011) compared confluent BOEC TER values across different membranes and reported < 500 Ω·cm^{-2} whereas at the upper end Keating & Quinlan (2008, 2012) define BOEC confluence as >1000 Ω·cm^{-2}. Palma-Vera *et al* (2014) defined confluence as ~600 Ω·cm^{-2} and Chen *et al* (2015) at 500 - 1100 Ω·cm^{-2}. Others simply report TER changes as a percentage of the initial reading (Ferrell *et al* 2010). Moreover given the observations in Figure 2-3 it seemed sensible to set a benchmark of confluence at 700 Ω·cm^{-2} as shortly after this point TER plateaus and the risk of over-confluence increases.

2.4.2. High Performance Liquid Chromatography (HPLC): Amino Acids

HPLC is a powerful analytical method primarily used in this project to quantitatively detect 18 amino acids (AA): aspartate, glutamate, asparagine, serine, histidine, glutamine, glycine, arginine, threonine, alanine, tyrosine,

tryptophan, methionine, valine, phenylalanine, isoleucine, leucine and lysine. The quantitative method has been previously described by Humpherson *et al* (2005) and Forde *et al* (2014) but in summary, amino acids present in *iv*DOF samples were beta mercaptoethanol derivatised with ortho-pthaldialdehyde (OPA) reagent supplemented with 1 $mg \cdot ml^{-1}$ beta mercaptoethanol (β-ME) (Figure 2-4) enabling the conjugates to emit fluorescence at 450 nm when excited at 330 nm (Sturmey *et al* 2010). For more detail please refer to Bartolomeo & Maisano (2006).

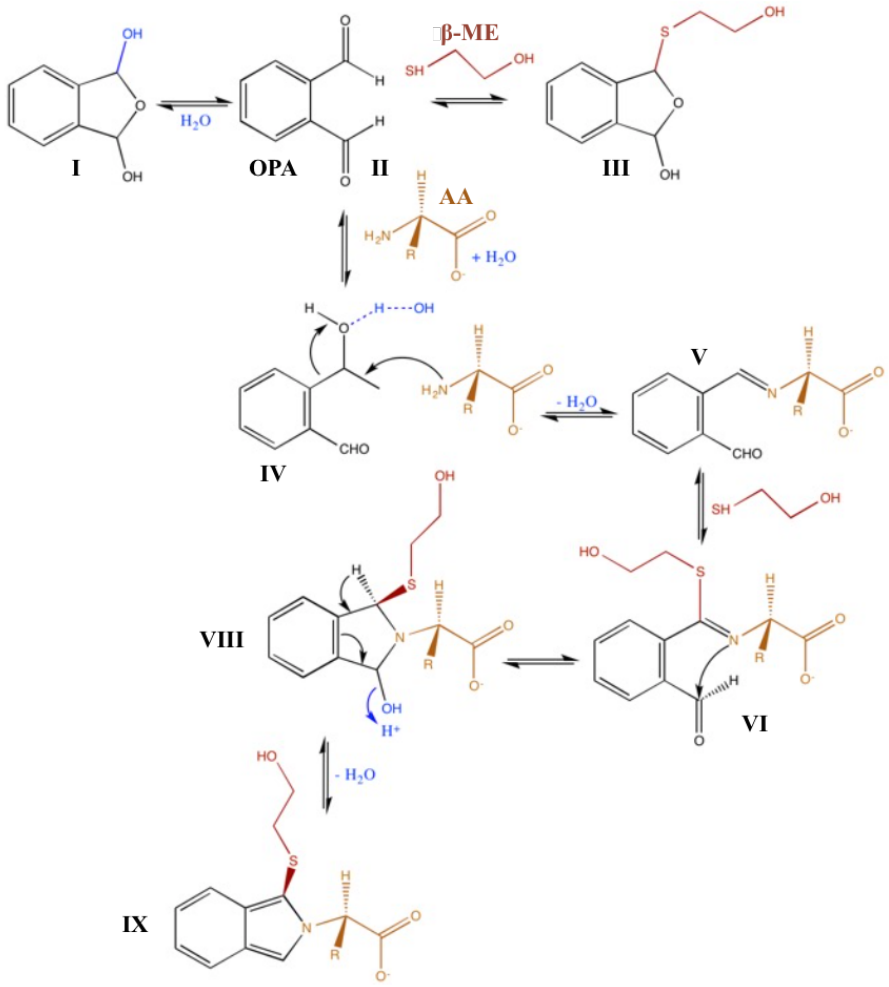

Figure 2-4 — The reaction mechanism for β-ME, OPA, and AA conjugation in an aqueous solution — adapted from Garcia *et al* 1989. Aqueous OPA (**II**) is in equilibrium with the cyclic hydrate (**I**) and thiol adduct (**III**). The most energetically favourable reaction is the nucleophilic attack of OPA by the AA amine (**IV**) resulting in an OPA-AA conjugate (**V**). Adduct cyclisation is initiated by the substitution of a hydrogen from OPA with an active thiol from β-ME. This creates an electropositive push towards the aldehydic carbonyl via the amino moiety of the thiocarbinomide intermediate (**VI**) resulting in the cyclisation of the conjugate and dehydration (**VIII**). This series of reactions results in the formation of a stable 1-aryl isoindole structure from o-amino-methyl benzophenone (OPA-β-ME-AA) derivative (**IX**).

Following amino acid derivatisation, reverse phase chromatography was performed through an Agilent 1100 HPLC coupled with a Phenomenex HyperClone® 5mm C-18 ODS 250 mm x 4.6 mm (extended) column. Gradient elution by two buffers: (**A**) 80% 83 mM sodium acetate (pH to 5.9 using glacial acetic acid), 19.5% methanol, 0.5% tetrahydrofuran (THF), and (**B**) 80% methanol and 20% 83 mM sodium acetate was used to separate OPA-amino acid derivatives at 30°C for 60 minutes at a flow rate of 1.3 $ml \cdot min^{-1}$. Amino acids were separated based on retention time — as detected by fluorescence (absorbance unit peak) and quantified based on peak area relative to known standards. The accuracy and precision of this method is exemplified by Figure 2-5.

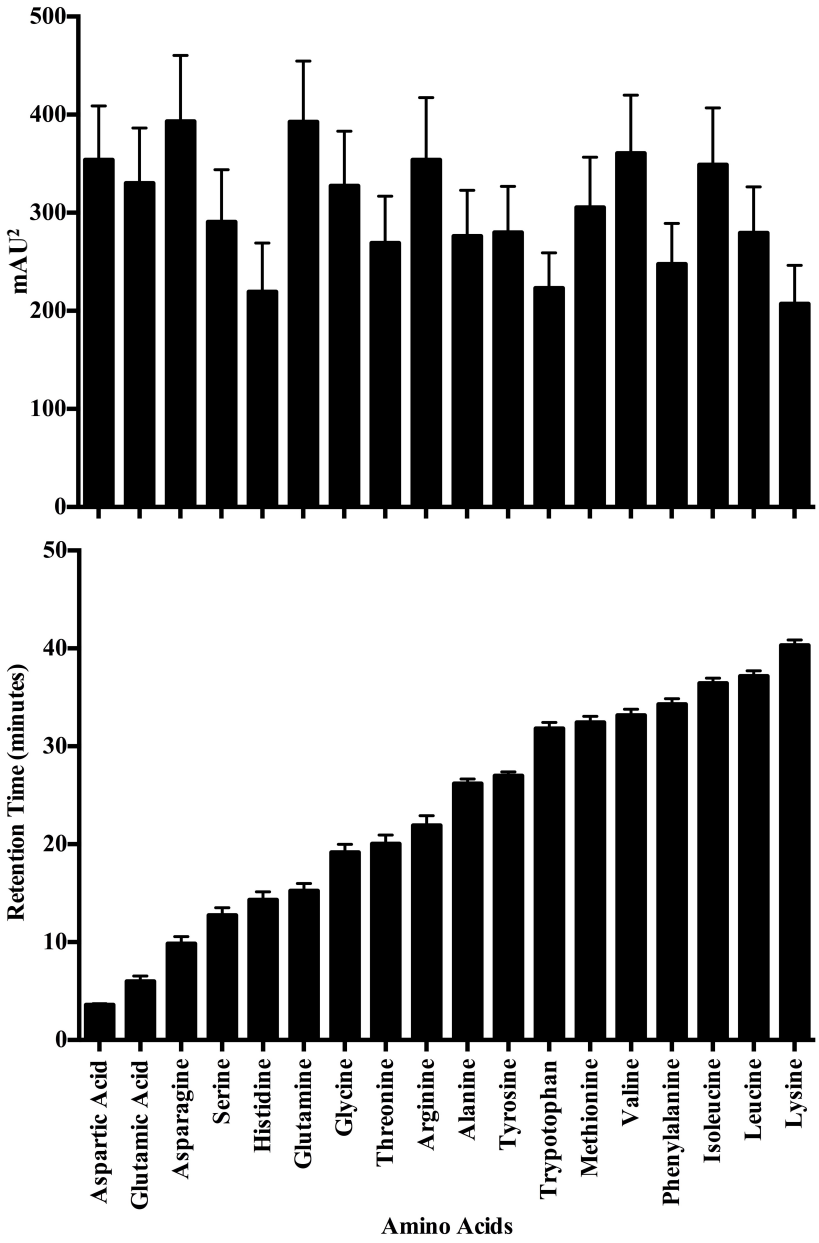

Figure 2-5 — **A**: The Absorbance Units (mAU^2) corresponding to 12.5 µM of all 18 amino acid standards as calculated from HPLC peak areas (n=82 ± SD), and **B**: the corresponding mean retention times of the amino acids (n=82 ± SD).

Based on these data, the percentage error in quantification by HPLC falls within 6% whereas the possibility for amino acid mistaken identity based on retention time is effectively zero. In other words if an amino acid concentration in an unknown sample is determined by HPLC as 12.5 µM this should be interpreted as 12.5 µM ± 0.75 µM factoring in inherent technical variability.

2.4.3. Polymerase Chain Reaction

2.4.3.1. mRNA Extraction

BOECs from flasks were incubated at 39°C under 5% CO_2, 95% air with 2 ml 1x Trypsin for approximately 5 minutes to enable cell detachment – as confirmed by visual assessment. Cells still adhering were lifted by scraping after which the trypsin was quenched with 8 ml culture medium. The cell suspension was subsequently centrifuged at 1000 g for 5 minutes and the supernatant discarded. To wash the cells from trypsin and media, the pellet was resuspended in 1 ml HBSS (without phenol red) and re-centrifuged at 1000 x g for 5 minutes three times at 4°C.

Once washed, the cellular pellet was kept on ice and taken to the fume hood where the pellet was resuspended in pre-chilled 1 ml Trizol and vortexed prior to the addition of 200 µl 100% pre-chilled chloroform. This mixture was vortexed, incubated at room temperature for 20 minutes, and centrifuged at 4°C for 5 minutes at 12,000 g. The lower (pink) organic (protein and lipid) phase and intermediary (white) interphase (DNA) were discarded. The upper transparent aqueous (mRNA containing) phase was collected, added to 1 ml pre-chilled isopropanol, vortexed, and incubated at -20°C for 20 minutes.

The mixture subsequently thawed on ice (~ 1 minute) and centrifuged at 12,000 x g for 40 minutes at 4°C prior to resuspension in 500 µl pre-chilled 70% ethanol 30% DEPC treated (nuclease free) water, and centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet left to air-dry (~ 10 minutes). Pellets were pulse-spun to check for any residual liquid and reconstituted in 50 µl pre-chilled DEPC water. The concentration ($ng \cdot ml^{-1}$) and purity (A_{260}/A_{280}) of mRNA isolated was determined on a NanoDrop spectrometer prior to sample storage at -20°C.

2.4.3.2. cDNA Synthesis

Global complementary DNA (cDNA) was synthesised by reverse transcription. To achieve this mRNA samples were thawed on ice and briefly pulse spun at 4°C. The reverse transcription reaction was performed using the High Capacity cDNA Reverse Transcription Kit. As per the manufacturer instructions, the following reverse transcription (RT) master mix was prepared per mRNA sample in 0.5 ml flat-cap PCR tubes:

10xRT Buffer	2.0 µl
25x dNTP Mix	0.8 µl
MultiScribe Reverse Transcriptase	1.0 µl
DEPC treated dH_2O	4.2 µl
Random Hexamers	2.0 µl
Total reaction volume	10.0 µl

A consistent volume of 10 µl and concentration of 10 $\mu g \cdot ml^{-1}$ of mRNA sample was mixed into the corresponding 10 µl of RT master mix on ice, avoiding the generation of bubbles. Following a brief pulse spin, reverse transcription was performed using a thermal cycler in accordance with the reaction below.

25 ºC	10 minutes
37 ºC	120 minutes
85 ºC	5 minutes
25 ºC	Hold (∞)

The concentration ($ng \cdot ml^{-1}$) and purity (A_{260}/A_{280}) of cDNA generated was subsequently determined using a NanoDrop spectrophotometer prior to sample storage at -20 °C.

2.4.3.3. Primer Design

Primers were designed using NCBI Blast software unless otherwise stated. Only exon spanning bovine specific primers were used. Thermal cycler optimisation curves for primers not previously evaluated in the literature are presented in Appendix 3. The primers used to quantify gene expression are provided below (Table 2-1).

Gene	Direction	Sequence	T_m (°C)	GC (%)
β-Actin	Forward (3' to 5')	TTCAACACCCCTGCCATG	59.64	56
β-Actin	Reverse (5' to 3')	TCACCGGAGTCCATCACGAT	59.73	55
OVGP1 *	Forward (3' to 5')	CTGAGCTCCATCCCCACTTG	57.20	60
OVGP1 *	Reverse (5' to 3')	GTTGCTCATCGAGGCAAAGG	57.10	55
ESR1 *	Forward (3' to 5')	AGGGAAGCTCCTATTTGCTCC	57.00	52
ESR1 *	Reverse (5' to 3')	CGGTGGATGTGGTCCTTCTCT	57.50	57
SLC1A1 †	Forward (3' to 5')	CACCGTCCTGAGTGGGCTTGC	61.30	67
SLC1A1 †	Reverse (5' to 3')	CAGAAGAGCCTGGGCCATTCCC	61.30	64
SLC38A2 †	Forward (3' to 5')	GAACCCAGACCACCAAGGCAG	58.10	62
SLC38A2 †	Reverse (5' to 3')	GTTGGGCAGCGGGAGGAATCG	61.80	67
SLC38A5 †	Forward (3' to 5')	TGGCCATCTCGTCTGCTGAGGG	63.20	64
SLC38A5 †	Reverse (5' to 3')	GCTCCTGCTCCACAGCATTCCC	62.00	64
SLC38A7 †	Forward (3' to 5')	CGGCAGCCCGAGGTGAAGAC	61.60	70
SLC38A7 †	Reverse (5' to 3')	GCCGCAGATACCTGTGCCCAT	60.90	62
SLC6A14 †	Forward (3' to 5')	TCGAGGGGCAACTCTGGAAGGT	60.80	59
SLC6A14 †	Reverse (5' to 3')	GGCAGCATCTTTCCAAACCTCAGCA	62.90	52
ZO1	Forward (3' to 5')	CTCTTCCTGCTTGACCTCCC	56.80	60
ZO1	Reverse (5' to 3')	TCCATAGGGAGATTCCTTCTCA	55.20	45

Table 2-1 — Comprehensive primers list including individual primer nucleotide base sequences, melting temperatures (T_m) and the ratio of the nitrogenous bases guanine (G) and cytosine (C) to total base content. Primers marked with the * symbol are taken from Ulbrich *et al* 2003 whereas primers marked † have been directly copied from Forde *et al* (2014).

2.4.3.4. PCR Amplification

To correct for cDNA loading all cDNA was diluted to 1 µg·ml^{-1} in 1.5 ml Eppendorf^{TM} tubes. The master mix was prepared on ice and the most effective reaction conditions were optimised according to the following volumes per well:

SYBR Green	12.5 µl
Forward Primer	2.75 µl
Reverse Primer	2.75 µl
cDNA [1 µg·ml^{-1}]	1.50 µl
DEPC treated dH_2O	5.50 µl
Total Reaction Volume	25.0 µl

Three technical replicates were prepared per sample in PCR-grade optical 96 well plates and sealed with corresponding adhesive films from Applied Biosystems (AB). Plates were subsequently pulse centrifuged before being loaded onto a Step-one Real-Time PCR machine (AB). Upon loading the most efficient cycling programme used throughout this thesis is outlined below:

Holding Stage	94 ºC	5 minutes	
Cycling Stage	94 ºC	30 seconds	Denaturation
	X ºC	45 seconds	Annealing
	72 ºC	45 seconds	Extension
Melt Curve	95 ºC	15 seconds	
	60 ºC	60 seconds	
	95 ºC	15 seconds	

The **X** ºC temperature varied for each primer pair used. This was 3 ºC below the lowest primer T_m hence in the case of β-actin gene expression analysis was 56.64 ºC. Holding stage and melt curves were performed as a single cycle whereas the cycling stage was consistently set at 55 cycles. Melt curves were performed for all genes as a more efficient alternative to conventional agarose electrophoresis for product size determination. Melt curves are an assessment of the temperature

related dissociation characteristics of DNA. The temperature at which 50% of DNA has dissociated is the *melting point* and is proportional to the size of the DNA product (Gigilio *et al* 2003).

2.4.3.5. qRT-PCR Analysis

Following the completion of the PCR run, the baseline threshold cycle (Ct) value was manually evaluated for each gene prior to the automatic determination of the relative rate of amplification using StepOne software V2.0. All genes studied were subsequently twice-normalised. Once against the housekeeping β-Actin gene and once against the expression of the same gene in untreated (native) BOECs in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al* 2009). All gene expression data presented have been determined by the ΔΔCt method (Livak and Schmittgen 2001) briefly outlined below.

2.4.3.6. qRT-PCR Interpretation by ΔΔCt

SYBR green is a cyanine dye which binds double stranded DNA (dsDNA) by intercalating between the major grooves of the dsDNA. The DNA-SYBR complex absorbs blue light (497 nm) and emits green light (520 nm), thus the degree of fluorescence is positively related to the concentration of dsDNA. As PCR thermally cycles and primer specific DNA is amplified the signal increases and can be detected in real time (RT-PCR).

The premise underlying quantitative real time PCR (qRT-PCR) and therefore the determination of relative gene expression by the ΔΔCt method is that the rate of DNA amplification (*i.e.* signal detection) is constant but the time it takes to reach a crossing threshold (Ct) is variable and directly proportional to the initial

concentration of cDNA present, which is, in turn, equal to the initial concentration of mRNA. More specifically, the rate of exponential amplification of DNA by PCR is given by:

Equation 2-2: $\boldsymbol{X}_n = \boldsymbol{X}_0 \times (1+E_X)^n$

where, $\boldsymbol{X}_n$ is the number of cDNA target of at cycle number n (of 55 in this project), $\boldsymbol{X}_o$ is the initial cDNA target. Therefore $\boldsymbol{X}_n = \boldsymbol{X}_o$ at cycle number 0. $\mathbf{E}_X$ represents the efficiency of cDNA target and is an experimentally determined constant.

Signal saturation will occur once all cDNA target has been amplified assuming other reaction parameters (*e.g.* primers) are not rate limiting. At this point the PCR run can be considered complete. 55 cycles were enough for all the genes analysed in this project to reach saturation, however, the Ct was always in the range of 25-35 cycles as depicted by Figures 2-6 and 2-7.

In contrast to signal saturation there is always an inherent degree of background signal, or noise, until exponential amplification begins — usually between cycles 5-10. The cycle number during which logarithmic signal amplification starts is the threshold cycle (Ct,X). Similarly $\boldsymbol{X}_T$ is the number of cDNA target present during the threshold cycle. The exponential amplification of cDNA during the threshold cycle can therefore be defined as:

Equation 2-3: $\boldsymbol{X}_T = \boldsymbol{X}_0 \times (1+E_X)^{-Ct,X} = \boldsymbol{K}_X$

where $\boldsymbol{K}_X$ is the experimentally determined constant describing the exponential amplification phase. All gene expression (proportional to signal amplification) data was normalised against the β-actin housekeeping gene which is basally

expressed regardless of cellular treatments. Thus β-actin served as the endogenous (internal) control or reference (R) whose amplification during the threshold cycle is expressed as:

Equation 2-4: $\boldsymbol{R}_T = \boldsymbol{R}_0 \times (1+E_{\boldsymbol{R}})^{Ct,\,\boldsymbol{R}} = \boldsymbol{K}_{\boldsymbol{R}}$

Therefore the normalisation formula to calculate target gene expression relative to an endogenous control is a straight division of the two equations:

Equation 2-5: $\boldsymbol{X}_T \div R_T = [\, \boldsymbol{X}_0 \times (1+E_{\boldsymbol{X}})^{Ct,\,\boldsymbol{X}}] \div [\, R_0 \times (1+E_R)^{Ct,\,\boldsymbol{R}}] = \boldsymbol{K}_{\boldsymbol{X}} \div \boldsymbol{K}_R$

The ΔΔCt analysis method assumes that the efficiencies of target cDNA amplification and reference cDNA amplification are identical ($E_X = E_X = E$) therefore:

Equation 2-6: $\boldsymbol{X}_0 \div R_0 \times (1+E)^{Ct,\,\boldsymbol{X} - Ct,\,\boldsymbol{R}} = \boldsymbol{K}$

The ratio of initial target cDNA ($\boldsymbol{X}_o$) to initial reference cDNA ($\boldsymbol{R}_o$) is defined as the normalised initial target ($\boldsymbol{X}_N$). Similarly Ct,X - Ct,R can be rewritten as ΔCt. Hence:

Equation 2-7: $\boldsymbol{X}_N = \boldsymbol{K} \times (1+E)^{-\Delta Ct}$

The second normalisation of data can now be conducted for the reference (first) normalised target gene (now labelled *q*) against the target gene from untreated cells, also known as the calibrator (*cb*). The amplification equation for the calibrator is identical to that of the reference. The second and final normalisation or calibration can therefore be expressed as a fraction of the two:

Equation 2-8: $\boldsymbol{X}_{N,q} \div \boldsymbol{X}_{N,cb} = \boldsymbol{K} \times (1+E_{\boldsymbol{X}})^{-\Delta Ct,\, q} \div \boldsymbol{K} \times (1+E_{\boldsymbol{X}})^{-\Delta Ct,\, cb}$

which simplifies to $\boldsymbol{X} = (1+E)^{-\Delta\Delta Ct}$. This formula for gene expression can be further simplified based on the assumption that E (the experimentally determined constant representing cDNA amplification efficiency) tends to 1 at maximum efficiency. Maximum efficiency can be assumed following protocol optimisation (Appendix 3) thereby reducing the amount of target gene relative to reference and calibration = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

2.4.3.7. Optimisation

As aforementioned in Table 2-1 several genes selected for analysis were already verified in *bos taurus* cells. Primers for *β-actin* and *ZO1* were designed *de novo* and optimisation curves were therefore performed (Figures 2-6 and 2-7 respectively). More specifically the β-actin primers (Table 2-1) showed 100.57% efficiency and an initial cDNA titration indicated that a loading concentration between log 1 $ng \cdot \mu l^{-1}$ and log 2 $ng \cdot \mu l^{-1}$ yields optimal amplification for the reaction conditions outlined in Section 2.4.3.4.

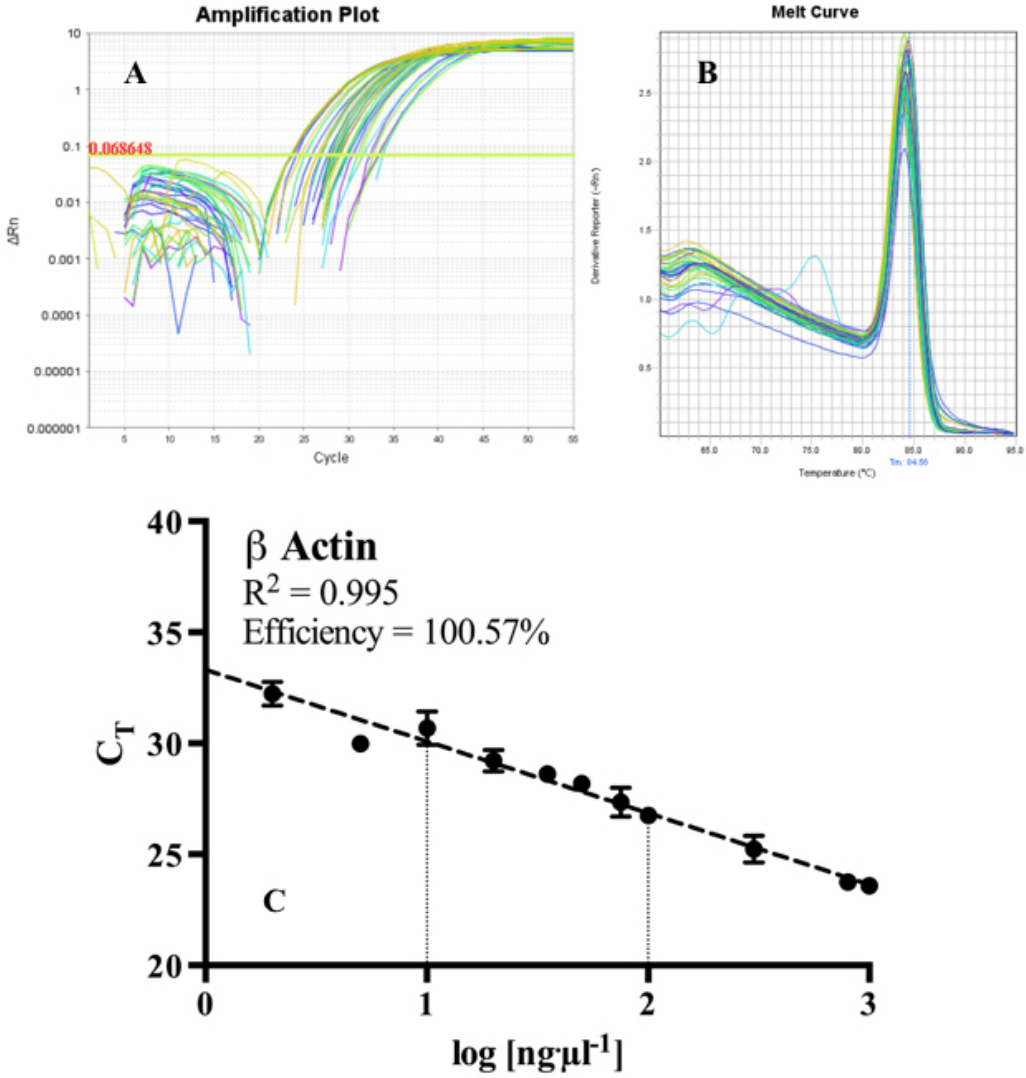

Figure 2-6 — Primer optimisation data for *β-actin* expression showing **A**: amplification plots with a clean Ct being reached between 23 and 35 cycles **B**: melt curves showing single product amplification at 84.56˚C and **C**: a linear relationship between the logarithmic initial sample concentration and the cycle at which the threshold was reached.

Similarly to *β-actin* the *ZO1* primers (Figure 2-7) gave 101.96% efficiency and an initial cDNA titration indicated that a loading concentration between log 1 ng·µl^{-1} and log 2 ng·µl^{-1} yields optimal amplification for the reaction conditions outlined in Section 2.4.3.4.

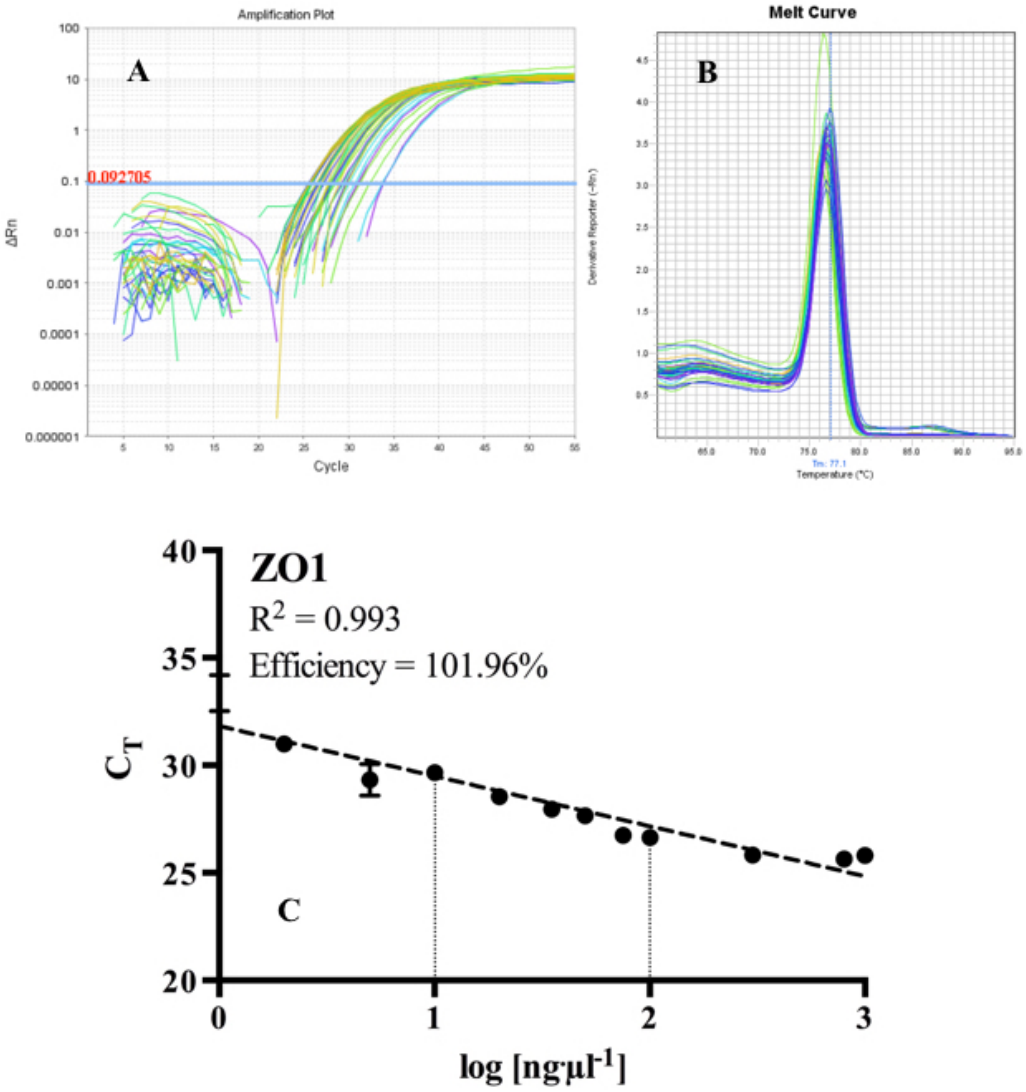

Figure 2-7 — Primer optimisation data for *ZO1* expression showing **A**: amplification plots with a clean Ct being reached between 25 and 35 cycles **B**: melt curves showing single product amplification at 77.1˚C and **C**: a linear relationship between the logarithmic initial sample concentration and the cycle at which the threshold was reached.

Unfortunately not all primers designed performed as well as these. In addition to the gene specific primers outlined in Table 2-1 additional primers were designed for several other solute carriers in addition to genes such as *Leptin* and *GLUT1*. However the amplification products obtained were far from pure as demonstrated by suboptimal melt curves. Hence these results had to be disregarded since no conclusions could be drawn from any data resulting from said analyses.

2.4.4. Micro-plate Reader Assays

Glucose, lactate, and pyruvate were quantified indirectly using enzyme-linked fluorometric assays akin to Leese (1983), Leese and Barton (1984), Gardner and Leese (1988, 1990), and Guerif *et al* (2013). The theory underpinning these assays is that the specific compound of interest is enzymatically converted with a nicotinamide structure produced as a reaction byproduct — which can be spectrophotometrically detected by micro-plate reader. The initial concentration of the specific compound of interest can hence be calculated by comparing resulting fluorescence intensity against a standard curve.

2.4.4.1. Glucose

Glucose was quantified indirectly by measuring at 450 nm the reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced by two sequential reactions wherein glucose and adenosine triphosphate (ATP) are catalysed by hexokinase to glucose-6-phosphate (G6P) and adenosine diphosphate (ADP) as in equation 2-9. Subsequently glucose-6-phosphate dehydrogenase converts G6P and the oxidised nicotinamide adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate (Gl6P), NADPH, and a free hydrogen ion (H^+) as shown in equation 2-10.

Equation 2-9: Glucose + ATP → G6P + ADP

Equation 2-10: $G6P + NADP^+ \rightarrow Gl6P + NADPH + H^+$

The inherent fluorescence of the v-bottomed 96-well plate and reaction mix (45 µl) was first detected by micro-plate reader prior to sample addition (5 µl) and incubation for 10 minutes at 37˚C in darkness. The measurements were then

repeated and the former values (background noise) were subtracted from those of the sample or standard readings. A representative standard curve is provided in Figure 2-8.

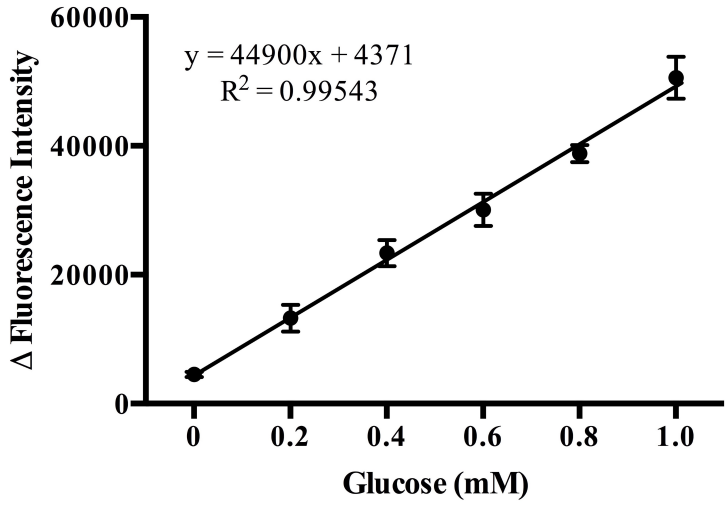

Figure 2-8 — Representative glucose standard curve (n=4 ± SEM).

The glucose assay **reaction mix** comprised: 0.4 mM Dithiothreitol (DTT), 3.07 mM $MgSO_4$, 0.42 mM ATP, and 1.25 mM $NADP^+$, 20 $IU \cdot ml^{-1}$ HK, and 20 $IU \cdot ml^{-1}$ G6PD, all in 4-(2-ethyl)-1-piperazinepropanesulfonic acid (EPPS) buffer at pH 8.0. This reaction mix was stored at -20˚C for up to 2 months.

2.4.4.2. Lactate

Lactate was quantified indirectly by measuring reduced nicotinamide adenine dinucleotide (NADH) at 450 nm produced from a reaction whereby **l**actate dehydrogenase catalyses the conversation of lactate and oxidised nicotinamide adenine dinucleotide (NAD^+) to pyruvate and NADH as outlined in equation 2-11.

Equation 2-11: Lactate + $NAD^+ \rightarrow$ Pyruvate + NADH + H^+

The lactate assay **reaction mix** comprised: 40 $IU \cdot ml^{-1}$ lactate dehydrogenase

(LDH) in a glycine-hydrazine buffer at pH 9.4. This reaction mix was stored at -20˚C for up to 2 months. The lactate assay was practically performed identically to the glucose assay with the exception of reaction mix and sample or standard incubation for 30 minutes. A representative standard curve is provided in Figure 2-9.

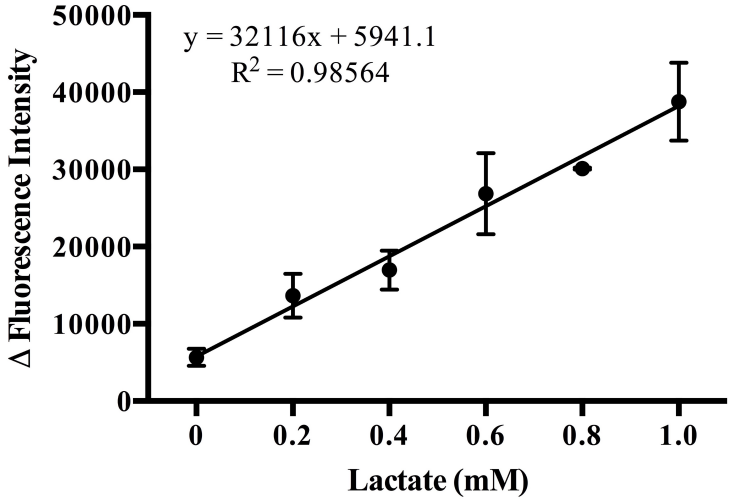

Figure 2-9 — Representative lactate standard curve (n=3 ± SEM).

2.4.4.3. Pyruvate

Pyruvate was quantified indirectly by measuring NAD^+ at 450 nm produced by lactate dehydrogenase in accordance with the reaction in equation 2-12.

Equation 2-12: Pyruvate + NADH + H^+ → Lactate + NAD^+

The pyruvate assay **reaction mix** comprised: 0.1 mM NADH and 40 $IU \cdot ml^{-1}$ lactate dehydrogenase in 4.6 mM EPPS buffer at pH 8.0. This reaction mix was stored at -20˚C for up to 2 months. The pyruvate assay was practically performed identically to the glucose and lactate assays with the exception of reaction mix and sample or standard incubation for 3 minutes. A representative standard curve is provided in Figure 2-10.

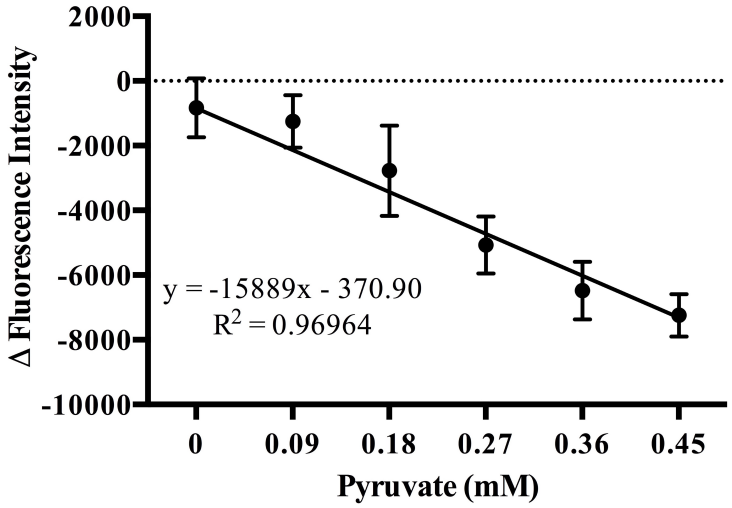

Figure 2-10 — Representative pyruvate standard curve (n=3 ± SEM).

2.5. Experimental Designs

Using primary cells from tissue at first passage has advantages and disadvantages over using established immortalised cell-lines. Advantages include being able to study cells in as close to their natural form *in vitro* since minimal opportunity to de-differentiate is afforded to them. Disadvantages include increased risks of infection, an inherent animal variability, and more often than not, low tissue retrieval from the abattoir. The latter presented a challenge when designing experiments around *iv*DOF.

Typically 4 reproductive tracts (8 oviducts) were retrieved from the abattoir which, when pooled together, yielded enough viable bovine oviduct epithelia to seed to 6 Transwell™ inserts. The volume of *iv*DOF obtained from each Transwell™ was usually 10 µl. The standard experimental design was to assign 3 Transwell™ inserts for treatment with the dependent experimental variable and the remaining 3 as a negative control. Thus ~30 µl from each group would usually be disposable for a subsequent analysis. This was defined as an n of 1.

Pooling tracts and *iv*DOF in addition to staggering biological replicates was necessary considering the volumes required for analyses. For instance amino acid analysis by HPLC used 25 µl of undiluted sample. Similarly to perform a single micro-plate reader (MPR) assay with technical triplicates required 15 µl. In summary, throughout this thesis n=3 is often presented. This is not suggestive of three animals but rather three independent abattoir collections and *iv*DOF isolations on a given date. Any remaining *iv*DOF was stored at -80˚C.

2.6. Statistical Methods

Statistical analyses were performed using Prism Graphpad 6 software for Apple Macintosh. Unless otherwise stated all statistical analysis were two way analysis of variance (ANOVA) followed by a Holm-Sidak non-parametric *post hoc* analysis where *a* = $p \leq 0.0001$ and *b* = $p \leq 0.001$ and *c* = $p \leq 0.01$ and *d* = $p \leq 0.05$.

2.6.1. Analysis of Variance

Two way ANOVA was chosen as it allows for multiple comparisons to be made for a single dependent variable (*e.g*. amino acid *a*) but between all independent experimental variables (*e.g.* treatment *x vs* treatment *y vs* treatment *z*). In other words two way ANOVA enabled multiple column comparisons within a given row to identify whether there is any relationship between the two independent variables on the dependent variable. Since ANOVA makes, amongst others, the assumptions that all sample populations are normally distributed — combined with the fact that *a priori* power analyses were not conducted — a non-parametric *post-hoc* was necessary.

2.6.3. Holm-Sidak *post hoc*

Deviation from the conventional *post hocs* (*e.g.* Bonferroni's and Tukey's tests) was because these tests are just a one-to-one function of the p-value attained from the two-way ANOVA. The advantage of these tests are that they compute confidence intervals *but* at the expense of statistical significance power. The Holm-Sidak test on the other hand is similar to the Bonferroni correction but a better fit for the data in this thesis because confidence intervals are irrelevant and the Holm-Sidak is more powerful and corrects for family-wise error, which is, simply put, the probability of making a type I error (false positive).

Chapter 3

Characterising the *in vitro* Oviduct Preparation

Chapter 3 — Characterising the *in vitro* Oviduct Preparation

3.1. Introduction

As described in Chapters 1 and 2 this thesis revolves around the discovery of an *in vitro* Derived Oviduct Fluid (*iv*DOF) which this chapter aims to characterise and profile. The extent to which an *in vitro* model can be used to understand biological processes *in situ* relies on defining the ways in which the preparation mirrors the behaviour of the cells *in vivo*. Since the oviduct is lined with a transporting epithelium, attention was focused on the way in which bovine oviduct epithelial cells *in vitro* transport nutrients from the basal to the apical compartment, with a considerable emphasis on amino acids (Table 3-1).

3.1.1. Amino Acids

Amino acids are a primary energy substrate as well as being central to fundamental processes including nitrogen metabolism, protein syntheses, cellular signalling, acid-base (pH) balance, osmolarity regulation, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, redox balance, and broader metabolic regulation (Wu 2010).

Amino acids may be categorised biochemically as either neutral, hydrophobic, cationic or anionic. Nutritionally however amino acids are categorised as essential (E) or non-essential (NE) with some sources including the category conditionally essential (CE). This classification is based on the requirements for inclusion in *in vitro* culture of cells (Eagle 1955). Essential amino acids cannot typically be synthesised by the body and supply is therefore diet dependent (Snell & Fell 1990). Non-essential amino acids may be synthesised intracellularly from readily available precursors to meet metabolic demands as well as being present in the diet. Conditionally essential amino acids are largely non-essential but may under

certain pathological or strenuous conditions not be produced at an equal or faster rate than depletion, thereby rendering them essential (Morris 2004).

The amino acids classified as E are: histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, valine and lysine. The NE are: alanine, aspartate, cysteine and glutamate. The 7 conditionally essential CE are: arginine, asparagine, glutamine, glycine, proline, serine, and tyrosine. In terms of the *in vitro* culture of mammalian cells (including oviduct epithelia) all nine essential amino acids are required in addition to the non-essential arginine, cysteine, glutamine, histidine and tyrosine (Eagle 1959; Rose 1968).

	Murine	Rabbit	Ewe	Porcine	Rabbit	Ewe	Mare	Human	Bovine	
Alanine (Ala)	0.506	0.475	0.241	0.171	0.46	0.440	0.140	0.112	1.263	0.532 - 0.6554
Arginine (Arg)	0.023	0.024	0.048	0.059	0.064	0.140	0.031	0.194	0.202	0.1138 - 0.1527
Asparagine (Asn)	0.014	0.036	0.016	0.011	ND	0.020	ND	0.046	0.012	0.036 - 0.0517
Aspartate (Asp)	0.215	0.025	0.021	0.004	0.024	0.120	0.022	0.027	0.061	0.1209 - 0.1714
Glutamate (Glu)	0.497	0.211	0.047	0.005	0.192	0.200	0.057	0.091	0.361	0.3052 - 0.4123
Glutamine (Gln)	0.347	0.272	0.058	0.034	ND	0.190	ND	0.038	0.143	0.1839 - 0.2097
Glycine (Gly)	0.586	2.889	2.288	0.875	2.766	2.300	0.263	0.035	2.601	1.3202 - 1.980
Histidine (His)	0.021	0.070	0.031	0.019	0.067	0.070	0.020	ND	0.152	0.058 - 0.073
Isoleucine (Ile)	0.024	0.053	0.036	0.024	0.069	0.130	0.025	0.019	0.179	0.0685 - 0.1002
Leucine (Leu)	0.043	0.099	0.083	0.033	0.129	0.250	0.053	0.059	0.352	0.1560 - 0.2206
Lysine (Lys)	0.093	0.060	0.090	0.066	0.165	0.210	0.053	0.052	0.417	0.1927 - 0.26
Methionine (Met)	0.029	0.032	0.014	0.007	0.022	0.050	0.014	0.013	0.075	0.0315 - 0.0488
Phenylalanine (Phe)	0.033	0.051	0.030	0.013	0.065	0.180	0.026	0.017	0.154	0.0522 - 0.0871
Serine (Ser)	0.136	0.201	0.024	0.050	0.318	0.040	0.051	0.032	0.121	0.1412 - 0.1954
Threonine (Thr)	0.187	0.154	0.013	0.020	0.125	ND	0.038	0.046	0.081	0.1198 - 0.2013
Tyrosine (Tyr)	0.032	0.066	0.032	0.014	0.079	0.150	0.041	0.037	0.123	0.0442 - 0.0618
Tryptophan (Trp)	0.001	0.008	0.002	0.000	ND	0.070	ND	0.015	0.016	0.0283 - 0.0465
Valine (Val)	0.104	0.169	0.079	0.036	0.172	0.250	0.041	0.026	0.250	0.1348 - 0.2022
Reference	1	1	1	1	2	3	4	5	1	6

Table 3-1 — Amino acid content (mM) of the oviduct lumen reported across species. Not determined is abbreviated to ND. Table has been adapted from Aguilar & Reyley (2005) and the References are [1] Guerin *et al* (1995), [2] Iritani *et al* (1971), [3] Moses *et al* (1997), [4] Engle *et al* (1984), [5] Tay *et al* (1997), and [6] Hugentobler *et al* (2007b).

3.1.1.1. Transport

The oviduct is primarily a secretory epithelium *i.e.* the net directional transport is from the bloodstream to the lumen – brought about by the asymmetrical distribution and activity of transporter proteins (Figures 1-10 and 3-1). The best characterised epithelia are predominantly absorptive (intestine, kidney, placenta). Primarily secretory epithelia include the trachea, stomach, pancreas, and oviduct. Cell membrane transporters are required to facilitate transport of predominantly

hydrophilic components such as amino acids (Kakuda & McLeod 1994). Moreover the majority of cell types synthesise proteins *de novo* and must internalise amino acids intracellularly usually against an extracellular concentration gradient (Kanai 1997). The main family of proteins responsible for amino acid transport are the solute carrier (SLC) proteins of which there are 43 familial subtypes and 1207 individual proteins identified to date (Table 3-2).

	Description	Total
SLC1	High-affinity glutamate and neutral amino acid transporter family	7
SLC2	Facilitative GLUT transporter family	14
SLC3	Heavy subunits of the heteromeric amino acid transporters	2
SLC4	Bicarbonate transporter family	10
SLC5	Sodium glucose cotransporter family	8
SLC6	Sodium and chloride dependent neurotransmitter transporter family	16
SLC7	Cationic amino acid transporter/glycoprotein-associated amino acid transporters	14
SLC8	Na^+/Ca^{2+} exchanger family	3
SLC9	Na^+/H^+ exchanger family	8
SLC10	Sodium bile salt cotransport family	6
SLC11	Proton coupled metal ion transporter family	2
SLC12	Electroneutral cation-Cl cotransporter family	9
SLC13	Human Na^+-sulfate/carboxylate cotransporter family	5
SLC14	Urea transporter family	2
SLC15	Proton oligopeptide cotransporter family	4
SLC16	Monocarboxylate transporter family	14
SLC17	Vesicular glutamate transporter family	8
SLC18	Vesicular amine transporter family	3
SLC19	Folate/thiamine transporter family	3
SLC20	Type-III sodium-phosphate co-transporter family	2
SLC21	Organic anion transporting family (also known as SLCO)	11
SLC22	Organic cation/anion/zwitterion transporter family	18
SLC23	Sodium-dependent ascorbic acid transporter family	4
SLC24	$Na^+/(Ca^{2+}\text{-}K^+)$ exchanger family	5
SLC25	Mitochondrial carrier family	27
SLC26	Multifunctional anion exchanger family	10

SLC27	Fatty acid transport protein family	6
SLC28	Sodium-coupled nucleoside transport family	3
SLC29	Facilitative nucleoside transporter family	4
SLC30	Zinc efflux family	9
SLC31	Copper transporter family	2
SLC32	Vesicular inhibitory amino acid transporter family	1
SLC33	Acetyl-CoA transporter family	1
SLC34	Type-II sodium-phosphate co-transporter family	3
SLC35	Nucleoside-sugar transporter family	17
SLC36	Proton-coupled amino acid transporter family	4
SLC37	Sugar-phosphate/phosphate exchanger family	4
SLC38	System A and N sodium-coupled neutral amino acid transporter family	6
SLC39	Metal ion transporter family	14
SLC40	Basolateral iron transporter family	1
SLC41	MgtE-like magnesium transporter family	3
SLC42	Rh ammonium transporter family (pending)	3
SLC43	Sodium-independent, system-L-like, amino acid transporter family	2

Table 3-2 — Solute Carrier (SLC) family compilation (Heidiger *et al* 2004).

It is important to note that not all SLC proteins transport amino acids. However the broad mechanisms of molecule transport by SLCs are conserved and apply to amino acids. Christensen *et al* (1964) first investigated the differential movement of amino acids kinetically and reported that more than one mechanism of amino acid flux existed. Considerable efforts have since uncovered the more detailed mechanisms underpinning SLC-mediated transport including amino acids.

Two broad categories of SLC linked amino acid movement exist: (***i***) Na^+ dependent and (***ii***) Na^+ independent – within which subdivisions define specific systems on the transport of cationic, anionic and neutral amino acids (Van Winkle 1993). Na^+ dependent systems include: $X^-_{AG,}$ ASC, GLY, β, PROT, $B^{o,+}$, B^0, A, N, IMINO, $ɣ^+$L whilst Na^+ independent systems include: $ɣ^+$, L, $b^{0,+}$, asc, x^-_c, imino, $ɣ^+$L. System $ɣ^+$L is common to both as it is Na^+ independent but transports cationic

amino acids in a Na^+ dependent manner (Kanai *et al* 2000). The elaborate biochemical reaction mechanisms underlying SLC-amino acid interactions are beyond the scope of this thesis however Figure 3-1 summarises the key SLC mediated transport systems pertinent to amino acid flux.

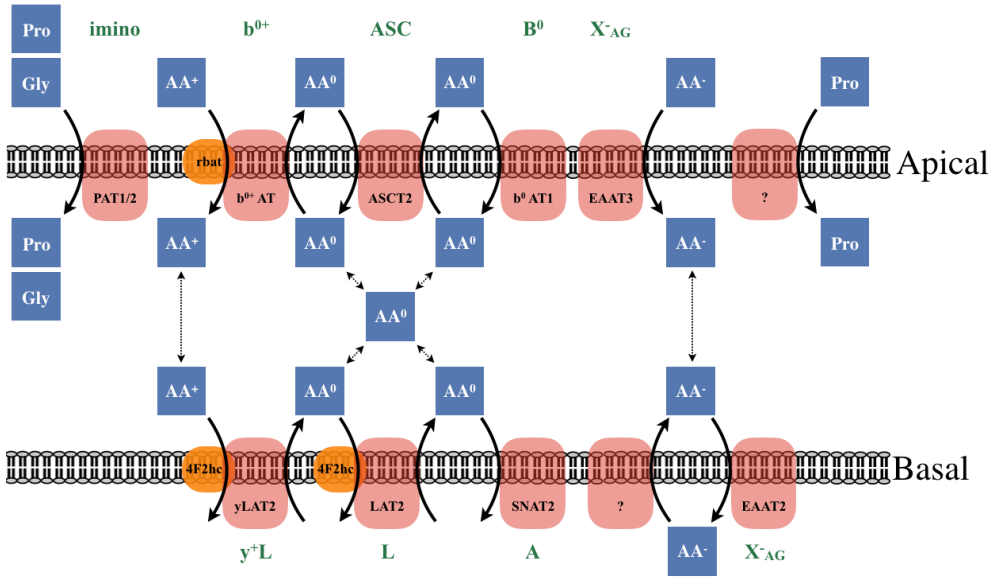

Figure 3-1 — Schematic depiction of the known proteins (red and orange) and their localisation (basal *vs* apical) responsible for cationic (AA^+), anionic (AA^-) and neutral (AA^0) amino acids (blue) transport across epithelia in addition to the corresponding mechanisms (green). Adapted from Whitear (2009) and Brosnsn & Brosnan (2006).

The most common means of amino acid flux regulation amongst epithelia is internal (adaptive) regulation (Guidotti *et al* 1978) – a feedback mechanism wherein an internal amino acid availability beyond a threshold concentration represses the influx of that same amino acid and *vice versa* (Ling *et al* 2001). For instance amino acid deprivation increases the activity the SLC38A2 (or SNAT2) system A protein (Franchi-Gazzola *et al* 1999) and induces further expression of the *slc38a2 gene* (Franchi-Gazzola *et al* 2001; Ling *et al* 2001). Similarly, amino acid starvation increases the expression of the *cat-1* (cationic amino acid transporter1) gene leading to an increase in the SLC7A1 system y^+ protein (Hyatt *et al* 1997).

It is worth noting that the internal adaptive regulation mechanisms of systems A and $ɣ^+$ differ. System A positive feedback occurs alongside a reduction in total amino acid availability whereas system $ɣ^+$ positive feedback can be mediated with the depletion of any *single* amino acid under its purview (Fernandez *et al* 2003). Systems L, $ɣ^+$ and ASC do not appear to be responsive to adaptive regulation (Gazzola *et al* 1973).

As an added level of control to internal (adaptive) regulation, amino acid transporters respond to environmental pressures. For instance, System N transporter activity is modulated by fluctuations in the ratio of intracellular to extracellular volume (Petronini *et al* 1994), glutamate transporter xCT expression is up-regulated by oxidative stress (Kim *et al* 2001), and *slc38a2* gene expression increases in response to osmotic stress (Alfieri *et al* 2001).

Cytokines and hormones further regulate amino acid transporter activity. For instance transforming growth factor beta [(TGF-β) (Boerner *et al* 1985)], glucagon (Fehlmann *et al* 1979), epidermal growth factor [(EGF) (Boerner *et al* 1985)], and platelet derived growth factor [(PDGF) (Owen *et al* 1982)] have all been shown to up-regulate System A transporters. Similarly, tumour necrosis factor alpha [(TNFα) (Irie *et al* 1997)], and interleukin β [(IL-β) (Visigalli *et al* 2004)] up-regulate System $ɣ^+$ transporters. For detailed reviews see Fafournoux *et al* (2000).

3.1.2. Aims and Objectives

The impact of hormones E2, P4, and T on SLC expression and amino acid flux is a common theme throughout this thesis. The specific aims for this chapter however are to characterise the *in vitro* oviduct preparation by **(*a*)** confirming the epithelial nature of the oviductal cells harvested (by flow assisted cell sorting,

transmission electron microcopy, light microscopy, and haematoxylin and eosin staining with bright-field microscopy), **(*b*)** determining OVGP1 presence in the *in vitro* Derived Oviduct Fluid (*iv*DOF), **(*c*)** measuring the sodium, potassium, chloride, bicarbonate, calcium, glucose, lactate, pyruvate, amino acid composition and osmolarity of *iv*DOF, and **(*d*)** performing functional assays on the *in vitro* oviduct preparation by investigating changes in *iv*DOF amino acid composition and BOEC gene expression in response to singular hormonal supplementation.

3.2. Materials and Methods

3.2.1. Flow Assisted Cell Sorting (FACS): BOEC Confirmation

To confirm the epithelial identity of cells harvested the total cellular material derived from four reproductive tracts was pooled and cultured in 9 separate T_{25} flasks. Following cells reaching confluence (assessed visually) all flasks were treated with trypsin for cell-flask detachment. Each flask was incubated at 39.0 °C for 5-10 minutes with 1.5 ml trypsin. Upon visual confirmation of cellular detachment each flask was supplemented with 8.5 ml culture medium to inactivate trypsin. Cell homogenates were then transferred to a corresponding falcon tube (not pooled). Each tube was subsequently centrifuged at 350 x *g* for 5 minutes and the supernatant was discarded. Each pellet was resuspended in pre-warmed (39.0 °C, 5% CO_2, 95% air) 2 ml PBSA (1% *w*/*v* BSA in PBS). This homogenate re-centrifuged at 350 x *g* for 5 minutes. The supernatant was discarded and the pellet was again resuspended in 2 ml PBSA.

Density and viability counts were performed as previously described (Section 2.3.3.) with populations averaging $64x10^6$ cells·ml^{-1} and a mean of 76.5% viability. Then 2ml pre-warmed FPBS (2% *v*/*v* FBS in PBS) was added to each cell population as antibody blocking solution prior to 30 minute incubation at 39.0 °C in 5% CO_2 in air. Post-incubation, all cells were centrifuged at 350 x *g* for 5 minutes and the supernatant was discarded prior to resuspension in 50 µl FPBS.

To identify BOECs selectively an anti-cytokeratin-18 (ã-CK18) antibody was selected since this polypeptide is ubiquitous in oviduct epithelia but not stromal cells (Section 1.5.4) (Moll *et al* 1983). An ã-Vimentin antibody was similarly chosen for BOFC identification as fibroblasts stain positive for this mesenchymal biological marker (Goodpaster *et al* 2008). Vimentin is moreover not present in bovine oviduct epithelia (Rottmayer *et al* 2006). Both primary (1º) antibodies

were mouse monoclonal and were confirmed or predicted by the manufacturer to react with bovine isoforms. For 1º antibody labelling a fluorescent goat anti-mouse Alexa Fluor® 488_{nm} secondary (2º) antibody was chosen. The experimental set up (*i.e.* cell allocation) for epithelial purity determination is provided below.

1. Cells
2. Cells + ã-Vimentin 1º Antibody
3. Cells + ã-CK18 1º Antibody
4. Cells + Alexa Fluor® 488_{nm} 2º
5. Cells + ã-Vimentin 1º Antibody + Alexa Fluor® 488_{nm} 2º Antibody
6. Cells + ã-CK18 1º Antibody + Alexa Fluor® 488_{nm} 2º Antibody
7. Cells + ã-Vimentin 1º Antibody + Alexa Fluor® 488_{nm} 2º Antibody
8. Cells + ã-CK18 1º Antibody + Alexa Fluor® 488_{nm} 2º Antibody
9. Cells + ã-Mouse IgG1 Antibody

The first cell population served as a negative control. The second and third were 2º antibody negative controls whereas the fourth was the 1º antibody negative control. The fifth and seventh cellular populations were allocated to fibroblast staining whilst the sixth and eighth were assigned to epithelial staining. The ninth was the non specific binding positive control — an ã-mouse IgG1 fluorescent antibody which does not exhibit specificity to any bovine reproductive tissue. This antibody therefore established the inherent technical fluorescence in the system (baseline).

Cell suspensions subjected to 1º binding were incubated with 5 µl of the antibody in question (1:10 dilution) for 60 minutes at 39.0 °C in 5% CO_2 in air. Cell-antibody suspensions were centrifuged at 350 x *g* for 5 minutes and the supernatant discarded. Each cell population was resuspended in pre-warmed 50 µl FPBS. BOEC samples assigned for incubation with the Alexa Fluor® 488_{nm} 2º were treated with 5 $\mu g \cdot ml^{-1}$ Alexa Fluor® 488_{nm} solution and incubated for 60 minutes (39.0 °C, 5% CO_2, 95% air) in near complete darkness. This staining

procedure applied for the sample labelled with the anti-mouse IgG1 antibody. All populations were finally prepared for FACS by centrifugation at 350 x *g* for 5 minutes and resuspension in 500 µl FPBS.

Samples were analysed on a BD FACSCalibur system running CellQuest software. To assess the total proportion of epithelial cells over 20,000 events were recorded. Both fibroblast and epithelial cells were gated in accordance with their side-scatter properties and mean fluorescence intensity at 488 nm — relative to that of the 1º negative control. This eliminated the risk of systematic and/or random errors arising from autofluorescence between and/or within subjects (Vince *et al* 2011). To test the BOFC isolation by differential adhesion time method described by Cronin *et al* (2012) the procedure was repeated identically for fibroblast purity determination (see Chapter 4).

3.2.2. Microscopy

3.2.2.1. Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was performed by the University of Hull Central Microscopy Facility. BOECs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in the same buffer, stained en-bloc in 1% uranyl acetate (aq) then serially dehydrated in ethanol before being embedded in Epon-Aradite resin. (All chemicals from Agar Scientific, Stansted, Essex). Subsequently 50nm sections were cut using a diamond knife on a Leica UC6 Ultramicrotome and collected on carbon-coated copper grids (EM Resolutions, Saffron Walden). Images were obtained using an Ultrascan 4000 digital camera (Gatan Inc, Pleasanton, Ca. USA) attached to a Jeol 2011 Transmission Electron Microscope (Jeol UK Ltd, Welwyn Garden City) running at 120 kV.

3.2.2.2. Light Microscopy

Light microscopy images were taken of confluent BOECs in T25 flasks using an Olympus IX51 inverted microscope with a x40 objective lens coupled with an F-view soft digital image correlation (DIC) imaging system and ImageJ software in line with Bolte & Cordelieres (2006).

3.2.2.3. Haematoxylin and Eosin

The protocol for haematoxylin and eosin (H&E) staining is as follows: BOECs were cultured on both Transwell™ inserts and glass cover slips as described in Section 2.3.4. and the Transwell™ membranes with cells were cut out using a blade once cells reached confluence. Cover slips and Transwell™ supports were rinsed three times in pre-equilibrated phosphate buffered saline (PBS) prior to 5 minute incubation at room temperature in 100% haematoxylin. Cells were then rinsed three times in 18.2 milliQ water and incubated for 5 minutes with 1% eosin. The stained cells were then rinsed once in 18.2 milliQ water and placed onto microscope slides. Each apical surface populated with stained epithelia was supplemented with two drops of Hydromount™ followed by the placement of a glass cover slip on top. The slides were then imaged on a Zeiss ApoTome 2 Observer Z1 microscope with x10 and x20 objective lenses and a Axiom 506 mono imager coupled with ZEN imaging software.

3.2.3. Western (Protein-Immuno) Blotting

Western (protein-immuno) blots were conducted to qualitatively evaluate the presence of OVGP1 in bovine oviduct epithelial cell lysates derived from both directly harvested tissue (for antibody quality assessment and method optimisation) and in *iv*DOF. Homogenates were divided equally into pre-chilled 1

ml Eppendorf tubes and centrifuged at 4 ºC for 15 minutes at 10,000 x *g* prior to resuspension in 1 ml cold PBS. This wash was repeated twice with the final resuspension being in 100 µl lysis buffer comprised of 150 mM NaCl, 10 mM Trizma base, 1 mM EGTA, 1 mM EDTA and 1% (*v*/*v*) Igepal. This lysis mix was incubated for 20 minutes prior to vortexing and protease inhibitor PMSF addition at 1 mM. Samples were stored at -20 ºC until ready to load for polyacrylamide gel electrophoresis.

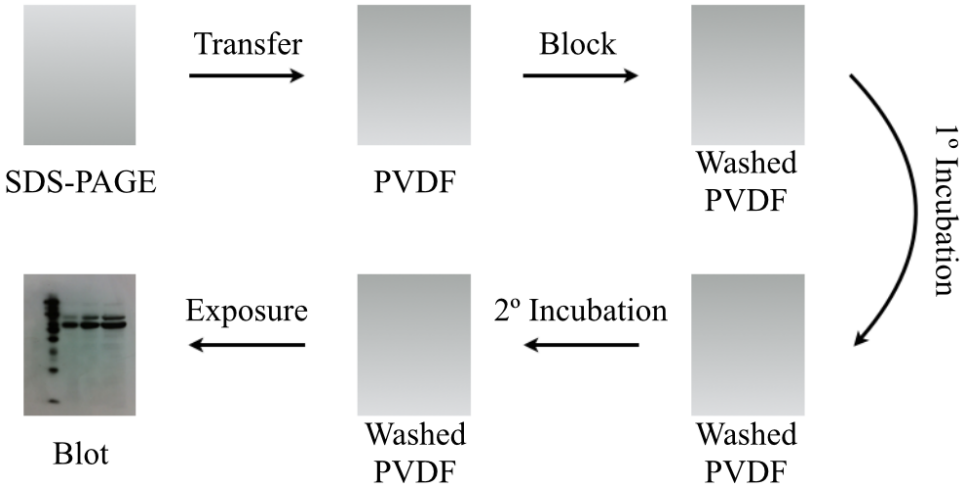

Figure 3-2 — A schematic overview of the western blotting process wherein protein samples are separated by size on a polyacrylamide gel by electrophoresis prior to transfer to a PVDF membrane. The membrane is then blocked with a high protein solution and washed before overnight incubation with the 1º antibody specific to the protein of interest. The PVDF membrane is subsequently washed and incubated with 2º antibody before band visualisation following enhanced chemiluminescent exposure.

Optimisation experiments revealed 10-18% acrylamide gradient resolving and stacking gels provided the best protein resolution from BOEC lysates and *iv*DOF. Gels were prepared as outlined in Table 3-3 below:

	10% Resolving Gel	18% Resolving Gel	4% Stacking Gel
30% Acrylamide	10 ml	18 ml	1.3 ml
1% Bisacrylamide	3.9 ml	3.1 ml	1.5 ml
1.5 M Tris (pH 8.7)	8.1 ml	8.1 ml	0 ml
0.5 M Tris (pH 6.7)	0 ml	0 ml	1.25 ml
20% SDS	200 µl	200 µl	65 µl
H_2O	7.9 ml	1.2 ml	5.725 ml
TEMED	10 µl	10 µl	10 µl
APS	225 µl	225 µl	150 µl

Table 3-3 — Composition of resolving and stacking polyacrylamide gels. Components of the 10% gel were added to BioRad sandwich assemblies followed by the 18%. Gels were levelled with pure methanol and left to set for 1 hour. Methanol was removed prior to the addition of the stacking gel with a 10-well comb inserted. This was left to set for 30 minutes then stored in moist tissue paper at 4 ºC until electrophoresis.

At the time of loading for SDS-PAGE the samples were thawed on ice and total protein concentration was determined for samples derived from harvested tissue. This involved 1:1 (*v*/*v*) mixing with Laemmli buffer and boiling to 100 ºC for 30 seconds followed by conventional Bradford assay (Lowry 1951). The mean concentration of protein was determined to be 0.7 $mg{\cdot}ml^{-1}$.

Bradford assays were not conducted on individual *iv*DOF samples owing to low volumes obtained (Section 2.5.). Thus blotting on *iv*DOF was entirely qualitative. Moreover the scope was not to quantify total protein but rather to observe the presence of OVGP1 *in vitro*.

Samples from *in vivo* derived material were loaded into wells at 10 mM (16.5 µl), 20 mM (33.3 µl) and 40 mM (66.7 µl). Samples from *iv*DOF were loaded at 40 µl. The first well of each gel contained a HRP-linked biotinylated protein ladder. After sample loading 1 L of electrophoresis (running) buffer consisting of 0.192

M Glycine, 0.025 M Tris Base and 0.1% (*w*/*v*) SDS was poured into the electrophoresis chamber and electrophoresis was conducted at 120 V for 80 minutes.

The proteins separated by electrophoresis and embedded in polyacrylamide gels were transferred to a 0.2 µm PVDF membrane by Trans-Blot® Turbo™ set to 2.5 A and 25 V for 25 minutes as per manufacturer guidelines. PVDF membranes were subsequently rinsed in TBS-Tween prior to blocking by incubation with milk for 30 minutes and rewashing in TBS-Tween. This blocking step saturated the PVDF membrane with protein such that any antibody addition afterwards would have to be specific to the protein of interest to observe clean bands.

Extensive protocol optimisation dictated that the ideal western blotting conditions were to incubate blocked PVDF membranes with a custom mouse ã-bovine 1º antibody for OVGP1, generously donated by Professor Susanne Ulbrich (Zurich) and Dr Thomas Fröhlich (Munich), at a concentration of 0.001% (*v*/*v*) or 1:1000 in milk. Incubation was conducted on a shaker at 4 ºC for 24 hours. Following 1º incubation the PVDF membranes were washed in TBS-Tween solution for 10 minutes three times.

The 2º antibody used for signal detection was a sheep anti-mouse horseradish peroxidase (HRP) linked antibody which was incubated with PVDF membranes at 1:10000 or 0.0001% (*v*/*v*) for 1 hour prior to washing in TBS-Tween solution for 10 minutes three times. Also incubated was 20 µl of an anti-biotin (not streptavidin) HRP linked 2º antibody for ladder detection. After 2º incubation and washing membranes were developed chemiluminescently. Enhanced chemiluminescent (ECL) buffers were prepared; the first comprised 2.5 mM luminol (in DMSO), 0.45 mM P-Coumoric (in DMSO), 0.1 M Tris Base and H_2O. The second 0.1% (*v*/*v*) H_2O_2, 0.1 M Tris base and H_2O. PVDF gels were shaken

for 5 minutes in a 1:1 ECL1:ECL2 solution in darkness. Membranes were then exposed to Hyperfilm™ for 5 minutes following immersion in a 20% (*v*/*v*) Kodak® autoradiography solution for 1 minute and shaking in Kodak® fixer solution. The blot was subsequently washed in water.

3.2.4. Ionic, Osmolarity, Carbohydrate, and Amino Acid Determination

Ionic analyses were performed at the Hull Royal Infirmary NHS laboratories whilst osmolarity was measured using a Gonotech Osmometer. Carbohydrate and amino acid analyses were conducted as described in Sections 2.4.4. and 2.4.2. respectively.

3.2.5. Hormone Supplementation

In order to determine the effects of endocrine parameters on the *in vitro* model, hormone stocks were prepared in ethanol prior to supplementation to the basal Transwell™ chamber. Steroid hormone [17β-oestradiol (E2), progesterone (P4), and testosterone (T)] concentrations were based on mean peripheral plasma levels in the bovine throughout the oestrous cycle as previously reported. E2 has been reported to circulate in concentrations between 5.3 $pg{\cdot}ml^{-1}$ (19.46 pM) and 13.5 $pg{\cdot}ml^{-1}$ (49.56 pM), P4 between 0.1 $ng{\cdot}ml^{-1}$ (0.32 nM) and 4.3 $ng{\cdot}ml^{-1}$ (13.67 nM), and T has been found ranging from 40.0 $pg{\cdot}ml^{-1}$ (138.7 pM) to 88.0 $pg{\cdot}ml^{-1}$ (305 pM) with one exception at 1809 $pg{\cdot}ml^{-1}$ (62.77 nM) (Kanchev *et al* 1976). These physiological *in vivo* considerations are reflected in the final concentrations added to the *in vitro* oviduct preparation (Table 3-4) whilst the concentration of insulin added *in vitro* [0.25 $ng{\cdot}ml^{-1}$ (172.2 pM)] is identical to previously reported bovine plasma measurements *in vivo* by Amstalden *et al* (2000).

Steroid hormones were added at a maximum volume of 9 µl in a total of 2000 µl of culture medium [0.45% (*v*/*v*)] to Transwell™ membranes or 22.6 µl in a total of 5000 µl of culture medium [0.45% (*v*/*v*)] to flasks. Insulin dissolved in ethanol was added to Transwell™ inserts at 20 µl to a total of 2000 µl medium [1% (*v*/*v*)] similarly to Bromberg & Klibanov (1995).

	Absolute [Hormone]	**Relative [Hormone] (*v*/*v*)**
17β-Oestradiol (E2)	29.37 pM	0.2%
Progesterone (P4)	6.36 nM	0.25%
Testosterone (T)	62.77 pM	0.45%
Insulin (I)	172.2 pM	1%

Table 3-4 — Concentration of hormones added to bovine oviduct epithelial cells cultured in Transwell™ inserts or flasks expressed in absolute units of molarity (M) and as percentages of total culture volume.

3.2.6. qRT-PCR

The impact of hormonal supplementation on BOEC gene expression was also examined as described in Sections 2.4.3. to 2.4.8. Expression profiles of oviduct epithelial characteristic genes *ESR1* (ERα) and *OVGP1* were determined by qRT-PCR (Figure 3-9) as were the expression profiles of six genes encoding solute carrier proteins involved in amino acid transport — *SLC1A1*, *SLC7A1*, *SLC38A2*, *SLC38A5*, *SLC38A7* and *SLC6A14* (Figure 3-11) following singular hormonal supplementation. BOECs were subjected to three treatment groups (***i***) 62.77 pM testosterone, (***ii***) 29.37 pM 17β-oestradiol, (***iii***) 6.36 nM progesterone and a vehicle control of 9 µl (0.45% *v*/*v*) ethanol – all for 24 hours. All genes analysed were normalised to *β-actin* whilst treatment impacts on gene expression were calculated relative to untreated BOECs.

3.2.7. Caco-2 Culture

As a 'negative control' the protocol for isolating fluid was repeated using cell-line derived absorptive colorectal epithelial adenocarcinoma cells (Caco-2). Specifically, cells from the primarily absorptive HTB-37 human colon carcinoma (Caco-2) line were routinely cultured in T75 polystyrene flasks at 37°C in 5% CO_2 in 95% air. Culture media comprised 12 ml high glucose DMEM supplemented with 15% foetal calf serum (*v*/*v*), 584 $mg{\cdot}l^{-1}$ glutamine, 1% minimum non-essential amino acids, 100 $U{\cdot}ml^{-1}$ PenStrep and 0.25 $\mu g{\cdot}ml^{-1}$ amphotericin B. Media was renewed every 48 hours until cells reached 90–95% confluence, at which point the cells were extracted with trypsin–EDTA solution (3 ml) from each flask and seeded to Transwell™ membranes at a density of $0.26x10^6$ $cells{\cdot}well^{-1}$. Media was replenished every 48 hours and for 20 days until TER was in excess of 900 $\Omega{\cdot}cm^{-2}$ — similarly to Section 2.3.4. and Courts (2013).

3.2.8. Electrophysiology

Electrophysiological studies were attempted on both whole oviduct sections and BOEC monolayers. To achieve the latter, BOECs were seeded to and cultured on Snapwell™ inserts as described in Sections 2.3.1. to 2.3.4. Upon reaching confluence BOECs were incubated for 20 minutes at 39°C in 5% CO_2 in normal Krebs Ringer (KR) bicarbonate solution consisting of 118 mM NaCl, 25 mM $NaHCO_3$, 4.74 mM KCl, 1.19 mM $MgSO_4$, 1.17 mM KH_2PO_4, 1.17 mM $CaCl_2$, and 1 mM glucose (Keating & Quinlan 2008). Snapwells™ were then clamped into modified Ussing chambers and supplemented apically and basally with KR medium, held 37°C heat block and gassed with 5% CO_2 (Figure 3-12).

Changes in transmembrane potential were observed using a DVC-100 V/I clamp (World Precision Instruments) whose probes were set to maintain voltage at 0 mV.

The BOEC monolayers were allowed to equilibrate in this system until a stable short circuit current (I_{SC}) was reached. Then 100 μM ATP dissolved in water supplemented to the basal Ussing chamber in attempts to establish and observe an adrenergic response [Cox & Leese (1995), Dickens *et al* (1993), Downing *et al* (1999; 2002), and Keating & Quinlan (2008; 2012)]. Following the addition of ATP the pore forming nystatin (500 IU/ml in 0.01% methanol) was added (Keating & Quinlan 2012) to disrupt the epithelial monolayer and thus raise the relative I_{SC}. Outputs from the DVC were obtained using an ADI PowerLab analogue-digital converter and visualised using LabChart 5 software.

The procedure for performing electrophysiological studies on whole oviduct sections was identical to the aforementioned with the exception of the use of a different Ussing chamber to accommodate whole tissue sections (Figure 3-12:B).

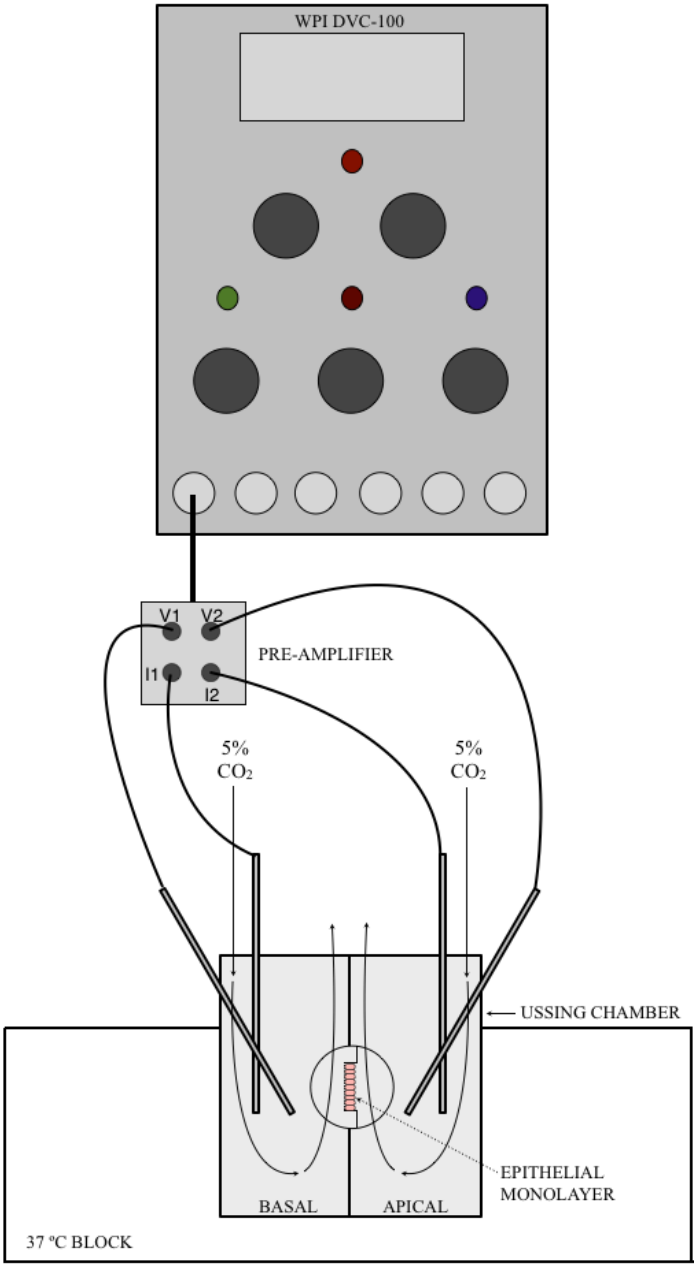

Figure 3-3 — A schematic depiction of the DVC-100 V/I clamp apparatus showing a BOEC-filled Snapwell™ sandwiched within a gassed and temperature controlled Ussing chamber. Probes protruding from the DVC preamplifier were inserted into the chamber to enable voltage manipulation and current measurements

3.3. Results

3.3.1. Cellular Identity Characterisation

The cell harvest and culture method (Section 2.3.) was first validated by Flow Assisted Cell Sorting (FACS); quantitatively confirming that cells harvested exhibited an abundance of the cytokeratin-18 (CK18) marker and were therefore epithelial in nature, as opposed to fibroblasts; the second most abundant cell type of the oviduct, displaying vimentin (Moll *et al* 1983; Goodpaster *et al* 2008; Rottmayer *et al* 2006) (Figure 3-4).

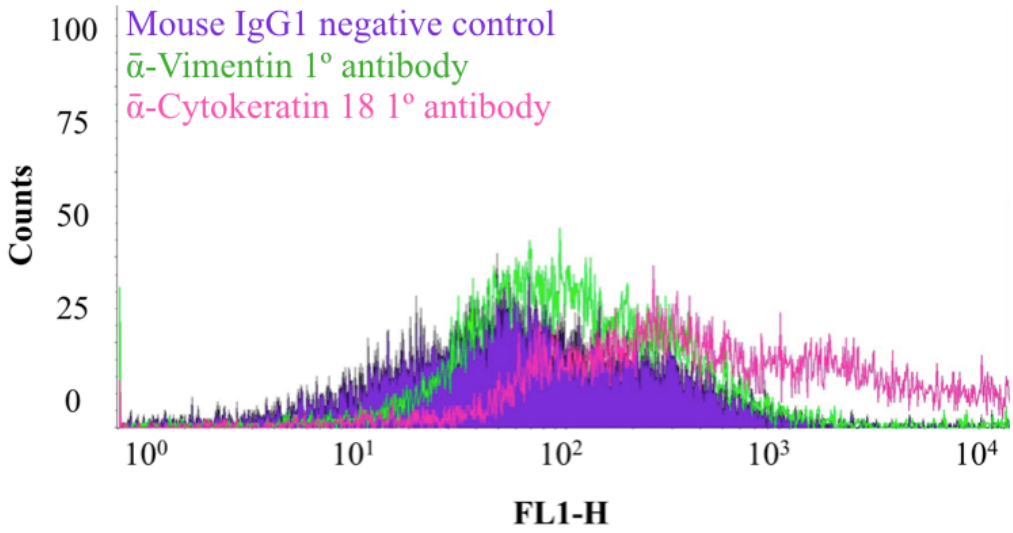

Figure 3-4 — Flow assisted cell sorting (FACS) analysis of cultured BOEC purity showing mouse IgG1 negative control (background noise), ᾱ-Vimentin 1º antibody (BOFC population), and ᾱ-Cytokeratin 18 1º Antibody (BOEC population) – all in combination with the Alexafluor 488 nm 2º antibody (representative of n=2).

Figure 3-4 shows the total of 2096.2 epithelial counts were obtained at a fluorescence (FL1-H) beyond those of the IgG1 negative control (background noise) and vimentin counts (over 10^3) thereby confirming very high epithelial purity. Owing to some (<100 counts) vimentin fluorescence at 10^2 FL1-H over that of the IgG1 it is not possible to confirm 100% epithelial purity but rather an excess of 95%.

To complement the FACS, TEM was performed to examine the ultrastructural features of cultured BOECs such as microvilli and secretory vesicles (Figure 3-5). The same cells were also photographed by light microscopy (Figure 3-6) and haematoxylin and eosin staining (Figure 3-7).

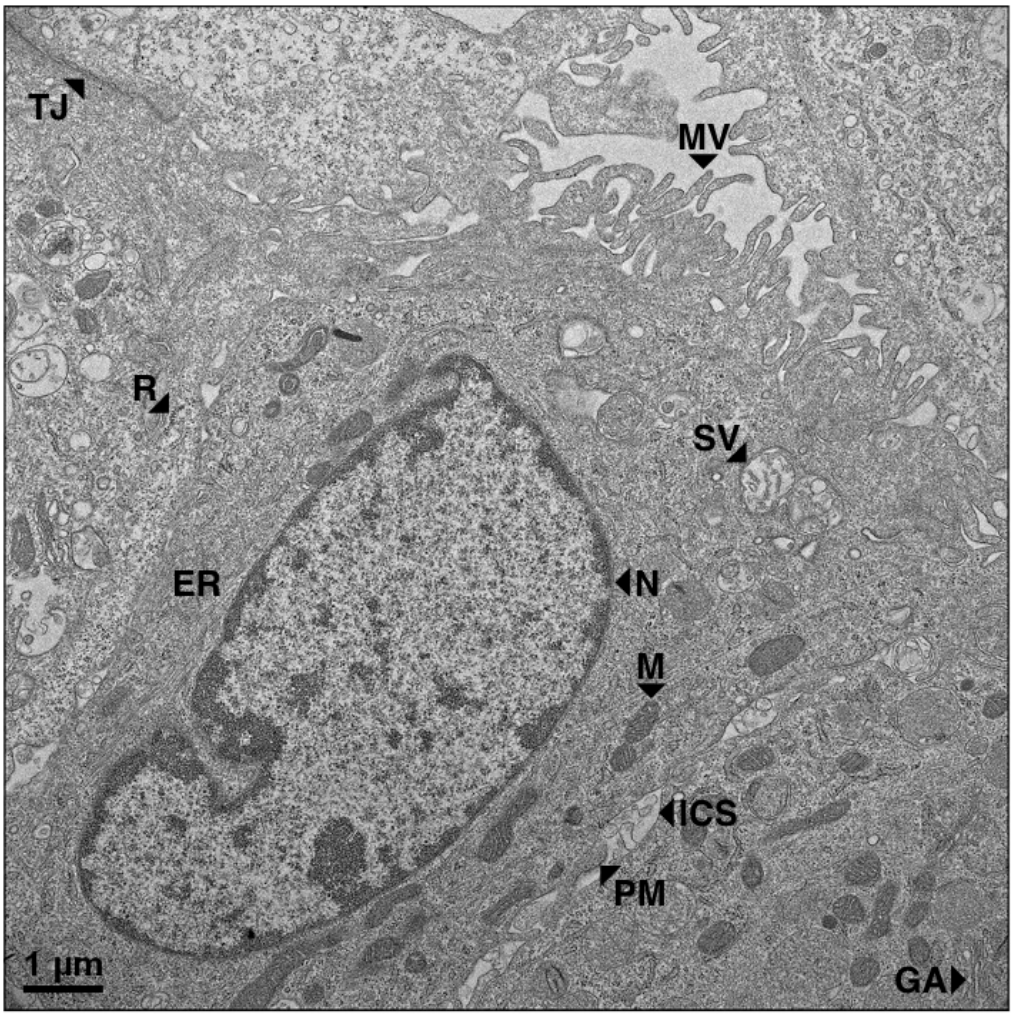

Figure 3-5 — Transmission electron microscopy (TEM) image of bovine oviduct epithelia showing the endoplasmic reticulum (ER), Golgi apparatus (GA), intracellular space (ICS), mitochondria (M), microvilli (MV), nucleus (N), plasma membrane (PM), ribosomes (R), a secretory vesicle (SV), and a tight junction (TJ).

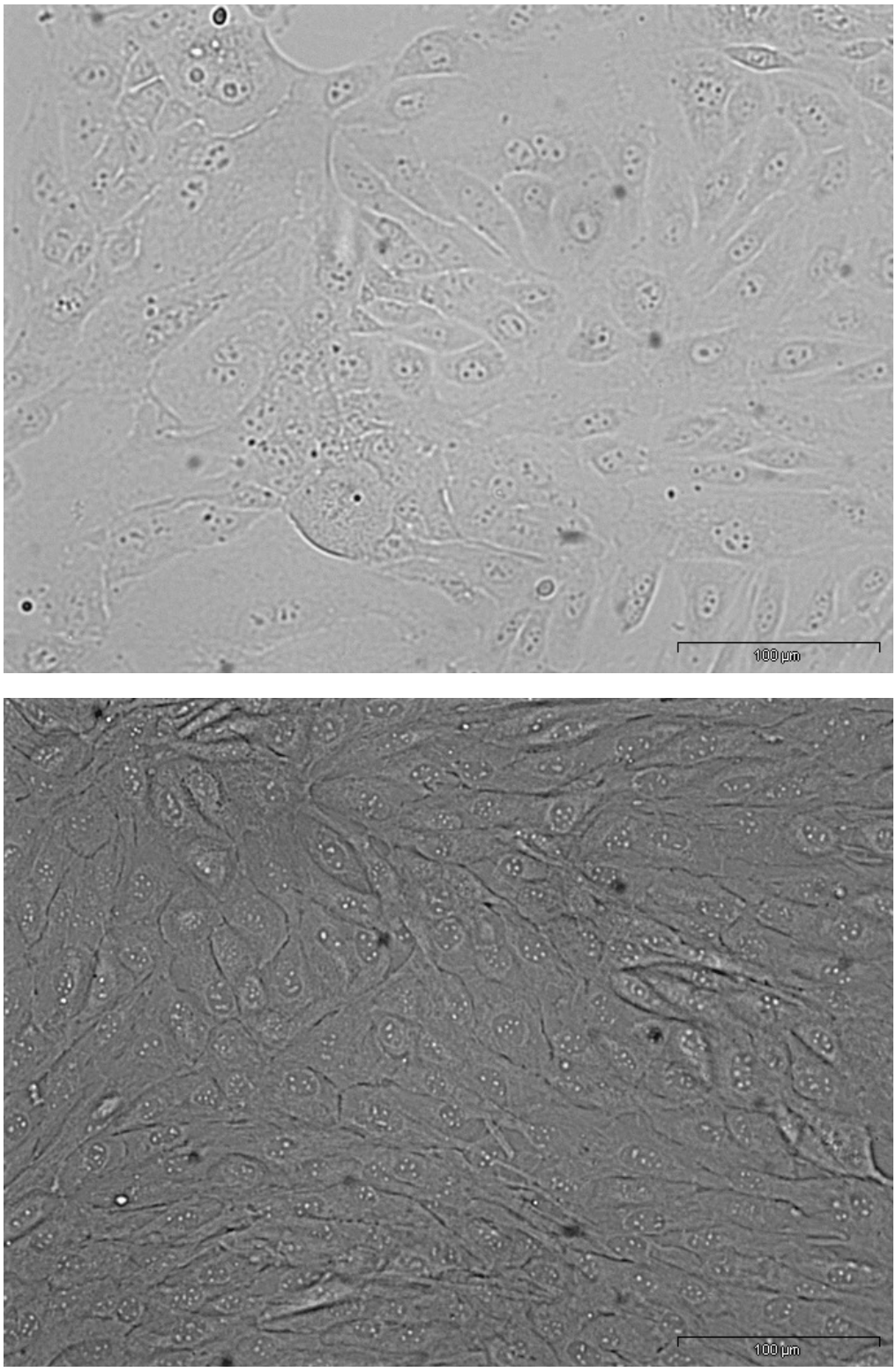

Figure 3-6 — Light microscopy images of confluent bovine oviduct epithelial cells in plastic flask culture at two different contrasts.

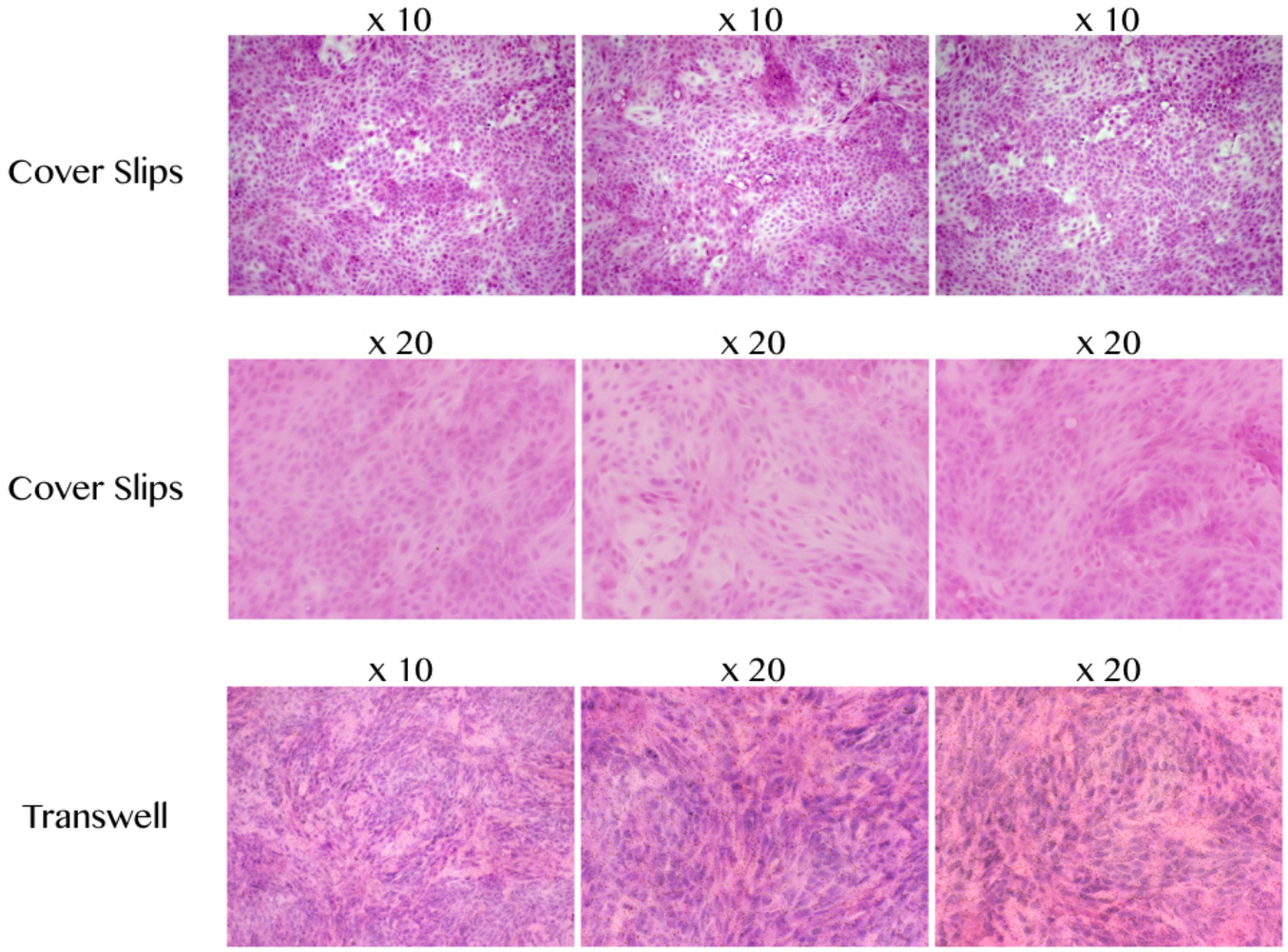

Figure 3-7 — Images of haematoxylin and eosin stained bovine oviduct epithelial cells cultured to confluence on cover slips and Transwell™ membranes and imaged at x10 and x20 magnification.

3.3.2. *iv*DOF Composition

3.3.2.1. OVGP1

BOECs were cultured as described in Section 2.3. Once confluence was confirmed by a TER in excess of 700 $\Omega \cdot cm^{-2}$ the apical media was removed and *iv*DOF accumulated for 24 hours (Figure 2-2). A qualitative western blot for bovine OVGP1 titrated from abattoir derived (fresh) *in vivo* oviduct fluid and cell lysates (Figure 3-8:A) showed two prominent bands, the most dominant around 80 kDa and the second around 90 kDa. These were therefore the glycosylated form of OVGP1 (Bauersachs *et al* 2004). A similar qualitative western blot for bovine OVGP1 from *in vitro* Derived Oviduct Fluid (*iv*DOF) was conducted showing a band solely at 60 kDa.

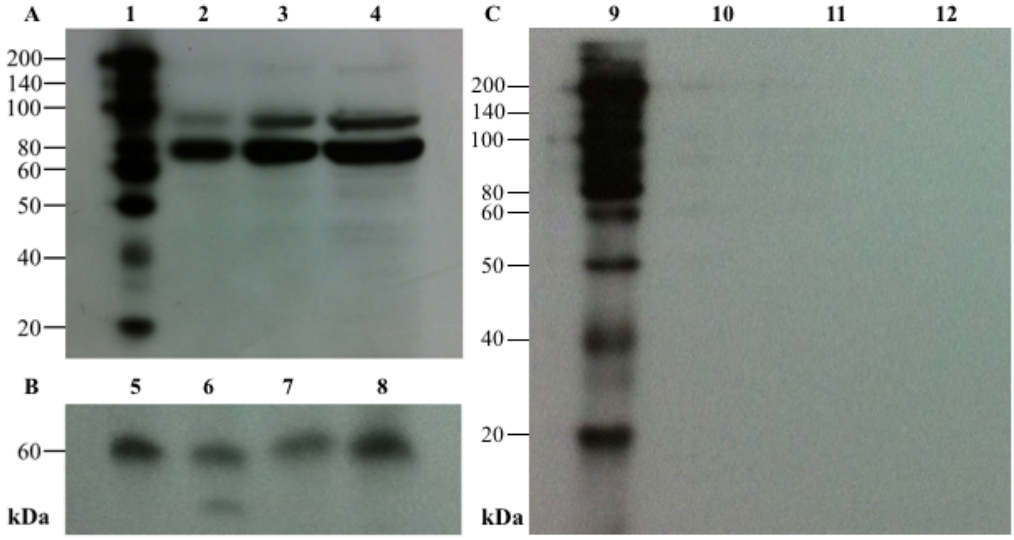

Figure 3-8 — Western blots for OVGP1 from **A**: *in vivo* derived oviduct fluid and cell lysates (n=1), **B**: native *iv*DOF (representative blot of n=4), and **C**: negative controls (n=1). Lanes **1** and **9** were loaded with a staggered 200 kDa HRP-linked biotinylated protein ladder. Lane **2** was loaded with 10 mM (16.5 µl) total protein, lane **3** with 20 mM (33.3 µl) and lane **4** with 40 mM (66.7 µl). Lanes **5-8** were loaded with 40 µl (arbitrary concentrations) of native *iv*DOF (from untreated BOECs). Lane **10** was loaded with *iv*DOF and the sheep anti-mouse horseradish peroxidase linked 2º antibody in isolation. Lane **11** was loaded with *iv*DOF and the custom mouse anti-bovine ã-OVGP1 1º antibody only. Lane 12 was loaded with with culture medium and both 1º and 2º antibodies.

3.3.2.2. Ionic Composition

In addition to OVGP1 protein, the sodium, potassium, chloride, bicarbonate, and calcium composition of culture medium and *iv*DOF was analysed (Table 3-5). This analysis was only available once hence reliable statistically significant differences could not be obtained. Moreover some ions were present in concentrations below the detection limits of the apparatus such as calcium (< 0.5 mM), bicarbonate (< 0.5 mM) and potassium (< 1.0 mM).

	CM	**E**	**N**	**E2**	**P4**	**T**
Sodium [Na^+]	122.0	161.0	113.0	159.0	149.0	146.0
Potassium [K^+]	3.6	1.0	3.3	< 1.0	1.5	1.5
Chloride [Cl^-]	100.0	156.0	92.0	157.0	140.0	138.0
Bicarbonate [HCO_3^-]	13.0	< 5.0	10.0	< 5.0	< 5.0	< 5.0
Calcium [Ca^{2+}]	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5

Table 3-5 — The ionic composition of culture medium (**CM**), 0.84% (*v*/*v*) ethanol (**E**) vehicle, untreated BOEC (**N**) *iv*DOF, 29.37 pM **E2** BOEC treated *iv*DOF, 6.36 nM **P4** BOEC treated *iv*DOF, and 62.77 pM pM **T** BOEC treated *iv*DOF (n=1). Concentrations given in mM.

3.3.2.3. Osmolarity, Glucose, Lactate, and Pyruvate.

BOECs were cultured as described in Section 2.3. Once confluence confirmed by a TER in excess of 700 $\Omega \cdot cm^{-2}$ the apical media was removed and *iv*DOF accumulated for 24 hours (Section 2.3.5). Osmolarity (Figure 3-9:A) and glucose, lactate, and pyruvate (Figure 3-9:B) of *iv*DOF were also measured. Specifically osmolarity was found to be 297 ± 12 mOsm (Figure 3-9:A) whereas glucose was 4.30 ± 1.18 mM, Lactate was 4.70 ± 0.68 and Pyruvate was 0.83 ± 0.34 (Figure 3-9:B).

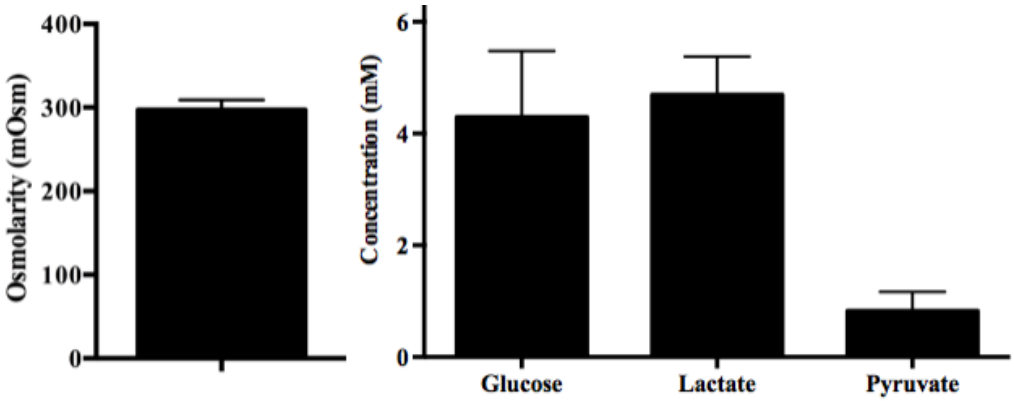

Figure 3-9 — The osmolarity of native *iv*DOF was determined to be 297 ± 12 mOsm (n=3 ± SD) in addition to the concentrations of glucose, lactate, and pyruvate in native *iv*DOF (n=3 ± SEM).

3.3.2.4. Amino Acids

Figure 3-10 shows that BOECs significantly modified the flux of six amino acids. Specifically, histidine, threonine and tyrosine were present in *iv*DOF in higher concentrations than basally supplied. Conversely, the concentrations of glutamine, arginine and tryptophan in native (*i.e.* untreated) *iv*DOF were lower than in standard culture medium supplied basally.

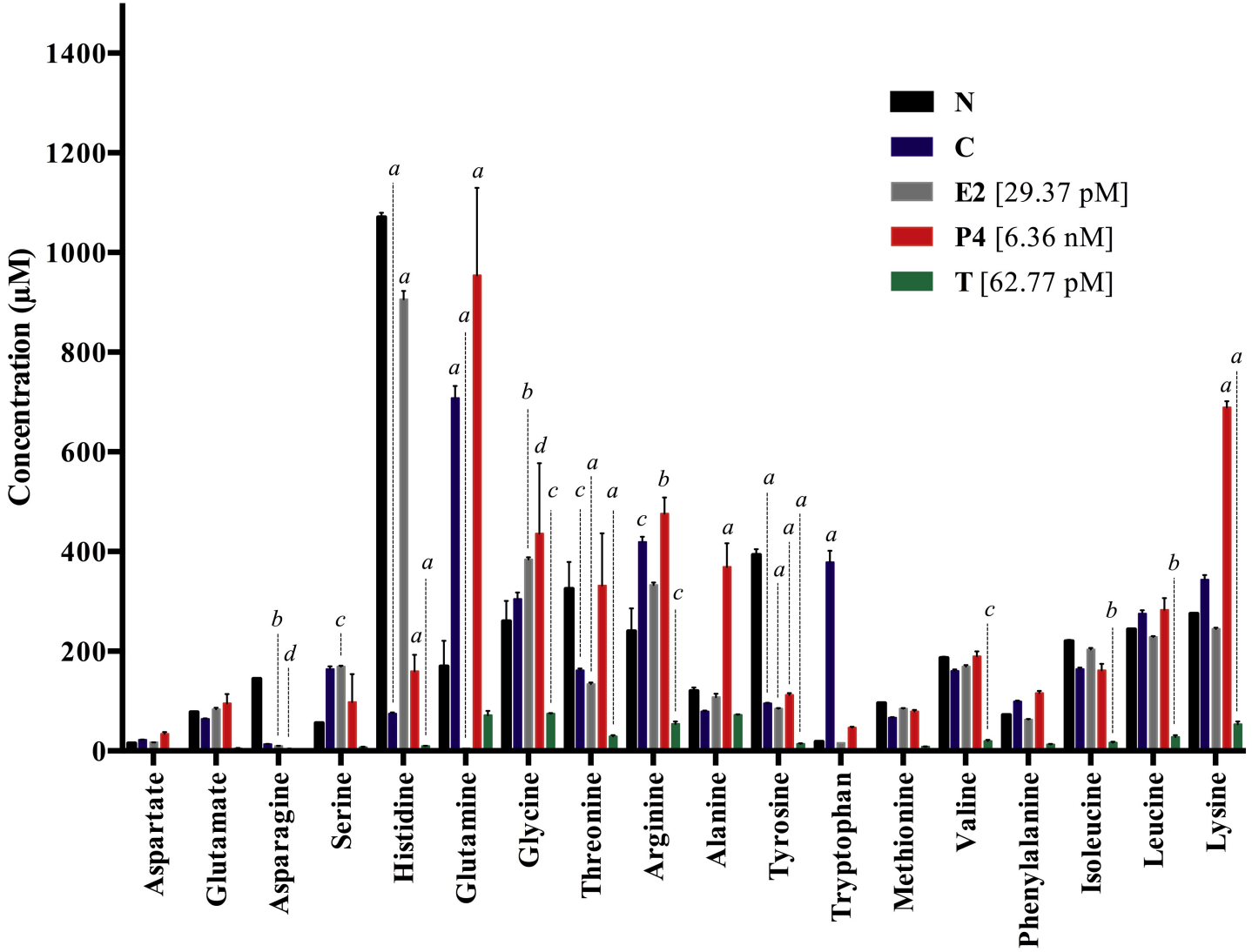

Figure 3-10 — The amino acid composition of *iv*DOF accumulated apically from untreated [native (**N**)] BOECs (n=12 ± SD) *vs* that of the culture medium [control (**C**)] supplied basally (n=3 ± SD) *vs iv*DOF derived from BOECs basally supplemented with 29.37 pM 17β-Oestradiol (**E2**) (n=6 ± SD) *vs iv*DOF from BOECs treated with 6.36 nM Progesterone (**P4**) (n=4 ± SD) *vs iv*DOF from BOECs basally supplemented with 62.77 pM Testosterone (**T**) (n=3 ± SD); *iv*DOF accumulated over 24 hours and statistically significant differences were determined by two-way ANOVA followed by a Holm-Sidak *post hoc* analysis where *a* = $p \leq 0.0001$, *b* = $p \leq 0.001$, *c* = $p \leq 0.01$, and *d* = $p \leq 0.05$.

3.3.3. Impact of Endocrine Stimulation on *iv*DOF

Following the assessment of native *iv*DOF the objective became to assess whether the *in vitro* oviduct preparation might be receptive to endocrine signals (hormones). As described in Section 3.2.5, BOECs were supplemented with physiological levels of 17β-oestradiol (E2), progesterone (P4), and testosterone (T) and the effects on the amino acid composition of *iv*DOF was evaluated. More specifically in response to basal supplementation with 29.37 pM E2, BOECs significantly modified the flux of 7 amino acids relative to native epithelia. Notably there was decreased apical flux of asparagine, histidine, glutamine, threonine and tyrosine. In contrast, serine and glycine were present in *iv*DOF from oestradiol treated BOECS in higher concentrations than in untreated BOECs (Figure 3-10). Basal addition of 6.36 nM progesterone (Section 3.2.5.) led to a decrease in histidine and tyrosine transport, and an increase in glutamine, glycine, arginine, alanine and lysine (Figure 3-10), and supplementation with 62.77 pM Testosterone (Section 3.2.5.) significantly decreased the concentration of 10 amino acids relative to native *iv*DOF. These were asparagine, histidine, glycine, threonine, arginine, tyrosine, valine, isoleucine, leucine and lysine. No amino acid exhibited an increased apical flux in comparison to native fluid following testosterone treatment (Figure 3-10).

3.3.4. Impact of Endocrine Stimulation on BOEC Gene Expression

Supplementation with E2 increased the gene expression of *ESR1* and *OVGP1* relative to all other treatments. E2 similarly increased the expression of *SLC38A7* whilst *SLC1A1* and *SLC6A14* expression correlated positively with T supplementation. Finally P treatment led to a rise in *SLC7A1* and *SLC38A5* gene expression whilst the vehicle control (ethanol) bore no impact on the expression of any of the genes investigated (Figure 3-11).

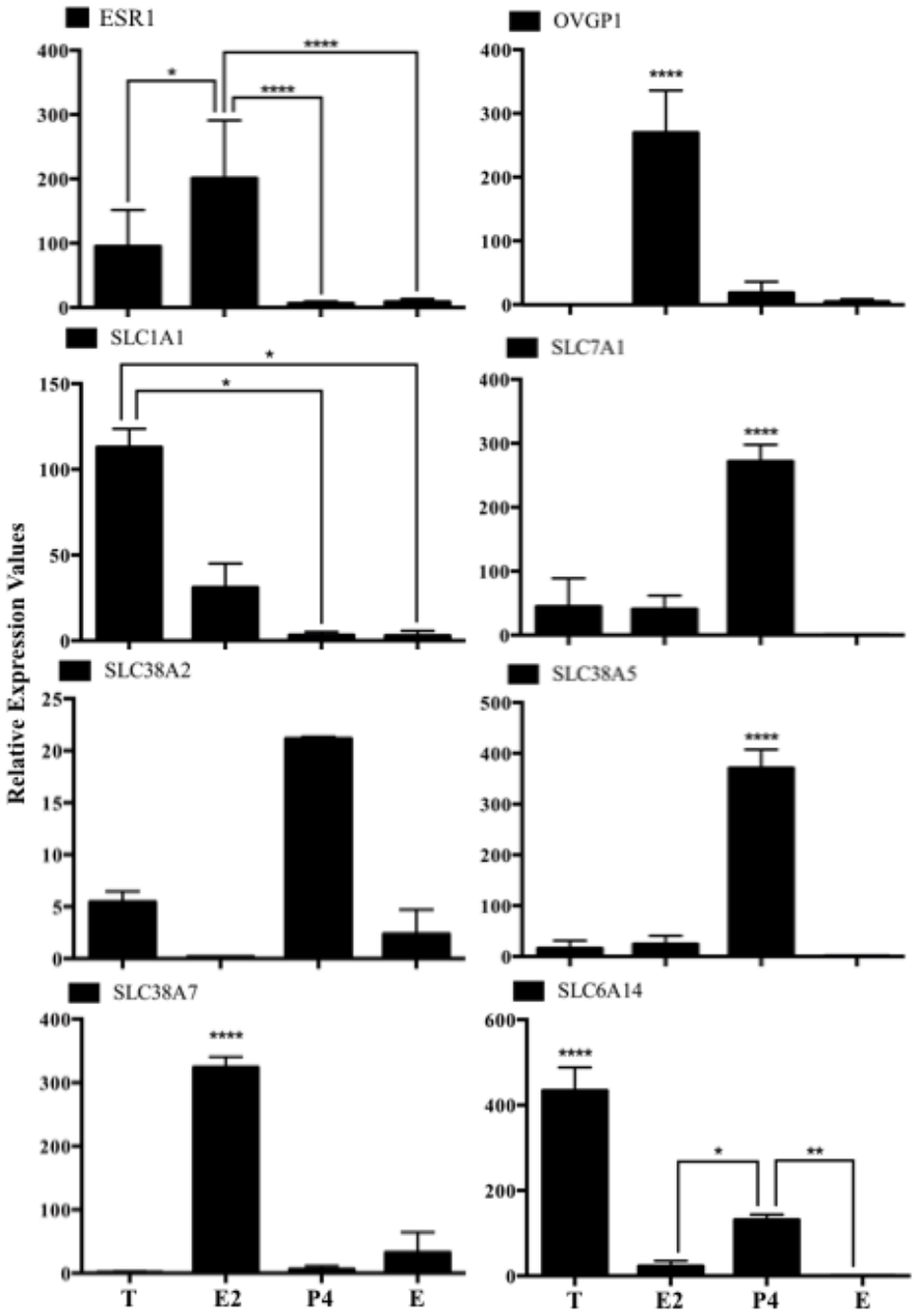

Figure 3-11 — Gene expression profiles of *oestrogen receptor a* (*ESR1*) and *oviduct specific glycoprotein 1* (*OVGP1*) in addition to solute carriers *SLC1A1*, *SLC7A1*, *SLC38A2*, *SLC38A5*, *SLC38A7* and *SLC6A14* as determined by qRT-PCR (n=3 ± SEM). BOECs were subjected to three treatment groups (***i***) 62.77 pM Testosterone (**T**) (***ii***) 29.37 pM 17β-oestradiol (**E2**), (***iii***) 6.36 nM Progesterone (**P4**) and a vehicle control of 9 µl (0.45% *v*/*v*) ethanol – all for 24 hours. All genes analysed were normalised to *β-actin* whilst the impact of treatment on gene expression was calculated relative to untreated BOECs. Statistically significant differences were determined by two way analysis of variance (ANOVA) followed by a Holm-Sidak *post hoc* where $a = p \leq 0.0001$, $b = p \leq 0.001$, $c = p \leq 0.01$, and $d = p \leq 0.05$.

3.3.5. Electrophysiology

Despite several attempts to generate electrophysiological data using BOEC monolayer cultures (Section 3.2.8.) this was not successfully achieved. However a purinergic response and membrane depolarisation curve was obtained using a whole oviduct section (Figure 3-12).

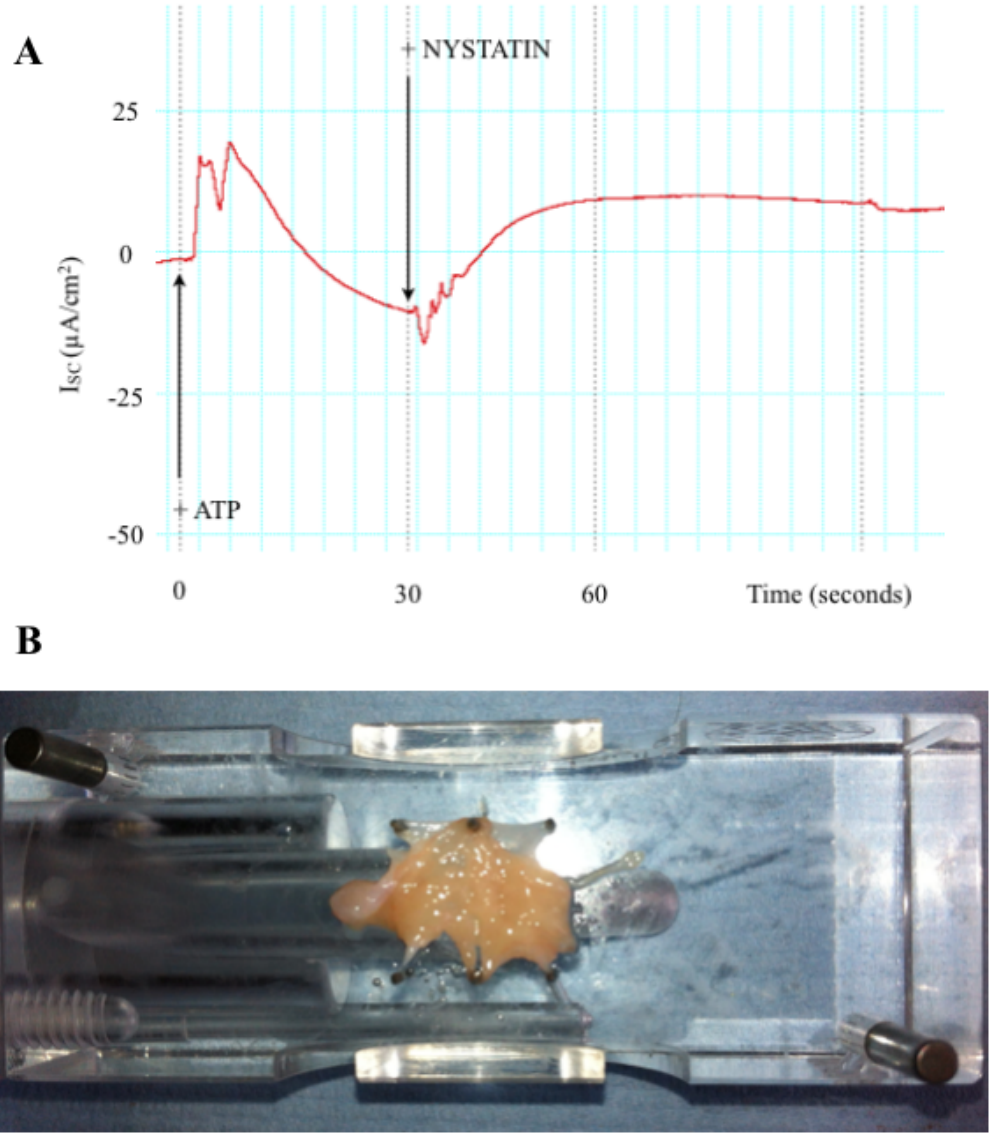

Figure 3-12 — **A**: Purinergic response (I_{SC}) of a whole oviduct segment to 100 µM ATP when the voltage was clamped to 0 mV. Subsequent supplementation with nystatin depolarised the membrane (n=1). **B**: Image of a bovine oviduct section (apical view) attached to one section of a modified Ussing chamber.

3.3.6. Additional Observations

3.3.6.1. Caco-2 Culture

As a *negative control* the protocol for isolating fluid was repeated using cell-line derived absorptive colorectal epithelial adenocarcinoma cells (Caco-2) (n=6). Upon reaching confluence and culture in an air-liquid interface for 24 hours, no fluid on the apical membrane was observed.

3.3.6.2. Transepithelial Electrochemical Resistance

As described in Section 2.4.1. Transepithelial Electrochemical Resistance (TER) was used as a measure of BOEC monolayer integrity. Cultures with TER values over 700 $\Omega \cdot cm^{-2}$ were considered confluent. TER was measured before and after all experiments and did not drop below 700 $\Omega \cdot cm^{-2}$ during any experiments pertaining to *iv*DOF collection and analysis in this Chapter.

3.3.6.3. Insulin

As described in Section 3.2.5. and Table 3-4 BOECs were supplemented with a physiological dose of insulin for 24 hours, the result of which was the lack of accumulation of any apical fluid (*iv*DOF) (n=3).

3.3.6.4. *iv*DOF Volumes

The volume of native *iv*DOF obtained was in the region of ~10 $\mu l \cdot Transwell^{-1}$. Although not measured systematically as a dependent experimental variable in this chapter there did not appear to be any differences in the volume of *iv*DOF obtained across treatments. The subject of *iv*DOF volume is discussed in depth in Chapter 4.

3.4. Discussion

This study presents the results of a new technique for investigating the formation and composition of bovine oviduct epithelial cell secretions [*in vitro* derived oviduct fluid (*iv*DOF)] from both untreated cells and from cells exposed to physiologically relevant hormone levels. A characterisation of this cell culture system is essential prior to studying the impact of the oviduct on key events such as fertilisation and maternal-embryo communication, in addition to improving *in vitro* embryo culture.

3.4.1. Cellular Identity Characterisation

The bovine oviduct epithelial cells (BOECs) of the *in vitro* oviduct model stained positive for the epithelial cell marker cytokeratin 18 (CK18) (Figures 3-4) and morphologically showed all the characteristics of oviduct epithelia expected in great detail by transmission electron microscopy (Figure 3-5). This image obtained is moreover reassuringly similar to those previously published of the rabbit oviduct epithelium by Dickens *et al* (1993). Moreover the light microscopy (Figure 3-6) in addition to the haematoxylin and eosin stained images (Figure 3-7) resemble bovine oviduct epithelia previously imaged by van Langendockt *et al* (1995), Abe & Hoshi (1997), Lee *et al* (2013), and human oviduct epithelial cells in Karst & Drapkin (2012) — in plastic culture (Figure 3-6), glass slide culture (Figure 3-7), and Transwell™ membrane culture (Figure 3-7). Perhaps the most reassuring indication of cellular identity however comes from the confirmation of OVGP1 protein in *iv*DOF (Figure 3-8:B). This is a specific marker of an oviduct epithelial cell owing to its cell type specificity and functional roles (discussed in Section 1.6.4.1.). BOECs moreover expressed the characteristic and oestrogen responsive genes *ESR1* (ERα) and *OVGP1* (Figure 3-11).

Building from this system validation was a more comprehensive analysis of the composition of *iv*DOF. The following section discusses these findings with a particular emphasis on comparing and contrasting these *in vitro* findings with what has been previously reported *in vivo*.

3.4.2. *In vitro vs in vivo*

3.4.2.1. Oviduct Specific Glycoprotein 1

Interestingly OVGP1 from fresh oviduct lysates (*in vivo* derived) (Figure 3-8:A) was located predominantly around the 80-90 kDa region consistent with previous findings (Boice *et al* 1990) and substantiating antibody specificity. However the OVGP1 secreted *in vitro* was ~60 kDa suggestive of the de-glycosylated form (Sendai *et al* 1994).

OVGP1 is secreted by granule exocytosis by oviduct epithelia and is presumed to aid sperm binding (McNutt *et al* 1992) and subsequent fertilisation (Gandolfi 1995). OVGP1 secretion *in vivo* correlates with cellular differentiation status in line with the stage of the oestrous cycle (Verhage *et al* 1998). OVGP1 secretion is increased during the follicular (oestradiol dominant) phase of the oestrous cycle, wherein the epithelia undergo hypertrophy. In contrast OVGP1 secretion decreases during the luteal (progesterone dominant) phase (Abe *et al* 1995; Abe 1996). A similar finding was observed in the *in vitro* model; *OVGP1* gene expression is significantly up-regulated in response to 17β-oestradiol (Figure 3-11), as is the case in the human (Briton-Jones *et al* 2004). Since OVGP1 is present in native *iv*DOF — *i.e.* from epithelia not exposed to any hormonal stimulus, it would be interesting to determine whether OVGP1 protein in *iv*DOF is influenced by hormonal supplementation, and whether 17β-oestradiol leads to OVGP1 glycosylation.

3.4.2.2. Ions and Osmolarity

Hugentobler *et al* (2007a) investigated the ionic composition of the oviduct by direct sampling from anaesthetised heifers during different stages of the oestrous cycle and reported concentrations of **Na^+** of 140 - 160 mM (*vs* 113 - 159 mM in *iv*DOF), **K^+** of 6-7 mM (*vs.* <1.0 mM - 3.3 mM in *iv*DOF), **Cl^-** of 80 - 100 mM (*vs* 92 - 157 mM in *iv*DOF), and **Ca^{2+}** of 3 - 4 mM (*vs* <0.5 mM in *iv*DOF). *In vivo* **HCO_3^-** data is unavailable.

The ionic composition of *iv*DOF analysis (Table 3-5) was cut short by our collaborators (n=1). Therefore the values for the ionic composition of *iv*DOF must be treated with caution. In spite of this it seems that the *in vivo vs in vitro* composition of Na^+ and Cl^- are broadly similar — unlike K^+ and Ca^{2+} which appear to be present in far lower concentrations *in vitro*.

Although definitive conclusions about changes in the ionic composition of *iv*DOF following BOEC hormonal supplementation cannot be reached it is interesting to compare this *iv*DOF data to that previously obtained from *in vivo* oviduct fluid sampling (Section 1.6.1.1.).

Osmolarity, a solute phenomenon to which embryos are sensitive (Baltz & Tartia 2010), was also measured in native ivDOF, and found to be 297 ± 12 mOsm (n=3 ± SD). The osmolarity of *in vivo* oviduct fluid has not been measured in the bovine but Li *et al* (2007) reported 314.75 ± 18.83 mOsm (n=4 ± SD) in the porcine. Moreover protocols for *in vitro* cattle embryo production state that synthetic oviduct fluid (SOF) should have an osmolarity between 270 - 300 mOsm (Sirard & Coenen 2006) — which is reassuringly within the range observed of native *iv*DOF.

3.4.2.3. Glucose, Lactate, and Pyruvate

Glucose, lactate and pyruvate are metabolites of paramount importance to both oviduct and embryo. Consequently it seemed logical to continue profiling *iv*DOF by measuring its glucose (G), lactate (L), and pyruvate (P) concentrations and to compare these findings (Figure 3-9:B) with previously reported *in vivo* data.

Similarly to Hugentobler *et al* (2007a), Hugentobler *et al* (2008) investigated the GLP composition of the bovine oviduct and reported concentrations of **G** of 3.17 ± 0.683 mM (n=7 ± SEM) [*vs* 4.30 ± 1.182 mM (n=4 ± SEM) in *iv*DOF]; **L** of 5.35 ± 0.888 mM (n=7 ± SEM) [*vs* 4.70 ± 0.681 mM (n=3 ± SEM) in *iv*DOF], and **P** of 0.11 ± 0.011 mM (n=7 ± SEM) [*vs* 0.83 ± 0.34 mM (n=3 ± SEM) in *iv*DOF]. Although glucose and pyruvate were 1.13 mM and 0.72 mM higher in *iv*DOF (respectively) and lactate 0.65 mM lower *in vitro*, multiple t-tests between *in vivo* and *in vitro* data for each metabolite showed no significant difference between the two groups.

3.4.2.4. Amino Acids

Much like glucose, lactate, and pyruvate, the overall amino acid composition of *iv*DOF was similar to that reported *in vivo* in Hugentobler *et al* (2007b) (Table 3-1). The general scale of the essential (E), non-essential (NE), and conditionally essential (CE) amino acid landscapes showed mean concentrations around the 200 µM region and maximums typically not exceeding 800 µM. Hugentobler *et al* (2007b) also showed that asparagine (CE), tyrosine (CE), and lysine (E) are present in the *in vivo* oviduct lumen in significantly higher concentrations than the plasma, much like observed in Figure 3-10. The native *in vitro* oviduct epithelium moreover apically secreted histidine (E) and threonine (E) in higher levels than provided basally.

3.4.3. Additional Observations

From a model characterisation perspective, the fact that apical accumulation of fluid was not detected in Caco-2 cells, an absorptive epithelium grown on Transwell™ membranes (Section 3.3.6.1.), provides confidence in the experimental protocol employed in addition to the secretory nature of the oviduct cells used in the *in vitro* oviduct model.

In addition to the fact that the *in vitro* oviduct secreted a fluid biochemically distinct to the culture medium provided basally (Figure 3-10), OVGP1 presence apically but not basally (Figure 3-8), and the fact that TER did not drop below the confluence threshold of 700 $\Omega \cdot cm^{-2}$ for the duration of the experiments conducted (Section 3.3.6.2.), strongly suggest that *iv*DOF is more than culture medium leak through.

An interesting additional observation was that insulin supplementation abrogated the formation of any fluid. Although such an insulin induced dry phenotype has yet to be published, two studies have established a link between insulin signalling and sodium transport. Deachapunya *et al* (1999) showed in porcine endometrial cells that insulin directly activates phosphatidylinositol-3-kinase (PI3K) which indirectly leads to the phosphorylation of the Na^+-K^+ ATPase α subunit thus increasing its affinity for Na^+ and resulting in increased Na^+-K^+ pump activity. The result of insulin supplementation in this case was therefore increased transepithelial sodium flux in an apical to basal direction. Wang *et al* (2001) also showed that insulin binding to its receptor activates the PI3K intracellular pathway. However the latter study showed that insulin acted by indirectly activating the epithelial Na^+ channel (ENaC) via serine-threonine kinase (SKG) in normal *Xenopus laevis* A6 kidney epithelial cells.

It therefore seems plausible to assume that the dry phenotype observed in the *in vitro* oviduct model could be by virtue of ENaC activation which would increase sodium import thus inhibiting Cl^- secretion, resulting in an intracellular accumulation of NaCl osmotically driving water in a basal direction (Figure 3-13).

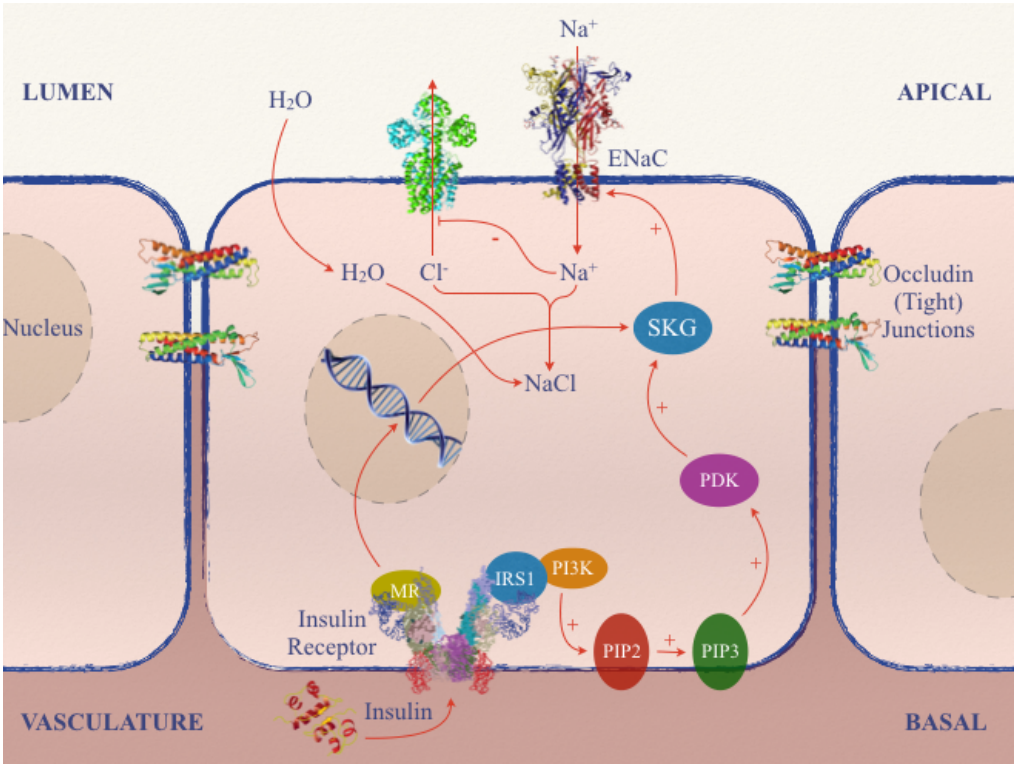

Figure 3-13 — Proposed mechanism for the dry oviduct phenotype observed following basal insulin supplementation involving the mineralocorticoid receptor (MR), insulin receptor substrate 1 (IRS1), phosphatidylinositol-3-kinase, (PI3K), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 3,4,5-triphosphate (PIP3), phosphatidylinositol dependent kinase (PDK), serine-threonine kinase (SKG), and epithelial Na^+ channel (ENaC), adapted from (Wang *et al* 2001).

Another observation was *iv*DOF volumes. Volume was not measured as an experimental variable in these experiments, however the volume of native *iv*DOF obtained during this set of experiments (with the exception of insulin supplementation) was in the region of ~10 µl·Transwell^{-1}. As single measurements of *iv*DOF at a 24 hour interval were made kinetic analyses are not possible. However if the 'rate' is loosely defined as 10 µl·24 hours^{-1} or 10 µl·1440 mins^{-1} or

0.007 µl·min^{-1} then this correlates poorly with the previously observed mean rate of *in vivo* bovine oviduct fluid formation of 1.505 ± 0.291 µl·min^{-1} (Hugentobler *et al* 2008). There are several potential explanations for this discrepancy which are discussed in more detail in Chapter 4.

3.4.4. Functional Assays and Analysis: Amino Acid Composition of *iv*DOF

Building on the confirmation of cellular identity and analysis of *iv*DOF composition, functional assays were performed to test the responsiveness of the *in vitro* epithelium to external parameters. Hormones were selected as independent experimental variables whilst amino acids were chosen as a dependent variable because of the recognised sensitivity of the oviduct to endocrine stimuli and the existing analyses of the amino acid composition of the *in vivo* oviduct (Table 3-1).

As previously discussed in Section 3.4.2.4. the amino acid profile of native *iv*DOF was comparable to previously published *in vivo* data. However the three notable exceptions to this trend were glutamine, histidine, and glycine.

3.4.4.1. Glutamine

Glutamine (CE) provides an interesting example of using the *in vitro* preparation to study oviduct fluid formation and regulation. It was present in the culture medium at 707.6 µM and native *iv*DOF at 170.0 µM (Figure 3-10) which is in line with previously reported *in vivo* oviduct luminal measurements between 143.0 - 209.7 µM (Table 3-1), even though the glutamine was the most abundant amino acid in the culture medium and present at a substantially higher concentration than the ~200 µM reported in physiological *in vivo* bovine plasma.

Intriguingly, 17β-oestradiol drastically reduced the glutamine concentration, from

170.0 μM (native) to 5.3 μM whilst testosterone had no significant impact (Figure 3-10) and progesterone treatment markedly increased glutamine content in *iv*DOF to 953.5 μM (Figure 3-10). One interpretation of these dramatic effects of 17β-oestradiol and progesterone towards glutamine secretion is in terms of the role of glutamine in the preimplantation embryo.

Glutamine improves preimplantation mouse embryo development *in vitro* (Rezk *et al* 2004; Smith & Sturmey 2013) hence it is unsurprising that progesterone, the levels of which rise in the luteal phase of the cycle, should result in elevated oviduct glutamine output. Although not the case in the bovine (Hugentobler *et al* 2007b), glutamine is the most abundant amino acid in human plasma (Lee *et al* 1998) and is a known primary embryo nutrient (Rieger *et al* 1992). It has also been observed that embryos cultured *in vitro* take up significantly less glutamine than those derived *in vivo* (van Winkle & Dickinson 1995).

Morbeck *et al* (2014) analysed the composition of commercial embryo culture media and reported that 9 of the 12 did not contain any glutamine, although the likelihood is that these media are supplemented with the dipeptide alanyl-glutamine (glutamax) which is less labile than glutamine. Two contained lower than physiological concentrations (26 μM and 30 μM) leaving only one (Origio ISM1) which contained glutamine at a concentration (778 μM) comparable to that observed in *iv*DOF from progesterone treated cells (953.5 μM). In the same study the majority of embryos cultured in Origio ISM1 arrested at the 2 and 4 cell stage (Morbeck *et al* 2014), however it is unknown whether glutamine was a factor. Nonetheless, these findings highlight the potential of the model oviduct preparation in understanding the dynamic periconceptual environment and in optimising embryo culture media.

3.4.4.2. Histidine

Histidine was perhaps the most striking amino acid as it was present in native *iv*DOF (1071.1 μM) at ~14.4 times the concentration in the culture medium provided basally (74.4 μM) (Figure 3-10) and at ~10 times the concentrations observed *in vivo* (Table 3-1). This unexpected observation raises two questions: (***i***) why the oviduct might secrete histidine in such amounts, and (***ii***) what are the origins of this essential amino acid.

Very little has been published on the role of histidine in early embryo development and it is therefore difficult to speculate as to why the *in vitro* oviduct behaves in this way. Professor John Cleland (Imperial College London) suggested an intriguing possibility for Figure 3-10: that histidine, as a positively charged imidazole, could act as an *iv*DOF pH buffer. The *in situ* bovine oviduct pH is 7.6 (Hugentobler *et al* 2004) whereas the BOECs were cultured at ~ pH 7.4. The case could therefore be that the native bovine oviduct epithelium secretes histidine *en masse* in an attempt to balance *iv*DOF pH.

Conventional biological buffers include bicarbonate ions [(HCO_3^-) alkaline] and carbon dioxide [(CO_2) acidic]; the latter of which remained constant *in vitro* at 5% (Section 2.3.4). HCO_3^- however was measured in native *iv*DOF at 10 mM (Table 3-5) which within a clean mixture would correspond to a pH of ~ 7.2 (using the Henderson Hasselbalch equation assuming 5% CO_2 = 40 mmHg pCO_2 and 10 mM HCO_3^- = 10 meq·L^{-1} HCO_3^-). This further supports the notion that histidine (with an amine pKa of 9.17) secretion might be a pH balancing mechanism. Moreover, although typically at the whole protein level, the regulation of pH by histidine is not uncommon (Borza & Morgan 1998; Williamson *et al* 2015; Sasaki *et al* 2015).

Another reason for the observed vast histidine secretion in native *iv*DOF could be as an *essential* amino acid for the developing embryo, despite only being present *in vivo* in significantly lower amounts (Table 3-1) and being only entirely present in 9 of the 12 embryo culture media analysed by Morbeck *et al* (2014).

The second question of the origins of histidine, an essential amino acid, is also rather important; although histidine has been shown to be consistently present in the *in vivo* oviduct lumen in higher levels than in the bloodstream, similar to Figure 3-10. This was also the case for other essential amino acids including threonine, methionine, tryptophan, valine, phenylalanine, isoleucine, leucine, and lysine (Hugentobler *et al* 2007b).

It should be noted that some discrepancies in the literature exist with some claiming that histidine is conditionally essential (Son *et al* 2005). Regardless this thesis takes the conventional view that histidine is essential and therefore cannot be readily synthesised *de novo*. Hence the origin of histidine in *iv*DOF might result from a high protein degradation rate with histidine remaining unincorporated in protein syntheses and/or metabolic pathways, resulting in a large free histidine amino acid pool coupled with the preferential transport of histidine across the monolayer. Another possibility could be the presence of histatins which are histidine-rich proteins usually found in parotid salivary cells and associated with anti-fungal properties (Oppenheim *et al* 1988; Kavanagh & Dowd 2010). This explanation however is unlikely as histatins have neither been identified in oviduct epithelia nor have been described as histidine reservoirs.

From a functional assay perspective, in response to endocrine stimuli, BOEC treatment with 17β-oestradiol brought the histidine content of *iv*DOF down slightly to 905.8 μM (Figure 3-10) whereas progesterone administration further decreased the concentration of histidine in *iv*DOF to 159.3 μM (Figure 3-10) —

more in line with concentrations observed *in vivo*. Interestingly, testosterone almost completely inhibited histidine secretion from 1071.1 μM to 9.7 μM (Figure 3-10). Thus histidine flux also appears to be subject to endocrine regulation.

It should be noted that the possibility of a technical error cannot be excluded even if the robustness of the method for amino acid quantification by HPLC has been established (Figure 2-5).

3.4.4.3. Glycine

Glycine (CE) tends to be present in the oviduct at the highest concentration of all the amino acids *in vivo* (Guerin *et al* 1995; Engle *et al* 1984; Moses *et al* 1997; Menezo & Laviolette 1971). This is unsurprising given the observations that glycine addition alone in culture improves embryo development (Lee & Fukui 1996; Baltz 2013).

Hugentobler *et al* (2007b) reported glycine concentrations of around 1550 μM in the *in vivo* bovine oviduct lumen whereas the present native *in vitro* value was almost five times lower; in the 300 μM region. However, treatment with 17β-oestradiol (Figure 3-10) and progesterone (Figure 3-10) significantly increased the concentration of luminal glycine relative to the native value. In contrast, testosterone supplementation (Figure 3-10) significantly decreased luminal glycine concentration. Although 17β-oestradiol and progesterone supplementation did not increase glycine secretion to levels comparable with the *in vivo* observations, it did appear that glycine flux was modulated, in part, hormonally. The following section investigates whether these phenomena might be regulated at the transcriptional level.

3.4.5. Functional Assays: Impact of Endocrine Stimulation on Gene Expression

Six solute carrier (amino acid transporter) genes were selected to discover the influence of endocrine stimulus on expression (Figure 3-11) in addition to contextualising the changes in the actual flux of amino acids (Section 3.1.1.1.).

SLC1A1 encodes the high affinity L-aspartate excitatory amino acid co-transporter 3 (EAAC3) and is known to respond positively to testosterone (Franklin *et al* 2006) as also observed in the *in vitro* model (Figure 3-11). *SLC1A1* did not respond to progesterone *in vitro*, in line with the observation that *SLC1A1* expression decreases in the bovine uterus during the progesterone dependent phase (day 16-20) of ruminant pregnancy (Forde *et al* 2014). Moreover *SLC1A1* which encodes a System X^-_{AG} (apically importing) transporter was significantly up-regulated following testosterone supplementation (Figure 3-11). It is unsurprising therefore that the apical accumulation of aspartate decreased to 0.61 μM from 15.2 μM (native) (Figure 3-10).

SLC7A1 encodes the arginine and lysine specific cationic amino acid transporter 1 (CAT1) (Broer 2008) whose expression positively correlated with progesterone *in vitro* (Figure 3-11), a result in agreement with *in vivo* data showing *SLC7A1* expression peaking between days 16-20 in the sheep endometrial epithelium (Gao *et al* 2009). SLC7A1 is a predominantly basally located (γ^+L) transporter hence although progesterone increased both *SLC7A1* expression *and* arginine and lysine flux (Figure 3-10), it is unlikely this was SLC7A1 directly mediated. Indirectly however it could be the case that an SLC7A1 mediated intracellular accumulation of arginine and lysine led to or even induced the subsequent arginine and lysine flux.

SLC38A2 encodes the sodium coupled neutral amino acid transporter 2 (SNAT2) protein which is ubiquitously found at the apical and basal membranes of kidney and intestinal epithelial cells (Broer 2008) and is responsible for shuttling several amino acids. The relative expression of *SNAT2*, although increased upon progesterone exposure (Figure 3-11), was still too low for any meaningful conclusions to be drawn.

SLC38A5 is the gene for SNAT5 which is the System A low affinity transporter for glutamine, asparagine, histidine, serine, alanine, and glycine (Broer 2008) and a known pH regulator (Baird *et al* 2006). Progesterone up-regulated the *SLC38A5* gene *in vitro* (Figure 3-11) however there are no data currently available reporting any hormonal response to *SNAT5* expression. SNAT5 is basally located and shuttles amino acids extracellularly. In spite of this alanine and glycine transport into the *in vitro* lumen increased, in line with *SLC38A5* expression, in response to progesterone. Histidine flux on the other hand was greatly reduced (Figure 3-10).

SLC38A7 similarly encodes SNAT7; a basally located System A transporter (Figure 3-1) for moving glutamine extracellularly (Hagglund *et al* 2011). *SLC38A7* was significantly up-regulated in response to 17β-oestradiol (Figure 3-11) and glutamine flux also decreased in *iv*DOF from BOECs subjected to 17β-oestradiol (Figure 3-10).

SLC6A14 expression is testosterone responsive and encodes the sodium-dependent neutral amino acid transporter protein (Broer 2008) predominantly responsible for the apical import of tryptophan (Noveski *et al* 2014). Although expression was consistent between the literature and the *in vitro* model (Figure 3-11) for *SLC6A14*, there was no significant difference in tryptophan transport following testosterone supplementation (Figure 3-10).

3.4.6. Summary

This chapter has both attempted to characterise a novel *in vitro* model of the oviduct epithelium and examine the functional impact of three hormones on the properties of the *in vitro* oviduct epithelium; in terms of the amino acid composition of *iv*DOF and gene expression profiling of some relevant solute carrier genes.The cellular identity of BOECs was confirmed by FACS, TEM, and light microscopy in addition to OVGP1 gene expression and protein presence in *iv*DOF. Furthermore the osmolarity, glucose, lactate, pyruvate, and amino acid composition of *iv*DOF correlated well with what has been previously reported *in vivo*.

From a functional standpoint, amino acid transporter gene expression generally responded as predicted in response to endocrine stimuli (*SLC1A1*, *SLC7A1*, *SLC38A2*, and *SLC38A7*). However the correlation between the expression of amino acid transporters and the actual movement of their respective amino acids in *iv*DOF is difficult to establish from Figure 3-10 alone. It is tempting to suggest that regulation of amino acid transport does not occur at a transcription level however this is not a feasible conclusion until the expression of all (apical and basal) SLC proteins (Table 3-2) pertinent to amino acid flux are investigated.

Moreover, to assess whether or not amino acid flux is regulated at the transcriptional level, protein immuno-blotting for the transporters (protein concentration and localisation to membranes), correcting amino acid concentrations for transporter flux kinetics, in addition to titrating the basal concentrations of amino acids and concentration of hormones added basally, would need to be conducted. It is however improbable that amino acid flux is entirely regulated at the transcriptional level given the range of protein-level regulation systems discussed in Section 3.1.1.1.

Together these data provide evidence for the physiological nature of the *in vitro* oviduct model and highlight its value in examining the mechanisms evolved by the oviduct to modulate its secretions. Limitations of the model and detailed future research questions are addressed in Chapter 6 whilst Chapter 4 uses the *in vitro* oviduct to model hyperandrogenaemia within the context of polycystic ovary syndrome (PCOS).

Chapter 4

Expanding the *in vitro* Model

Chapter 4 — Expanding the *in vitro* model

4.1. Introduction

The model presented in Chapter 3 enables novel investigations to be made into the responses of the oviduct epithelium to a plethora of stimuli. This chapter builds on these findings to investigate the impact of pathophysiological endocrine stimulation and pharmacological agent supplementation on BOEC secretion composition and cellular physiology. The barrier properties of the *in vitro* oviduct are moreover tested against carnitine, and the effects of the addition of a stromal cell component in terms of underlying fibroblasts (dual culture) to the epithelium are also examined.

4.1.1. Endocrine Effects

A primary component of impaired fertility is hormonal (Unuane *et al* 2011). With this positioning in mind the following builds on Sections1.8 and 1.9. and outlines some key aspects of pathological reproductive endocrinology.

4.1.1.1. Polycystic Ovary Syndrome

An association between hyperandrogenism and diabetes was first described by Achard & Thiers (1921) although the polycystic ovary (PCO) phenotype was not physically and clinically characterised until Stein & Leventhal (1935). Scientific interest in the associated hypothalamic-pituitary-ovarian axis imbalances in PCO women subsequently arose following the finding of increased serum LH and elevated LH:FSH in such females (Rebar *et al* 1976) (Figure 1-2 *vs* Figure 4-1) in addition to the description of a link between hyperandrogenism and hyperinsulinemia (Burghen *et al* 1980).

Technological advances enabled Swanson *et al* (1981) and Adams *et al* (1985) to better describe and diagnose *Stein-Leventhal Syndrome* by ultrasound. These events led to a characterisation of Polycystic Ovary Syndrome (PCOS) by the United States National Institute of Health (NIH) in 1990, however an international consensus on a definition of this complex syndrome was not formally decided until 2003 in Rotterdam. Diagnostic indicators of PCOS are thus known as The Rotterdam Criteria and are — following the exclusion of related pathologies such as Cushing's syndrome, hyperprolactinemia, and congenital adrenal hyperplasia — a female fulfilling two of the following three criteria: (*i*) oligoovulation or anovulation, (*ii*) clinical or biochemical signs of hyperandrogenism and (*iii*) polycystic ovaries (Azziz 2006).

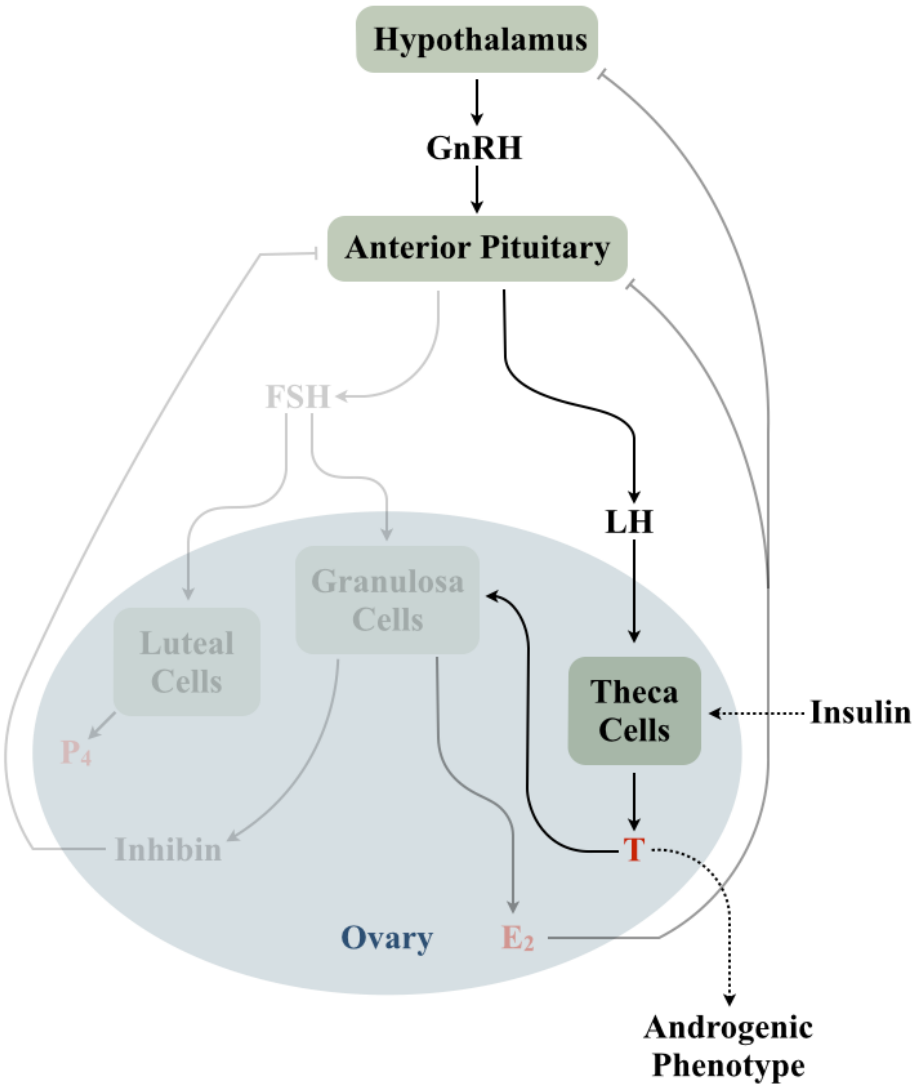

Figure 4-1 — Proposed mechanism of PCOS-induced hyperandrogenism wherein a defect in the granulosa aromatase enzyme responsible for the conversion of testosterone to E2 causes the accumulation of testosterone which is further accentuated by hyperinsulinemia. Increased T:E2 leads to a vicious cycle wherein negative feedback is perturbed (adapted from Doi *et al* 2005). Although not shown in this diagram, the problem is exacerbated by excess androgen secretion from the ovaries which passes to peripheral tissues, such as adipose tissue, which expresses functional aromatase. High concentrations of E2 are therefore produced causing waves of abnormal hypothalamic GnRH inhibition, whilst LH in circulation remains high. This mechanism of hypothalamic-ovarian axis imbalance is a dominant theory for observed hyperandrogenemia in PCOS states (Doi *et al* 2005).

As a consequence of the underlying presentation of PCOS, 74% of sufferers are infertile, 29% exhibit dysfunctional menstrual bleeding, 51% amenorrhoea, 69% hyperandrogenism, 21% virilisation, 65% are obese, and 60% display obesity-

independent insulin resistance (DeLeo *et al* 2003; Ehrmann *et al* 1999; Dunaif *et al* 1989; Chang *et al* 1983; Ciaraldi *et al* 1992; O'Driscoll *et al* 1994; Legro *et al* 2004; Sheehan 2004; Garad *et al* 2011).

PCOS is now recognised as the most common endocrine disorder in women of reproductive age (Yildiz *et al* 2004) affecting approximately 8% of the global female population. Of particular interest to this project is the subfertile and infertile pathology of females with PCOS. Given the broad diagnosis of PCOS, reflective of the complex phenotype, several mechanisms are likely to be responsible for PCOS related subfertility; a notion elaborated upon in Chapter 7.

In summary, endocrine imbalances like hyperandrogenism can disrupt the oestrous and menstrual cycles in addition to presenting other pathological symptoms . Oviduct epithelia expresses receptors for E2, P4 and T (Kawashima *et al* 1996) (Chapter 3); it is therefore plausible to postulate that the oviduct too is susceptible to endocrine imbalances. For this reason hyperandrogenism was mimicked the *in vitro* bovine oviduct model described in Chapter 3 with the aim of evaluating the extent to which this pathophysiology affects oviduct epithelial cell physiology and secretion composition.

4.1.1.2. Female Androgen Deficiency Syndrome

On the other end of the T spectrum lies the female androgen deficiency syndrome (FADS) or hypoandrogenism. FADS has been characterised to a far lesser extent than hyperandrogenism despite the significant pathological consequences (Bachmann 2002).

FADS is associated with women who are naturally or surgically menopausal (Bachmann 2001) whilst chemotherapy and radiotherapy have also been shown to

cause androgen deficiency even post-treatment (Bachmann 2002). It has moreover been hypothesised that oral contraceptives may lead to FADS since low-androgenic progestin and oestrogen replacement therapies reduce free T in hyperandrogenic females (Derman 1995). However FADS also presents itself idiopathically (Bachmann 2002).

Whichever the aetiology, symptoms of FADS include a reduction in bone and muscle mass, and increased incidences of fatigue, insomnia, headaches, and depression (Bachmann 2002). Reproductive issues associated with FADS include diminished sexual motivation, fantasy, enjoyment and arousal, reduced vaginal vasocongestion (Bachmann 2002), and a diminished functional ovarian reserve (Gleicher *et al* 2013) presumably owing to an inherent decrease in stromal androgen production.

The impact of FADS on the oviduct has not been investigated. From a clinical perspective one would think *for good reason* since FADS has not been directly linked to impaired fertility. However diabetes has; and one study in the rat has shown that induced diabetes caused hypoandrogenism (Anderson *et al* 1987). Therefore it seemed worth exploring the link highlighted by Figure 4-2 — specifically the research question: could FADS blunt oviduct epithelial function?

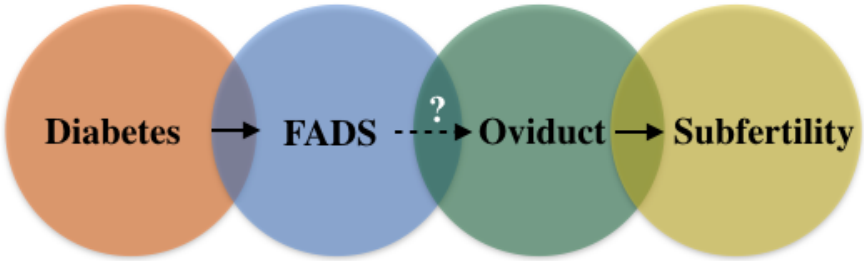

Figure 4-2 — Potential macro-mechanism underpinning diabetes and subfertility in some women.

4.1.2. Pharmacology

In addition to investigating the impact of pathophysiological endocrine states on the oviduct, the *in vitro* model lends itself to examining the effects of pharmacological compounds. The influence of three drugs (metformin, clomifene, and diclofenac) on the amino acid composition of *iv*DOF was explored.

4.1.2.1. Metformin

Metformin (Figure 4-3:A) is commonly administered to type II diabetics and in the treatment of PCOS (Zhai *et al* 2012). Metformin is primarily effective because it decreases hepatic glucose production whilst increases gluconeogenesis in peripheral tissues (Viollet *et al* 2012). As discussed above insulin resistance a common feature in PCOS females however the role of metformin in alleviating the symptoms of PCOS are more complex. Metformin reduces ovarian testosterone synthesis by directly suppressing androstenedione production (Figure 1-3) and decreasing aromatase activity in ovarian theca cells (Palomba *et al* 2009). Although the detailed biochemical mechanisms underlying metformin action remain unclear (Viollet *et al* 2012), owing to its widespread prescription in females already predisposed to a subfertile phenotype (PCOS) it seemed

worthwhile to test the impact of this drug on the amino acid composition of *iv*DOF.

4.1.2.2. Clomifene

Clomifene (Figure 4-3:B) was of interest as it is a non-steroidal selective oestrogen receptor modulator (SERM) and commonly administered to females with PCOS (Legro *et al* 2007). It is most commonly prescribed since it has been shown to induce ovulation in 60-85% of cases studied (Kousta *et al* 1997). The detailed mechanisms of action are unclear however clomifene has been shown to elevate FSH and LH circulation (Adashi *et al* 1984). It is thought that clomifene acts by inhibiting hypothalamic E2 negative feedback of GnRH release (Kamath & George 2011). Unlike metformin, some studies looking into the impact of clomifene on the oviduct have been conducted with findings showing that clomifene causes aberrant apoptosis and activates ERα in the rat (Shao *et al* 2009), induces a burst in secretory cell activity at doses above 5 mg in rabbits (Birkenfeld *et al* 1985), and caused isthmic damage, as determined by aberrant haematoxylin and eosin staining, when supplemented at 1 mg/kg/day over 4 days in the rat (Mahdy 2014). Thus it was of interest to investigate the effects of clomifene on the amino acid composition of *iv*DOF.

4.1.2.3. Diclofenac

Diclofenac is a common non-steroidal anti-inflammatory drug (NSAID) commercially known as Voltaren™ and typically used as a painkiller in addition to inflammation reduction. It is widely used to treat pelvic pain (Downing *et al* 1999). Unlike metformin and clomifene, upon ingestion, diclofenac is metabolised to several derivatives, the most abundant of which is 4-hydroxy-diclofenac (4-OH DFC; Figure 4-3:C) (Bort *et al* 1999; Kumar *et al* 2002). The primary mechanism

of action of diclofenac and its metabolites is presumed to be via cyclooxygenase (COX) enzyme inhibition thereby repressing inflammatory prostaglandin synthesis (Hawkey 1999). A recent study showed that women taking diclofenac for moderate back pain showed a reversible reduction in ovulation rates (Sherif *et al* 2014). It is presumed this is because uterine endometrial COX-2 inhibition by diclofenac reduced PGF2α synthesis thereby impairing ovarian follicle rupture. However, despite widespread use, there is no knowledge of how diclofenac affects the oviduct milieu.

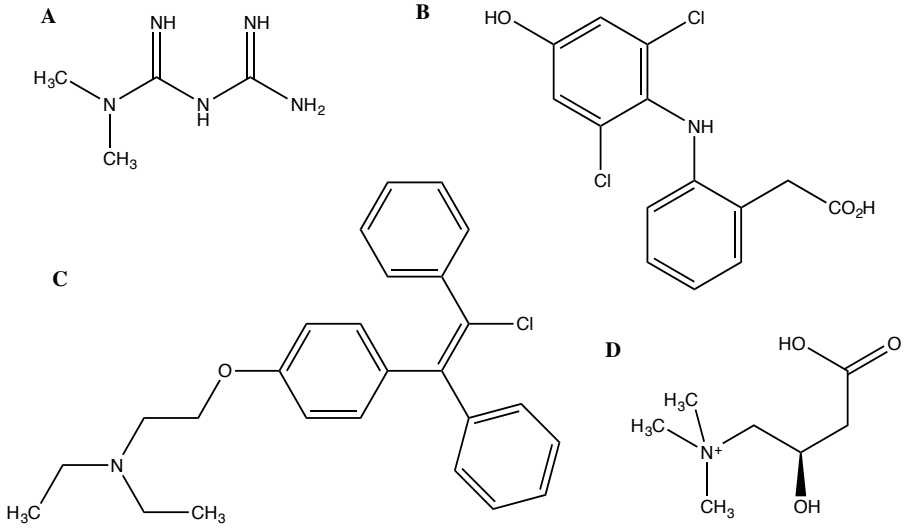

Figure 4-3 — The chemical structures of (**A**) metformin, (**B**) clomifene, (**C**) 4-hydroxy-diclofenac, and (**D**) carnitine.

4.1.3. Carnitine

One of the many functions of the oviduct is selecting which compounds can transverse into the lumen. Thus in addition to investigating the impact of pathophysiological endocrine stimuli and pharmacological agents on *iv*DOF composition the model was expanded further to test the barrier capacity of the oviduct epithelial monolayer. An ongoing project within the lab was embryo fatty acid metabolism regulation by carnitine (Figure 4-3:D) — an amino acid derivative and nutrient. However since carnitine has never been identified in oviduct fluid *in vivo* or *ex situ* it seemed logical to complement this existing work

by establishing whether or not these studies were physiologically relevant by employing the *in vitro* oviduct model to investigate the permeability of the oviduct epithelium to carnitine.

4.1.4. Dual Culture

Finally to expand the model further a multicellular model was investigated. More specifically, directly beneath the oviduct epithelium *in vivo* is the stroma (Section 1.5.3.), a biologically complex lattice of irregular connective tissue created and maintained by a dense population of fibroblast cells (Fazleabas et al 1997). Fibroblast-epithelial communication has been extensively studied in the cells of the airways across species (Parrinello *et al* 2005; Noble 2008; Woodward *et al* 1998; Srisuma *et al* 2010; Ohshima 2009; Chhetri *et al* 2012; Nishioka 2015; Knight 2001; Sakai & Tager 2013) but fibroblast-epithelial interactions have been investigated to a much lesser extent in the oviduct. In the *in vivo* murine uterine endometrium however, Field *et al* 2015 recently showed for the first time that several epithelial and stromal immune related genes were modulated *post coitum*, many of which suggest that epithelial-stromal interaction pathways are activated. One of the greatest challenges in studying the impact of BOFCs on BOECs has been the lack of an *in vitro* culture system enabling the culture of BOFCs basally adjacent to BOECs but within a separate chamber, replicating the *in vivo* environment (Figures 1-6:B and 1-6:D); coupled with a measurable output of BOEC function such as *iv*DOF.

4.1.5. Aims and Objectives

This chapter has four principal aims: 1. To investigate the impact of hypoandrogenism and hyperandrogenism on (*i*) *iv*DOF composition and volume, and (*ii*) epithelial gene expression and TER physiology; 2. To explore the effects

of three drugs (metformin, diclofenac, and clomiphene) on *iv*DOF amino acid content; 3. To evaluate whether or not carnitine crosses the epithelial monolayer, and 4. To evaluate the impact of fibroblast dual culture on *iv*DOF amino acid composition.

4.2. Materials and Methods

Bovine oviduct epithelial cell (BOEC) culture, transepithelial electrochemical resistance (TER) measurements, high performance liquid chromatography (HPLC), and quantitative real-time polymerase gene expression (qRT-PCR) were conducted as described in Sections 2.3.4, 2.4.1, 2.4.2 and 2.4.3, respectively. Moreover the experimental design described in Section 2.5 applies throughout this chapter unless otherwise stated as do the statistical analyses explained in Section 2.6. Methods specific to this Chapter are provided below.

4.2.1. Combinatorial Hormonal Supplementation

To determine the effects of *physiological*, *hypoandrogenic* and *hyperandrogenic* endocrine states on the *in vitro* model, hormone stocks were prepared in ethanol prior to supplementation to the basal Transwell™ chamber similarly to Section 3.2.3. and in accordance with Table 4-1. Hormone concentrations were selected to represent a mean physiological and maximum pathophysiological ranges about the T:E2 ratio similarly to reports by Kanchev *et al* (1976), Pastor *et al* (1998), Balen (2004), Di Sarra *et al* (2013), and O'Reilly *et al* (2014). Hormones were added to Transwell™ inserts at a maximum volume of 16.7 µl in a total of 2000 µl of culture medium [0.835% (*v*/*v*)] or 41.8 µl in a total of 5000 µl of culture medium [0.835% (*v*/*v*)] to flasks. As described in Bromberg & Klibanov (1995) and depicted in Figure 4-14 — this solvent contribution had no significant effect on of *iv*DOF composition when added alone as a vehicle control.

	17β-Oestradiol (E2)	Progesterone (P4)	Testosterone (T)
Hyperandrogenic (H)	19.46 pM	6.36 nM	6.27 nM
Physiological (P)	29.37 pM	6.36 nM	208 pM
Hypoandrogenic 1 (F1)	19.46 pM	6.36 nM	62.77 pM
Hypoandrogenic 2 (F2)	19.46 pM	6.36 nM	34.7 pM
Hypoandrogenic 3 (F3)	29.37 pM	6.36 nM	2.43 pM

Table 4-1 — Hormone concentrations added to bovine oviduct epithelia cultured on Transwell™ membranes and in flasks to mimic physiological and pathophysiological endocrine states *in vitro*.

4.2.2. Carbohydrate, Amino Acid, Gene Expression, and TER Measurements

Glucose, lactate any pyruvate analyses were conducted by fluorometric enzyme linked assays as per Section 2.4.4. Amino acids were quantified by HPLC as described in Section 2.4.2. Gene expression profiles were obtained by qRT-PCR as outlined in Section 2.4.3., and the method for measuring TER is provided in Section 2.4.1.

4.2.3. Pharmacological Supplementation

An analysis of metformin serum levels in type II diabetics showed that the drug circulated in concentrations of 7.71 μM with a median intra-individual overall coefficient of variation of 29.4% (Frid *et al* 2010). This broad range of 7.71 ± 2.3 μM is consistent across other similar studies including Kadhim *et al* (2012), Liu & Coleman (2009), and Huttunen *et al* (2009). In line with this a metformin stock of 7.8 mM was prepared by dissolving one 500 mg tablet of metformin hydrochloride in 500 ml of water thus enabling the subsequent addition of metformin to basal Transwell™ culture at a concentration of 7.75 μM and an associated aqueous vehicle contribution of less than 0.1% (*v*/*v*).

A 20 µM clomifene stock was prepared by crushing a 50 mg Clomid™ tablet and dissolving 0.0194 g into 1054 ml ethanol. Circulating concentrations of clomifene are typically in the region of 100 nM (Young *et al* 1999; Rostami-Hodjegan *et al* 2004). Thus clomifene was supplemented to epithelial cultures at a final concentration of 99 nM with an associated vehicle contribution of less than 0.5% (*v/v*).

As diclofenac is readily metabolised into several derivatives, a range of circulating concentrations for the dominant metabolite 4-hydroxy-diclofenac (4OH-DFC) have been reported (Aithal *et al* 2004; Miyatake *et al* 2009) ranging from 3.2 µM to 6.4 µM. To this end a 1.92 mM 4OH-DFC stock was prepared by dissolving 1 mg of 4OH-DFC in 1.67 ml ethanol Thus enabling Transwell™ supplementation with 4OH-DFC at both concentrations of 3.2 µM and 6.4 µM with respective solvent contributions of 0.17% and 0.33% (*v/v*).

4.2.4. Carnitine Transport and Detection

For carnitine transport experiments BOECs were apically and basally incubated with 2 ml pre-equilibrated normal Krebs Ringer medium for 20 minutes at 39°C in 5% CO2 prior to basal carnitine supplementation. Free carnitine has been reported to circulate in the vasculature in concentrations ranging between 25.8 µM - 60.0 µM (Rebouche 1992; Miyata & Shimomura 2013) thus it was decided that carnitine would added to BOEC culture at 40 µM. This was achieved by preparing a 24 mM carnitine stock in water similarly to Vilskersts *et al* (2011). The time of carnitine addition was defined as 0 minutes and 10 µl samples from both apical and basal chambers were taken at 15 minute intervals for 2 hours. TER was measured at 0 minutes [1080.3 ± 260.5 Ω·cm-2 (n=3 ± SD)] and 120 minutes [1046.23 ± 247.4 Ω·cm-2 (n=3 ± SD)] showing no sign of compromised monolayer integrity for the duration of the experiments.

Carnitine was detected indirectly by fluorometric assay similarly to the carbohydrate assays described in Section 2.4.9. The assay was performed in accordance with the manufacturer instructions (Section 2.2.) but in brief the inherent fluorescence of the clear v-bottomed 96-well plate and reaction mix (5 µl) was first detected by micro-plate reader set to 535/587 nm prior to sample addition (3 µl) and incubation for 30 minutes at 37˚C in darkness. Measurements were then repeated and the former values (background noise) were subtracted from those of the sample or standard readings. Triplicate technical and biological replicates were conducted and sample concentrations were determined a standard curve (Figure 4-7).

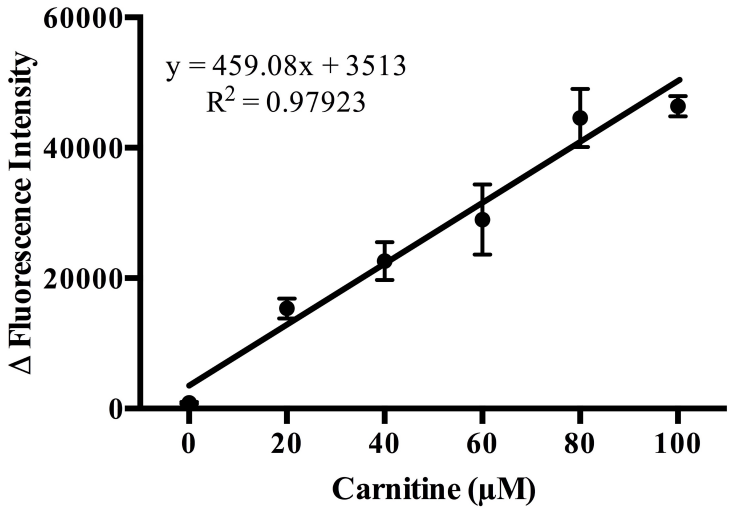

Figure 4-7 — Representative carnitine assay standard curve (n=3 ± SD).

4.2.5. Flow Assisted Cell Sorting: BOFC Confirmation

In order to confirm the stromal nature of fibroblasts isolated by differential seeding adhesion times (Section 2.3.2.) (Cronin *et al* 2012) the Flow Assisted Cell Sorting (FACS) protocol presented in Section 3.2.1. was repeated, with the difference that an ã-vimentin antibody was chosen for BOFC identification since fibroblasts stain positive for this mesenchymal biological marker (Goodpaster *et*

al 2008). Vimentin is also not present in bovine oviduct epithelia (Rottmayer *et al* 2006).

4.2.6. Dual Culture

BOECs and BOFCs were isolated based on their differential adhesion times. as described by Cronin *et al* (2012). The two cell types were initially seeded together in T75 flasks and following 18 hours of culture, BOECs (un-adhered; in suspension) were removed, leaving BOFCs adhered to the surface of the plastic.

Following BOEC and BOFC separation, the fibroblast populations filled the flask growth area after approximately 5 days of culture with media supplementation every 48 hours. At this time a T75 flask contained ~8.4x10^6 fibroblast cells; sufficient for 8 Transwell™ inserts at just over 1x10^6 fibroblasts per well. Upon filling flask growth area, fibroblast cell homogenates (T75 flask) were incubated at 37 ºC with 4 ml 10x Trypsin for 10 minutes to detach the cells.

Once fibroblasts were in suspension, as confirmed by visual microscopy, 4 ml of culture medium was added to the suspension to inactivate Trypsin. The homogenate was centrifuged for 5 minutes at 350 x g prior to re-suspending the pellet in 8 ml pre-incubated (39.0 °C, 5% CO2, 95% air) culture medium. This gave a cellular density of ~1.05x10^6 fibroblast cells.ml-1 thereby enabling seeding to 8 culture inserts by adding 1 ml per basal fascias of Transwell™ semi-permeable supports.

This was achieved by fastening a cylindrical polyprolene scaffold around the polyethylene terephthalate base of the apically laminin coated polyester membranes, to which fibroblasts were seeded and allowed to proliferate. The dual

culture scaffold was capped with a double threaded polyprolene lid designed to aerate the cell homogenate whilst maintaining sterility (Figure 4-5).

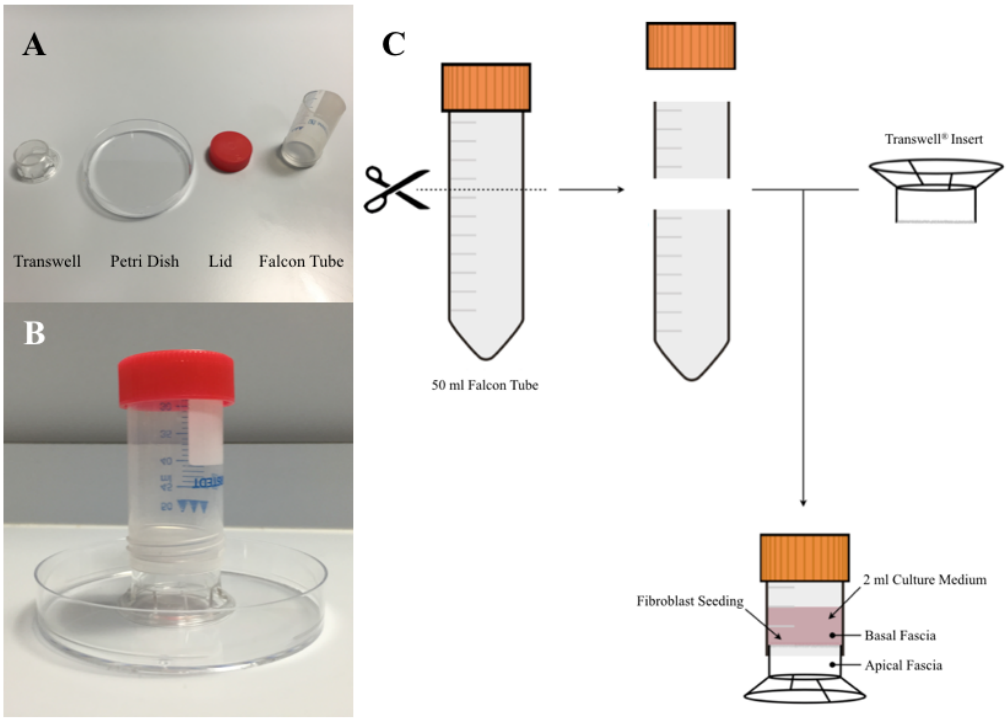

Figure 4-5 — **A**: The individual apparatus required to achieve dual culture and B: its assembly. **C**: The technical method and apparatus innovated for seeding fibroblasts to the basal fascia of Transwell™ membranes for establishing dual culture. Specifically large (50 ml) Falcon tubes were cut two thirds from the base and the caps removed. The top end of the Falcon tube was manually fastened over the inverted Transwell™ support whilst the cap was placed over the severed end of the tube. This scaffold could then support cell proliferation on the basal fascia of the semi-permeable membrane.

Fibroblasts were maintained in this manner for ~ 5 days at which point they fully populated their growth area — confirmed visually by light microscopy. The Transwells™ were then reorientated and BOECs introduced to the apical fascia as described in Section 2.3.4. This method has been termed *dual culture* as opposed to *co-culture* which, in the context of research on early development, implies epithelial cell culture with embryos (Orsi & Reischl 2007).

4.3. Results

4.3.1. Impact of Hormone Supplementation on *iv*DOF

Hyperandrogenic stimulation led to a significant increase in *iv*DOF production (Figure 4-6), compared to physiological, hypoandrogenic, and native (untreated) conditions.

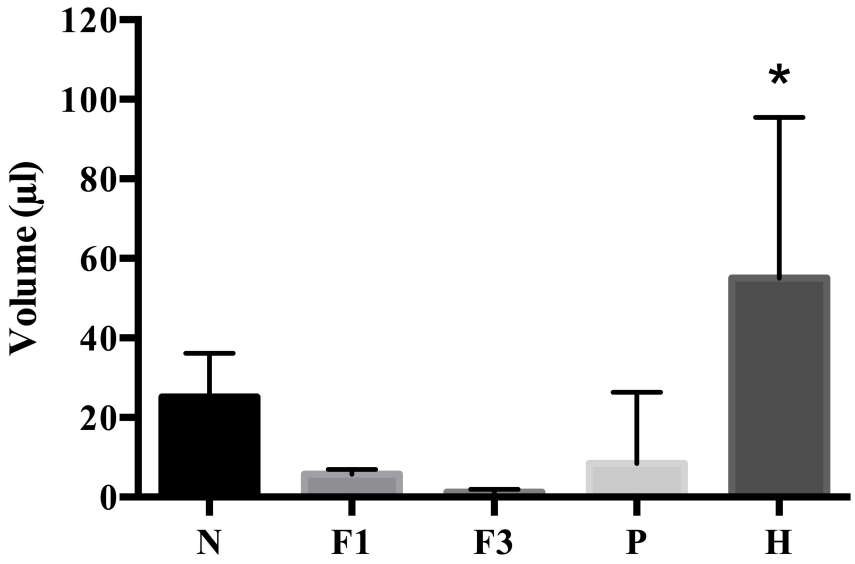

Figure 4-6 — Volumes of *iv*DOF obtained from native (**N**) cell populations (n=6 ± SD), *hypoandrogenic* (**F**) supplemented epithelia (n=3 ± SD), *hyperandrogenic* (**H**) treated epithelia (n=4 ± SD), and from cells supplemented with a *physiological* (**P**) hormonal profile (n=3 ± SD). Hyperandrogenic stimulation led to an significant increase in *iv*DOF production (p=0.0354).

Figure 4-7 shows that endocrinological supplementation had little impact on the glucose, lactate, and pyruvate composition of *iv*DOF when an unpaired t-tests were conducted.

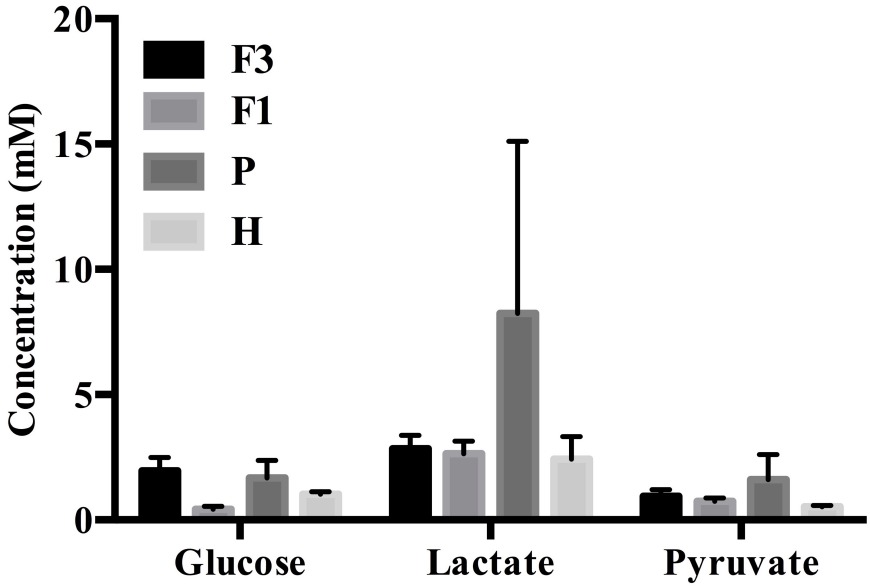

Figure 4-7 — The carbohydrate composition of *iv*DOF from native *iv*DOF (**N**: n=3 ± SD), *hypoandrogenic* supplemented epithelia (**F1** and **F3**; n=3 ± SD), *hyperandrogenic* treated epithelia (**H**; n=3 ± SD), and from cells supplemented with a *physiological* hormonal profile (**P**: n=3 ± SD).

Next the amino acid composition of *iv*DOF was determined (Figure 4-8) with the data showing that introducing a physiological hormonal milieu into the *in vitro* oviduct preparation altered the flux of 6 amino acids relative to native. Specifically histidine flux was decreased whereas glutamine, glycine, arginine, alanine, and lysine transport increased.

A *severe* hypoandrogenic endocrine profile (F3) provided basally for 24 hours disrupted the transport of six amino acids relative to native. Specifically, histidine, threonine, tyrosine, and isoleucine flux was decreased whilst glutamine and glycine were elevated in F3 treated populations relative to untreated. Interestingly the F3 treatment reduced histidine and threonine concentrations in *iv*DOF to levels below the limit of detection. As the concentration of T increased, histidine and tyrosine levels rose, approaching those seen in native *iv*DOF. Glutamine and glycine on the other hand were reduced as T levels rose. Moreover

hypoandrogenism led to a decrease in glutamine, glycine, threonine, arginine, alanine, and lysine whilst increasing histidine compared to untreated cells.

By contrast, a hyperandrogenic endocrine profile reduced histidine, and tyrosine flux whilst elevating glutamine, glycine, arginine, alanine, and lysine. Comparing the amino acids profiles of physiological *vs* hyperandrogenic BOECs however showed that hyperandrogenism led to increased glycine secretion and a parallel reduction in histidine and arginine flux.

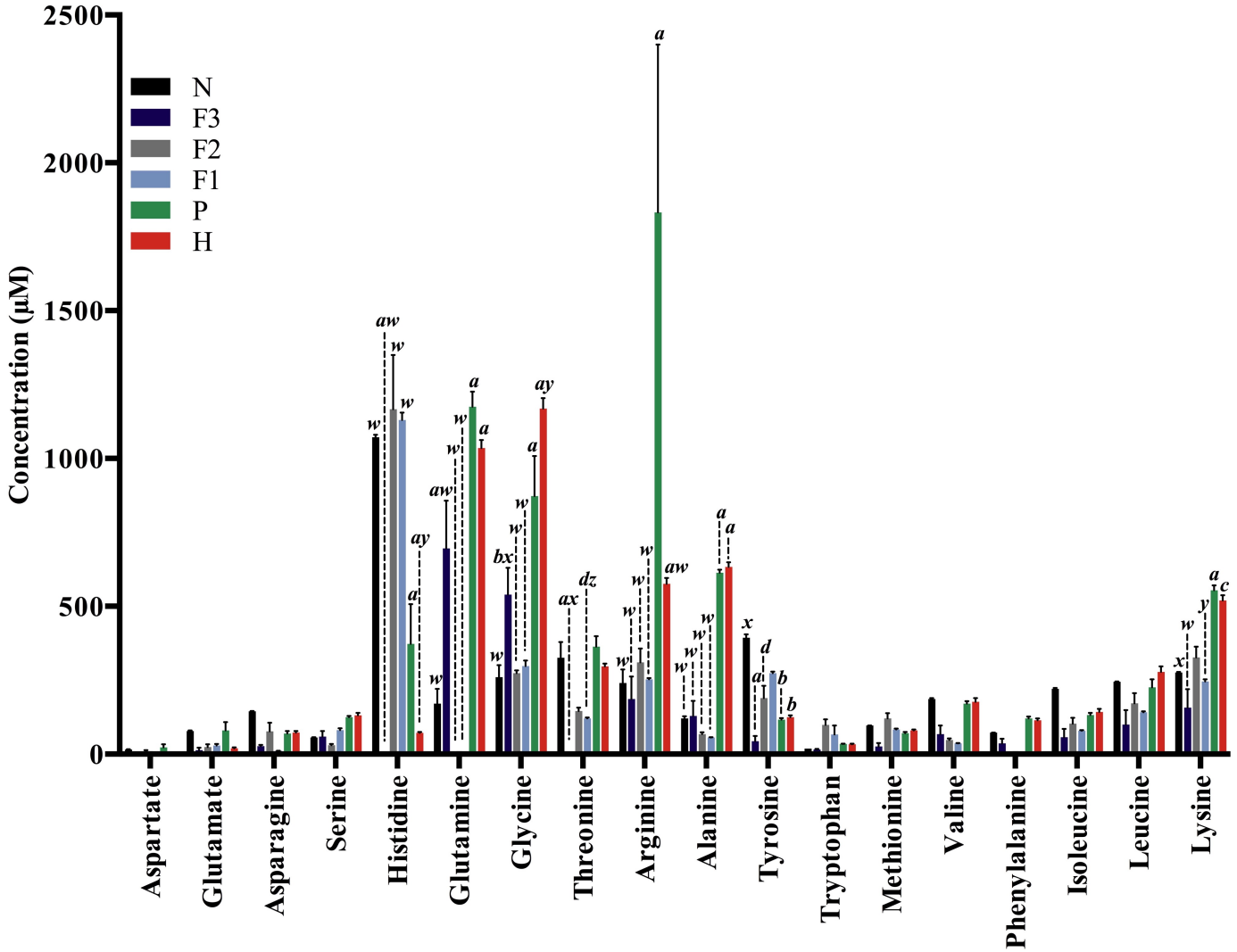

Figure 4-8 — Native *iv*DOF (**N**; n=12 ± SD) *vs iv*DOF from *physiologically* treated epithelia (**P**; n=3 ± SD) *vs iv*DOF from BOECs subjected to a *hypoandrogenic* profiles (**F1-F3**; n=3 ± SD) *vs iv*DOF from cells supplemented with *hyperandrogenic* levels of 17β-oestradiol, progesterone and testosterone (**H**; n=3 ± SD); *iv*DOF accumulated over 24 hours. Statistically significant differences were determined by two-way ANOVA and a Holm-Sidak *post hoc* analysis. Significant differences between the native treatment are denoted by *a* = $p \leq 0.0001$, *b* = $p \leq 0.001$, *c* = $p \leq 0.01$, and *d* = $p \leq 0.05$ whilst differences between the physiological treatment are presented as *w* = $p \leq 0.0001$, *x* = $p \leq 0.001$, *y* = $p \leq 0.01$, and *z* = $p \leq 0.05$.

4.3.2. Impact of Hormone Supplementation on Gene Expression

The pattern is that mRNA was generally ~ 10-20 times higher (and always statistically significantly different) in the physiologically (P) and hyperandrogenic (H) experimental cohorts compared to all the others [ethanol (E) vehicle control, F1, F2, and F3] (Figure 4-9).

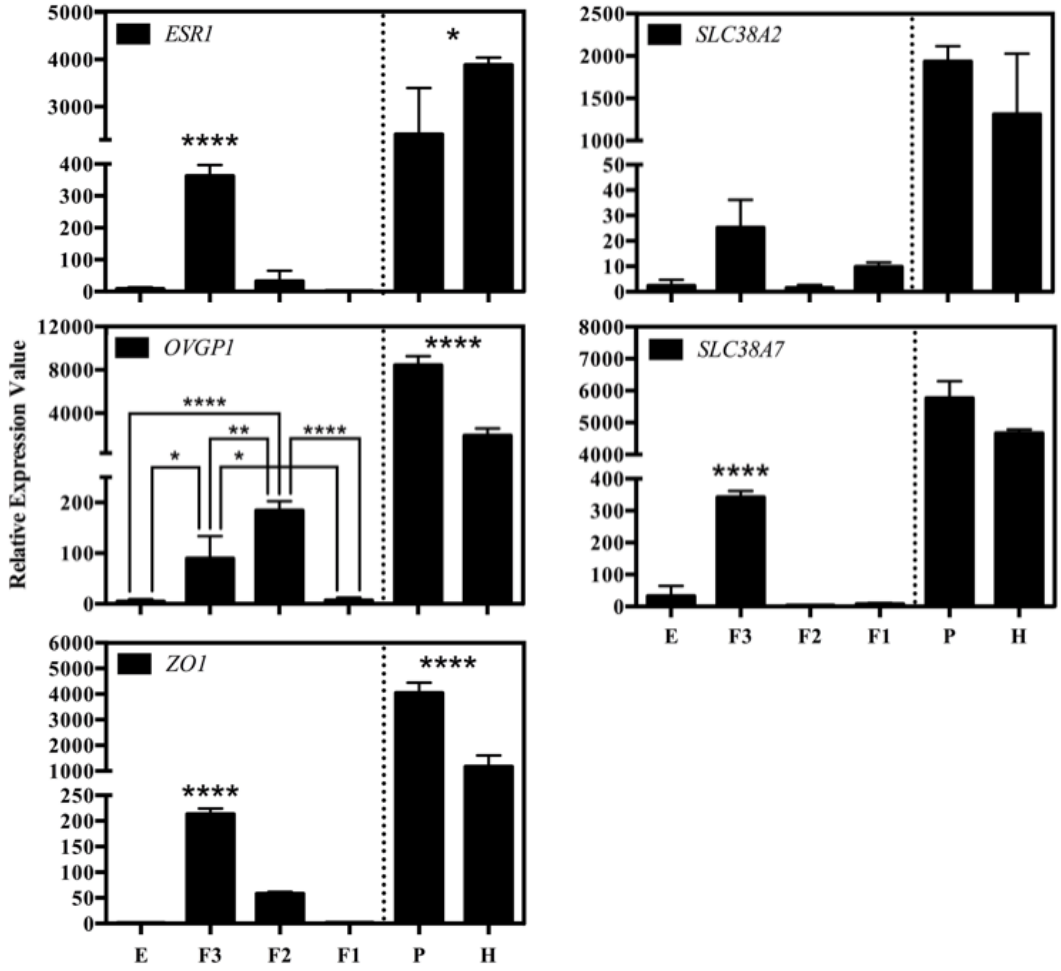

Figure 4-9 — The relative gene expression of oestrogen receptor alpha (*ESR1*), oviduct specific glycoprotein 1 (*OVGP1*), zona occludin 1 (*ZO1*), and solute carrier proteins A2 (*SLC38A2*) and A7 (*SLC38A7*) following incubation with 6 treatments for 24 hours — ethanol [(**E**) 0.835% (*v*/*v*)] as a vehicle control, *hypoandrogenic 1* [(**F1**) 19.46 pM E2, 6.36 nM P4, 62.77 pM T], *hypoandrogenic 2* [(**F2**) 19.46 pM E2, 6.36 nM P4, 34.7 pM T], *hypoandrogenic 3* [(**F3**) 29.37 pM E2, 6.36 nM P4, 2.43 pM T], *physiological* [(**P**) 29.37 pM E2, 6.36 nM P4, 208 pM T], and *hyperandrogenic* [(**H**) 19.46 pM E2, 6.36 nM P4, 6.27 nM T]; all (n=3 ± SEM). Statistically significant differences were determined separately between **P** and **H** and again between **E**, **F3**, **F2**, and **F2** (as depicted by the dotted line) by two-way ANOVA followed by a Holm-Sidak *post hoc* analysis; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.3.3. Impact of Hyperandrogenism on TER

Figure 4-10 shows that bovine oviduct epithelial cells exhibited a significant reduction in TER when incubated for 24 hours with a hyperandrogenic (H) and hypoandrogenic (F1) hormonal mix.

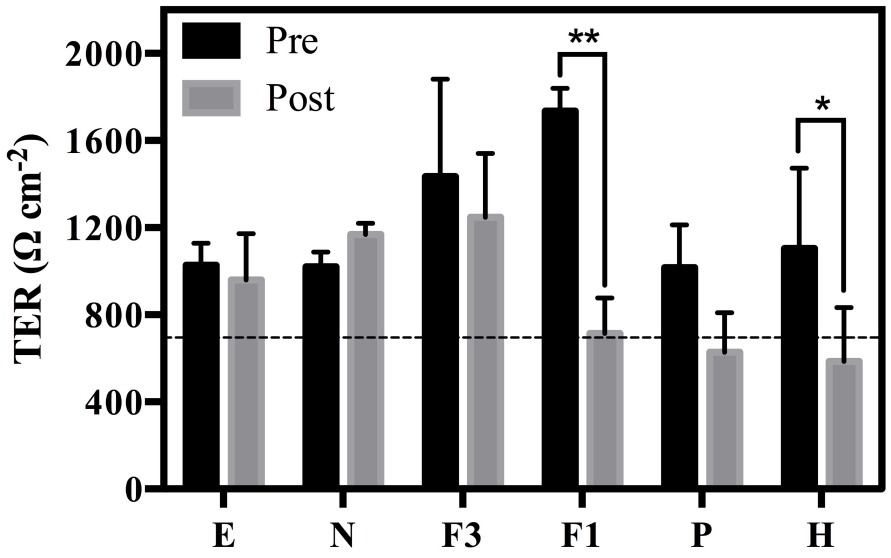

Figure 4-10 — TER values from BOECs before and after exposure to 4 endocrine conditions — *hypoandrogenic* (**F1** and **F3**), *physiological* (**P**), and *hyperandrogenic* (**H**) in addition to ethanol (**E**) (vehicle control) and native (**N**) (negative control); all n=3 ± SD.

4.3.4. Impact of Pharmacological Supplementation on *iv*DOF

Basally supplementing confluent BOECs with metformin for 24 hours significantly reduced the concentrations of glutamine, glycine, and alanine in *iv*DOF whilst increasing threonine (Figure 4-11). In contrast, treatment with clomifene (Figure 4-12) and 4OH-DFC (Figure 4-13) had no impact on the amino acid composition of *iv*DOF. This was also the case for the ethanol vehicle control (Figure 4-14).

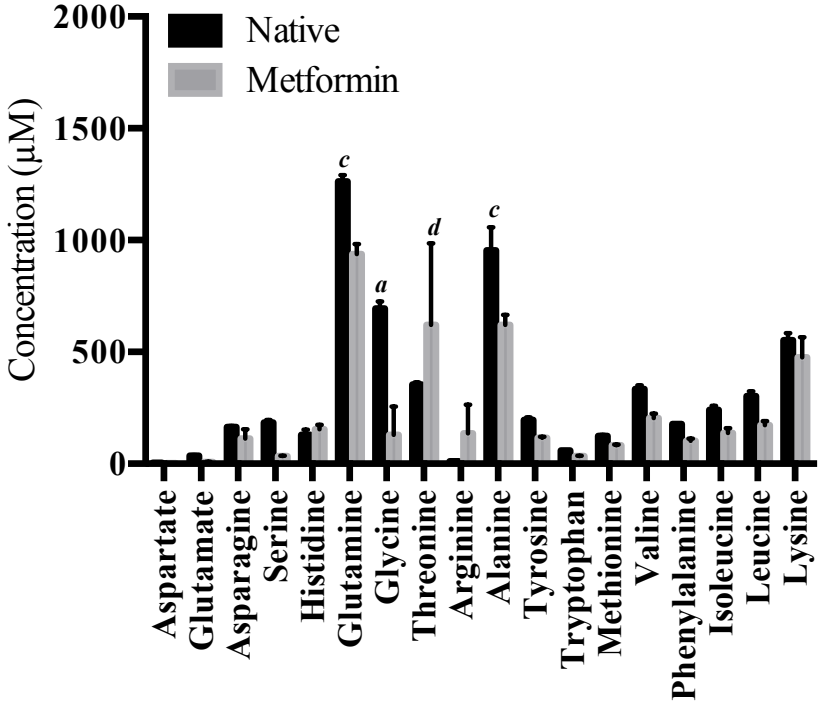

Figure 4-11 — Native *iv*DOF (n=3 ± SD) *vs iv*DOF from bovine oviduct epithelia basally treated with 7.75 μM metformin for 24 hours (n=2 ± SD). Statistically significant differences were determined by two-way ANOVA followed by a Holm-Sidak *post hoc* analysis; *a* = $p \leq 0.0001$, *b* = $p \leq 0.001$, *c* = $p \leq 0.01$, and *d* = $p \leq 0.05$.

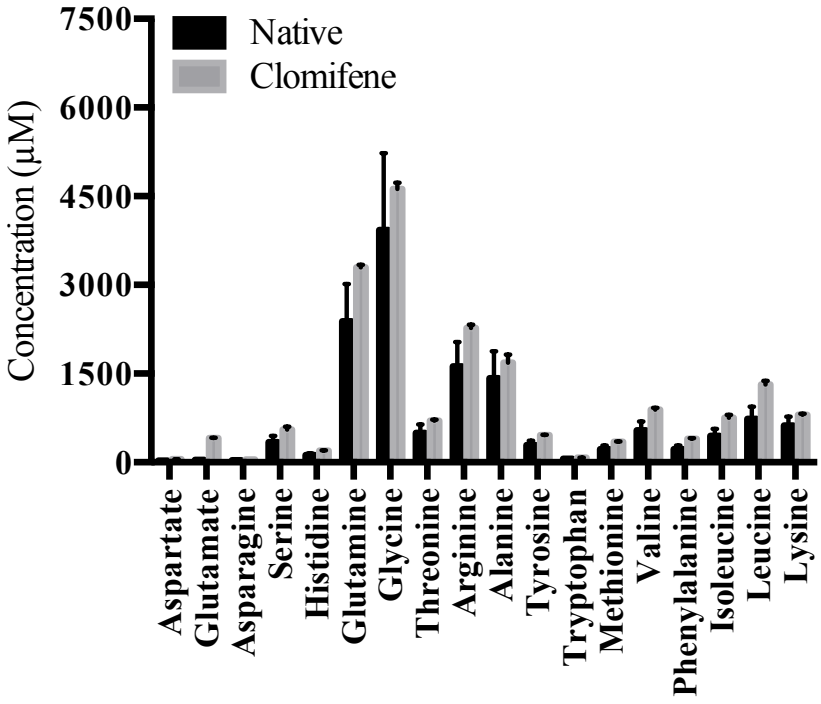

Figure 4-12 — Native *iv*DOF (n=3 ± SD) *vs iv*DOF from cells basally supplemented with 99 nM clomifene for 24 hours (n=3 ± SD). No statistically significant differences were observed.

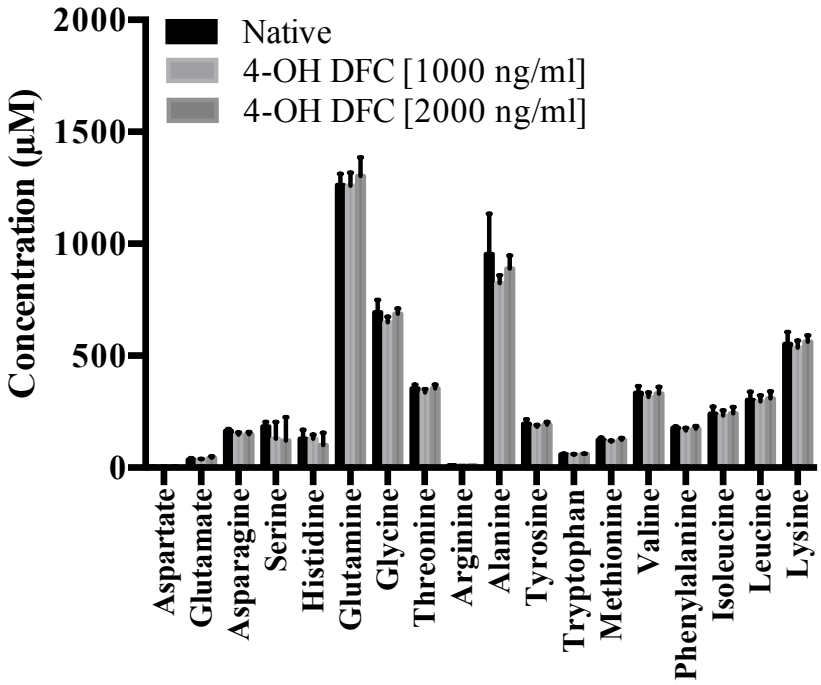

Figure 4-13 — Native *iv*DOF (n=3 ± SD) *vs iv*DOF from oviduct epithelia supplemented with 3.2 µM [1000 ng/ml (n=2 ± SD)] and 6.4 µM [2000 ng/ml (n=3 ± SD)] 4OH-DFC for 24 hours. No statistically significant differences were observed.

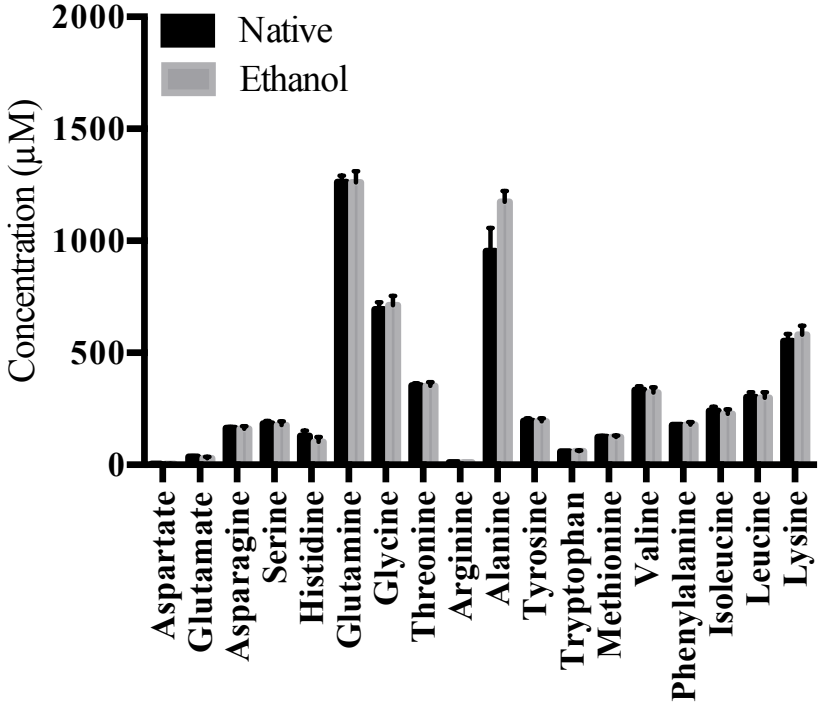

Figure 4-14 — Native *iv*DOF (n=3 ± SD) *vs iv*DOF from bovine oviduct epithelial cells basally supplemented with 1% (*v*/*v*) ethanol for 24 hours. No statistically significant differences were observed (n=3 ± SD).

4.3.5. Carnitine Transport

In line with the third objective of this chapter being to evaluate whether carnitine transverses the bovine oviduct epithelial cell monolayer, the data show that carnitine did indeed cross the epithelial barrier (Figure 4-15). Total carnitine (Σ) is the sum of each 'apical accumulation' and 'basal depletion' time points.

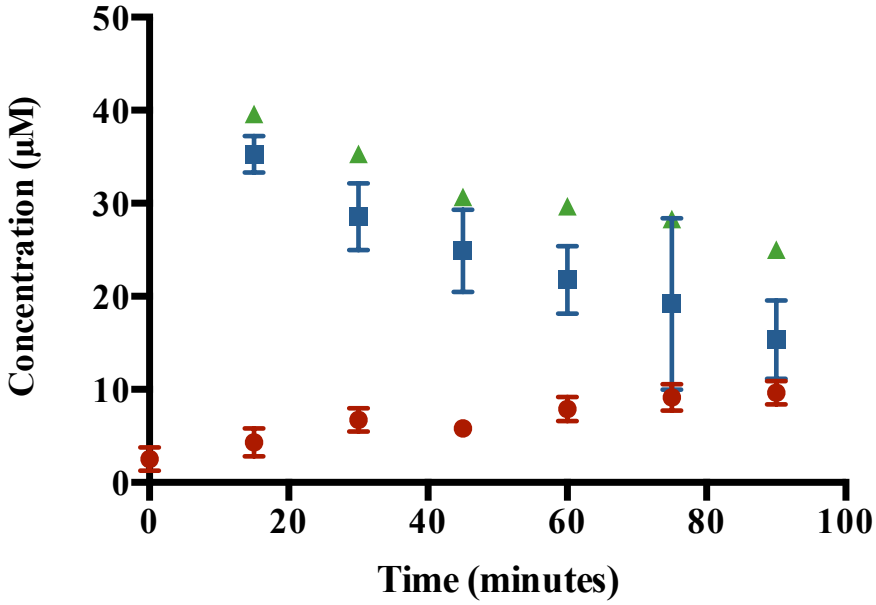

Figure 4-15 — The flux of 40 µM of carnitine from a basal (blue squares) to apical (red circles) direction across the *in vitro* bovine oviduct epithelial monolayer over 120 minutes (n=3 + SEM). The green triangles are the sum of the basal and apical values for each time point showing the temporal depletion of total paracellular carnitine.

4.3.6. Dual Culture: FACS Confirmation of BOFCs

Figure 4-16 shows that over 99% stained positive for vimentin, indicating a high degree of fibroblast purity isolation, and therefore a high degree of purity for dual culture (Section 4.2.5.).

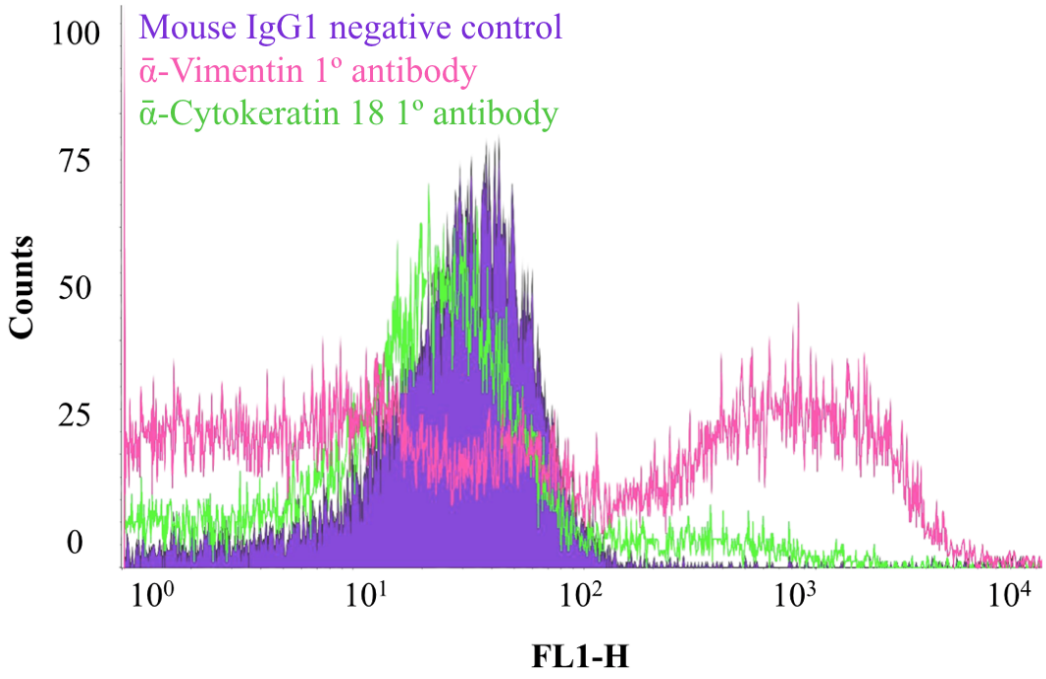

Figure 4-16 – FACS analysis of cultured BOFC purity showing mouse IgG1 negative control (background noise), ᾱ-Cytokeratin 18 1° antibody (BOEC population), and ᾱ-Vimentin 1° Antibody (BOFC population) in excess of 99% stromal purity (n=1).

4.3.7. Impact of Dual Culture on *iv*DOF

Having established the purity of the oviduct epithelial cells (Figure 3-4) and fibroblasts (Figure 4-16), the two cell types were cultured together according to Figure 4-5. The composition of the resulting *iv*DOF was examined and the data provided in Figure 4-17 showed that BOECs cultured with BOFCs significantly modified the flux of seven amino acids. Specifically asparagine, histidine, threonine, and tyrosine, were present in dual culture *iv*DOF in lower concentrations than native *iv*DOF. By contrast, the concentrations of glutamine, arginine and tryptophan were higher.

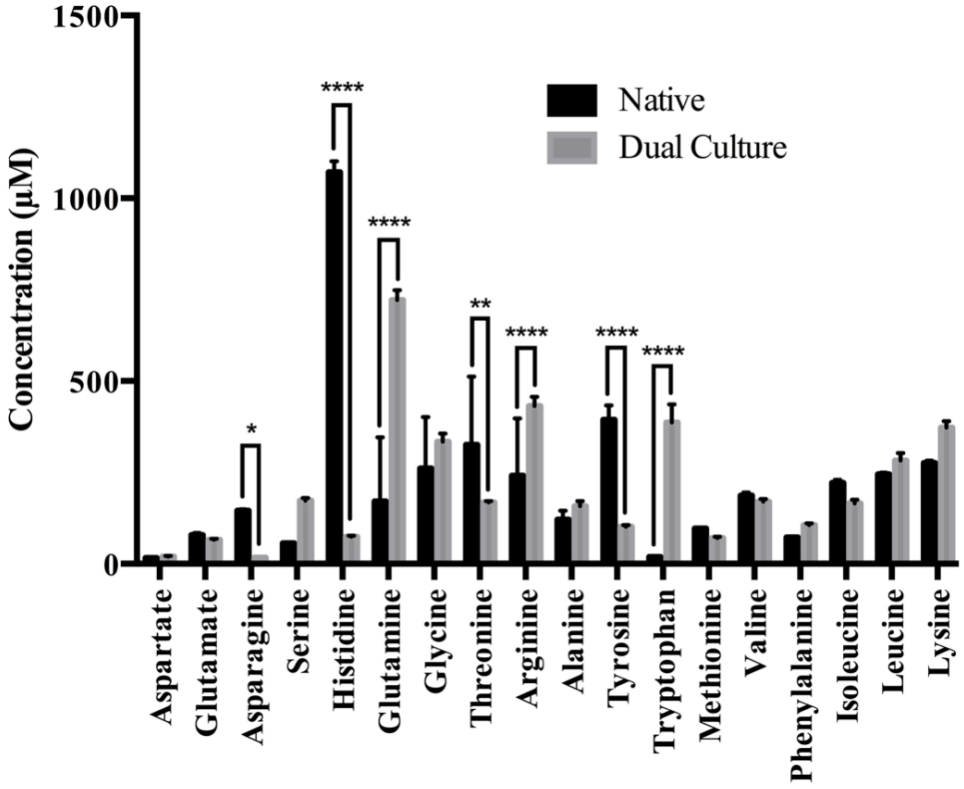

Figure 4-17 — The amino acid composition of native *iv*DOF (n=12 ± SD) *vs iv*DOF from BOECs cultured with BOFCs basally adjacent in the dual culture arrangement (n=4 ± SD). *iv*DOF accumulated over 24 hours. Statistically significant differences were determined by two-way ANOVA followed by a Holm-Sidak *post hoc* analysis where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.4. Discussion

In this chapter the *in vitro* oviduct model presented in Chapter 3 has been expanded to examine the effects of physiological, hypoandrogenic, and hyperandrogenemic mimicking endocrine environments on *iv*DOF composition, BOEC gene expression, and epithelial TER. The impact of three drugs on *iv*DOF amino acid content has also been investigated in addition to the transport properties of carnitine. Finally the effects of epithelial dual culture with fibroblasts on *iv*DOF were also assessed.

4.4.1. Volume and Carbohydrate Content

Interestingly the volume of *iv*DOF accumulated apically only increased following hyperandrogenic supplementation (Figure 4-6). This could be attributable to an increase in active fluid secretion by the epithelium or an increase in passive leak-though of fluid from the basal chamber. This concept of a *leaky oviduct* phenotype is discussed below

Carbohydrate metabolism has been investigated comprehensively in liver and muscle cells — particularly in response to pancreatic (*e.g.* insulin and glucagon) and adrenal (*e.g.* adrenaline and cortisol) hormones. By contrast the literature on carbohydrate utilisation by reproductive tissues in response to steroid hormones remains sparse. Nonetheless owing to the general phenomenon that sex hormones mediate protein anabolism, an energetically demanding process, (Miers & Barrett 1998) it was expected that hyperandrogenic treatment would reduce glucose and increase lactate concentrations in *iv*DOF. However Figure 4-7 shows that the glucose, lactate, and pyruvate content of *iv*DOF remained unchanged across treatments. These data alone are insufficient to claim that the broad metabolic profile of the oviduct epithelium remains unaltered following pathological

endocrine supplementation but is an interesting area for future research given that the corresponding amino acid composition of *iv*DOF varied.

4.4.2. Amino acids: N *vs* P

The first broad observation was that BOEC exposure to more complex combinatorial hormonal endocrine profile (Figure 4-8) did not seem to affect amino acid secretion as much as when provided in isolation (Figure 3-10), since in *iv*DOF from physiologically treated epithelia the transport of only six amino acids changed relative to the native control. Notably, the fact that histidine (Section 3.4.4.2.) was brought back down to levels more in line with what has been previously reported *in vivo* (Tay *et al* 1997) was reassuring. Similarly alanine (NE) and lysine (E) were elevated to levels directly comparable with *in vivo* observations (Hugentobler *et al* 2007b; Tay *et al* 1997). Glycine (Section 3.4.4.3.) was greatly increased in *physiologically* derived *iv*DOF bringing it to levels slightly closer to what have been observed *in vivo* (Hugentobler *et al* 2007b).

In contrast glutamine was elevated to levels beyond what have been previously observed *in vivo*. As discussed in Section 3.4.4.1. glutamine improves preimplantation mouse embryo development *in vitro* (Rezk *et al* 2004; Smith & Sturmey 2013) and is a known primary embryo metabolite (Rieger *et al* 1992; Mathew *et al* 1993) whose catabolism increases as the embryo progresses to the blastocyst stage (Tiffin *et al* 1991). In light of this is tempting to speculate then that the oviduct may respond to maternal endocrine status as opposed to embryo presence — a notion supported by the recent finding that amino acid and carbohydrate composition in the oviduct varied between ampulla and isthmus independently of embryo presence (Maillo *et al* 2016; unpublished).

The most notable finding however was that of arginine which was elevated to levels beyond what have been previously observed *in vivo* and *in vitro* (Chapter 3) Arginine is a conditionally essential amino acid whose role in reproduction has been investigated to a great extent although not within an oviductal context. For instance it is well known that arginine is a common substrate for NO and polyamide synthesis — both crucial for mammalian placental angiogenesis (Che *et al* 2013).

A study by Saevre *et al* (2011) found that elevated circulating maternal arginine increased the overall pregnancy rate in the ewe and the authors even suggested that "treatment with arginine surrounding the time of maternal recognition of pregnancy may have prevented pregnancy loss in some ewes." Other data such as the finding that mouse embryo pluripotency is regulated by histone arginine methylation (Torres-Padilla *et al* 2007) suggest that arginine is also important for the embryo in addition to uterine receptivity. This is supported by Sturmey *et al* (2010) who reported that arginine was one of two amino acids consistently depleted by bovine blastocysts regardless of derivation (*i.e. in vitro* and *in vivo*), and Leary (2015) who observed that human embryos which developed beyond the unexpanded blastocyst stage consumed significant amounts of arginine.

It is unsurprising given all of the above that the oviduct epithelium would elevate arginine secretion following physiological hormonal stimulation, especially since arginine can be readily synthesised from glutamate via ornithine (Wu 2010) — *i.e.* under P stimulation the oviduct epithelium secreted 436.9 times the concentration of arginine present in basal culture medium (Figure 3-10) whilst presumably still catering for its own needs.

4.4.3. Amino acids: N *vs* F3

Glutamine and glycine were elevated in the *iv*DOF produced under hypoandrogenic conditions relative to native whilst tyrosine was reduced. Surprisingly histidine (E) and threonine (E) were absent from in *iv*DOF when the oviduct epithelia were supplemented with the F3 hormonal milieu. It is noteworthy that the histidine content of native *iv*DOF (Figure 3-10) was approximately ten times greater than the concentrations previously observed *in vivo* (Hugentobler *et al* 2007b; Guerin *et al* 1995). This unexpected finding warrants further investigation. One explanation for this absence of histidine and threonine in *iv*DOF could be the metabolic needs of the oviduct epithelia. It has been established that sex hormones promote protein synthesis (Miers & Barrett 1998) with other hormones such as cortisol and prolactin shown to stimulate epithelial amino acid uptake (Anderson & Rillema 1976).

4.4.4. Amino acids: P *vs* H and F

Exposing the preparation to androgenic extremes H and F3 considerably impacted the amino acid profile of the *in vitro* oviduct secretome relative to P. Specifically H endocrine stimuli led to increased glycine secretion and parallel reductions in histidine and arginine flux. H moreover appeared to have a lesser impact on amino acid flux regulation that F3 as the latter reduced the movement of 7 amino acids relative to P. These were histidine, glutamine, glycine, threonine, arginine, alanine, lysine.

The H increase in glycine was unexpected because T supplementation alone in Figure 3-10 reduced glycine flux. Two explanations can be put forward; the first is that under H conditions BOECs become hypotonic. Figure 4-6 could support this concept since *iv*DOF volume significantly increased following H incubation.

Therefore, to retain adequate intracellular osmolarity, glycine may have been apically expelled by the cells to move water and reestablish isotonicity. The second possibility is the differential hormonal regulation of glycine transporters.

4.4.5. Effects of Hormonal Supplementation on Gene Expression

To complement the gene expression data provided in Chapter 3 (Figure 3-11) and to gain some insight into the potential mechanisms underlying the differences in amino acid flux observed following different endocrine stimuli, solute carrier expression was investigated. Owing to vastly inconsistent technical replicates, it was not possible to present a more comprehensive panel of genes in this chapter. Moreover the levels of mRNA detected by qRT-PCR were ~ 10-20 times higher (and always statistically significantly different) in the P and hyperandrogenic H experimental cohorts compared to all the others (Figure 4-11). It should be noted that although it could conceivably be the case that under P and H supplementation the genes analysed are over-expressed by an order of magnitude, these experiments and analyses were performed with some years in between wherein a number of variables can change. Thus comparisons are made within the experimental cohorts as depicted by the dotted lines in Figure 4-11 — *i.e.* E *vs* F3 *vs* F2 *vs* F1 and P *vs* H.

In terms of individual genes analysed, and similarly to Chapter 3, the expression of the characteristic genes *ESR1* and *OVGP1* were quantitatively evaluated to discover whether the cell cultures were responsive to combinatorial hormone supplementation and as an indication of oviductal epithelial nature, in addition to being important functional genes. The data show that within the hypoandrogenic cohort *ESR1* mRNA was determined to be present at highest levels following F3 treatment wherein T was lowest. This was also the case for all other genes

analysed with the exception of *OVGP1* which was expressed in greatest levels with F2 hormonal incubation, and *SLC38A2* where differences were insignificant.

The relative decrease in expression of *ESR1* from F3 to F2 and F1 treatments can be largely attributed to the decrease in the concentration of E2 in cultures from 29.37 pM to 19.46 pM (33.7% decrease). However the relative decline in *ESR1* expression was over ten times greater (340 %). Moreover the concentration of E2 in the F2 conditions was identical (19.46 pM) to that of the F1 treatment. T on the other hand increased from 34.7 pM to 62.77 pM (Table 4-1) and the expression of *ESR1* decreased from 32.8 relative expression value units to 1.6 despite E2 remaining constant. This identical phenomenon of potential E2 desensitisation was also observed with *SLC38A7*. Whilst it appears from these finding that E2 signalling is heightened in a low T environment, the fact that *ESR1* expression increased following H incubation (relative to P) despite the 33.7% decrease in E2 suggests that a hyperbolic relationship exists — whereby E2 responses are heightened in both abnormally low and abnormally high T environments. This could be explained by the fact that T has a low affinity for the oestrogen receptor *in vitro* which is not enough to elicit oestrogen receptor localisation to the nucleus but is enough to decrease the association rate of E2 on ERα (Rochefort *et al* 1976) — a so called anti-oestrogenic effect. Although not directly comparable, it is worth noting that P4 insensitivity has been observed in uterine endometria of human PCOS patients (Savaris *et al* 2011; Aghajanova *et al* 2010).

SLC38A7 encodes SNAT7 the basally located System A transporter (Figure 3-1) responsible for moving glutamine extracellularly (Hagglund *et al* 2011). In chapter 3 SNAT7 was up-regulated following E2 supplementation (Figure 3-11) thus the finding that SNAT7 expression was greatest following F3 hormonal supplementation was expected (Figure 4-9). On the other hand, and as with *ESR1* one would expect *SLC38A7* expression to be elevated following P treatment.

However this was not the case further supporting the possibility that E2 signalling is perturbed under abnormal T levels.

Moreover *OVGP1* expression was higher in F3 treated BOECs compared to F1 treated epithelia but highest following F2 supplementation (Figure 4-9). This seemingly anomalous finding inconsistent with previous reports that *OVGP1* expression is elevated during oestrus (Chen *et al* 2013) at which time E2 levels are high (Figure 1-1); although this was reassuringly observed when comparing *OVGP1* expression in P *vs* H cell cohorts. Moreover *SLC38A2* seemed to follow the pattern of over-expression in F3 treated cells but expression levels across all treatments were too low for any meaningful conclusions to be drawn.

In terms of relative amino acid flux the investigation of only 2 solute carrier proteins makes this analysis weak but extending from Chapter 3 and in summary the gene expression data once again do not correlate well with the observed amino acid transport.

Exposing BOECs to F1 and F2 conditions for 24 hours led to a significant decrease in *zona occludin 1* (*ZO1*) gene expression relative to F3 (Figure 4-9) or more specifically the F3 endocrine profile elevated *ZO1* expression relative to F1 and F2. However more interestingly — a H hormonal supplementation reduced *ZO1* expression relative to P. Although an oestrogen response element (ERE) has yet to be formally associated with *ZO1* it is widely accepted that *ZO1* expression is responsive to ERα and ERβ activity (Weihua *et al* 2003). Di Leva *et al* (2013) showed in breast cancer cells that E2 activates a cluster of micro RNAs (miR-191/425) that up-regulate *ZO1* expression. The abundance or lack of this miRNA cluster has not been linked to hyperandrogenism or PCOS to date (Sorensen *et al* 2014) however Zeng *et al* (2004) observed an increase in occludin expression following E2 supplementation. Thus it is logical to conclude that the

increase in *ZO1* expression in P supplemented cells is likely to be attributable to the elevated E2 in culture.

4.4.6 Effects of Hormonal Supplementation on TER

As TER is proportional to epithelial monolayer integrity, which is in turn proportional to *ZO1* expression (Sultana *et al* 2013) (Section 1.5.4.1.), it was investigated as an independent experimental variable. Consistent with the *ZO1* data it was assumed that TER would increase following P treatment This was not the case; Figure 4-10 shows that the TER of BOECs supplemented with a P endocrine profile was reduced from 1017.5 to 628.1 although not significant. Moreover a H endocrine profile resulted in a significant reduction in TER from 1104.0 to 585.8 $\Omega \cdot cm^{-2}$ — a value also below the 700 $\Omega \cdot cm^{-2}$ threshold established in Section 2.4.1. as an 'acceptable' confluence despite this baseline being lower for some (Tahir *et al* 2011; Palma-Vera *et al* 2014).

This combined phenomenon of altered *ZO1* expression and TER reduction in H *vs* P epithelia paralleled with an increase in *iv*DOF volume following H supplementation (Figure 4-6) is referred to as the *leaky oviduct* phenotype. In future this could be further tested by using non-metabolisable fluorescent substrates such as fluorescein isothiocyanate (FITC)–dextran and fluorescein to test the relative permeability of the monolayer. However part of the problem in assessing the merits of this phenotype is that TER cannot be measured *in vivo* thus there is no true physiological comparison. These data hence raise the question of whether an inherent 'leakiness' of the oviduct is normal? If this assumption holds true then one conclusion is that a P endocrine profile restores TER whilst H may reduce TER too much. This would also hold true for F1 and F2 with F3 having no effect on restoring TER. Either way it seems that T plays a more dominant role in

reducing TER than E2 does in elevating it. Liu *et al* (1999) also reported that testosterone reduced TER in the Caco-2 cell line.

4.4.7. Effects of Pharmacological Supplementation on *iv*DOF

Next the value of using the *in vitro* oviduct model to test the impact of drugs on secretion composition was evaluated. Specifically the impact of metformin, clomifene and 4OH-DFC on the amino acid composition of *iv*DOF was investigated.

Metformin supplementation decreased the transport of glutamine (CE), glycine (NE), and alanine (NE) whilst increasing threonine (E) flux (Figure 4-11). Metformin has been shown to interfere with numerous central cellular processes including phosphoinositide 3 kinase (PI3K) inhibition (Slomovitz & Coleman 2012), protein kinase B (Akt) inhibition (Hawley *et al* 2002), adenosine monophosphate activated protein kinase (AMPK) activation (Zhou *et al* 2001), and mechanistic target of rapamycin (mTOR) inhibition (Nair *et al* 2014). Thus speculation as to how exactly metformin impacts the flux of these four amino acids is challenging. However the mTOR pathway is a likely candidate as it is involved in amino acid sensing via mitogen activated protein kinase kinase kinase kinase 3 (MAP4K3) (Kim 2009). Moreover an interesting clinical study by Krakoff *et al* (2010) showed that metformin indirectly reduced serum alanine amino transferase activity.

Metformin has previously been shown to cross the placenta (Lautatzis *et al* 2013) and although it is not formally licensed for use during pregnancy in the UK the NHS guidelines state that it can be prescribed off licence if deemed necessary. Nne clinical trial has shown that metformin administration during pregnancy in females with PCOS reduced first-trimester pregnancy loss (Jakubowicz *et al*

2002). Although only two biological replicates were conducted here (Figure 4-11) metformin altered the flux of 4 amino acids relative to native. In future it would be more clinically relevant to investigate the impact of metformin on the composition of *iv*DOF from hyperandrogenic epithelia to see whether metformin supplementation can restore physiological secretions in diseased mimicked states.

In contrast to metformin, clomifene supplementation did not impact the amino acid composition of *iv*DOF. Clomifene an interesting drug as it is specifically a racemic mixture of z-clomifene (cis-isomer with anti-oestrogenic *and* oestrogenic activity) and e-clomiphene (trans-isomer with anti-oestrogenic activity) (Tarlatzis & Grimbizis 1998). From Figure 4-12 it is clear that clomifene did not exert any oestrogenic effects on the oviduct epithelium but in future it would be interesting to basally supplement BOECs with both E2 and clomifene to elucidate whether the anti-oestrogenic activity of clomifene applies to the oviduct epithelium.

As discussed in Section 4.1.2.3. diclofenac is a popular anti-inflammatory drug whose predominant metabolite is 4OH-DFC which appears to have no real reproductive significance in the literature. Thus it was unsurprising that 4OH-DFC did not alter the amino acid composition of *iv*DOF (Figure 4-13) and serves as a useful negative control of sorts.

4.4.8. Carnitine Transport

Figure 4-15 shows that carnitine transversed the bovine oviduct epithelial monolayer *in vitro* thus concluding that (*i*) this model is useful in detecting the movement of compounds into the lumen, and (*ii*) the early embryo is most likely exposed to carnitine *in vivo*. When the total carnitine present in the basal and apical chambers of the preparation were summed it became apparent that total free carnitine in solution depleted over time to just over half the initial concentration.

This phenomenon is likely explained by BOEC metabolism of carnitine — specifically the conversion of carnitine to acylcarnitine by outer carnitine acyltransferase which simultaneously catalyses the breakdown of acyl-CoA to acyl and CoA (Bremer 1983). Regardless, this simple *proof of principle* experiment shows the potential of the model in investigating the transport properties of the oviduct epithelial monolayer which is the subject of Chapter 5.

4.4.9. Effect of Dual Culture on *iv*DOF Composition

Following confirmations of BOFC and BOEC purity (Figure 4-16) the two cell types were cultured in the same Transwell™ with the epithelial cells on the apical side of the membrane and the fibroblasts directly underneath BOECs (Section 4.2.6.). With this dual culture preparation, the composition of the *iv*DOF was changed; specifically by increased appearance of 7 of 18 amino acids relative to native control.

This was an encouraging finding and established partial proof of principle of this experimental dual culture approach. The presence of underlying fibroblast cells would be expected to provide a closer to physiological environment for the oviduct epithelial cells (Section 1.5.4.1.). To pursue further studies it would be necessary to measure a wider range of constituents in *iv*DOF under different hormonal conditions. Such experiments might then provide clues as to the identity of paracrine mediators of secretion into *iv*DOF. For instance it would be of interest to examine the composition of cytokines and signal molecules in the culture medium, such as transcription growth factor beta (TGFβ), which is known to be secreted by human cancer associated fibroblasts (CAFs) to stimulate epithelial cell proliferation (Kalluri & Zeisberg 2006). Moreover, there is a scarcity of literature on fibroblast-epithelial signalling in a physiological context.

Furthermore, because quiescent and proliferating fibroblasts exhibit very high metabolic activity and amino acid turnover (Lemons *et al* 2010), the native *vs* dual culture profiles contrasted in Figure 4-17 are not strictly comparable on the simple rationale that there are double the total number of cells present to consume and affect culture media amino acid composition, and potentially *iv*DOF. Thus in further studies it would be interesting to compare the effect of irradiated fibroblasts on *iv*DOF.

4.4.10. Summary

Exposing the *in vitro* oviduct model to pathogenic hormonal concentrations did not alter the amino acid composition in *iv*DOF (Figure 4-8) as markedly as when compared with singular hormonal supplementation in Chapter 3. Moreover amino acid flux into *iv*DOF was inconsistent with the gene expression profiles of corresponding solute carriers (Figure 4-9). However the gene expression profiles of *SLC38A7* and *ESR1* suggest that a hyperbolic relationship exists whereby ERE sensitivity is heightened in both abnormally low and abnormally high T environments. Similarly the decreased expression of *ZO1* in BOECs treated with a hyperandrogenic hormonal mixture was consistent with a *leaky oviduct* phenotype, further demonstrated by a significant reduction in TER (Section 4.4.6.), and an increase in *iv*DOF accumulation (Figure 4-6). Finally the dual cell experimental model of the bovine oviduct provides a novel means of exploring oviduct function. Within the wider context of biology the results demonstrate the potential use of this *in vitro* model in characterising the transport or barrier properties of the oviduct towards a range of circulating xenobiotics. The broader impact of these findings is discussed in Chapter 7.

Chapter 5

Case Study of the Impact of Genistein on the *in vitro* Preparation

Chapter 5 — Case Study of the Impact of Genistein on the *in vitro* Preparation.

5.1. Introduction

Building on the characterisation of the *in vitro* oviduct (Chapter 3) and the investigation into the barrier properties of the model to carnitine (Chapter 4), the aim of this chapter was to (***i***) investigate the transport of the dietary derived compound genistein across the oviduct cells and (***ii***) determine its effect on the secretion of amino acids in *iv*DOF.

5.1.1. Genistein

Genistein-7-glucoside (Figure 5-1:A), or genistin, is a natural conjugated isoflavone. Isoflavones are plant derived polyphenolic molecules capable of exerting oestrogenic effects; otherwise known as phytoestrogens. Genistin is found in a range of foods including lentils, soybeans and coffee (Coward *et al* 1993; Wiseman *et al* 2002).

Following ingestion, genistein-7-glucoside is hydrolysed in the intestine by mammalian lactase phlorizin hydrolase (Rowland *et al* 2003) to release the aglycone form genistein (Figure 5-1:B). This aglycone is rapidly absorbed by the upper intestine (Izumi *et al* 2000) and circulates readily in the plasma (Clarke *et al* 2002; Coldham *et al* 2002). Genistein-7-glucoside is not present in human plasma (Setchell *et al* 2002) but genistein has been observed in concentrations ranging from the low nM range (Setchell *et al* 2001) to low µM (Iwasaki *et al* 2008 in Yang *et al* 2012) to low mM ranges (Rajah *et al* 2012).

Soya derivatives are a major component of several foodstuffs, including human milk-replacements, which are high in genistein-7-glucoside. Furthermore, soy has long been popular in eastern societies with intake rapidly increasing in the west

(Clarke *et al* 2004). The 1998 UK Total Diet Survey estimated that the average adult daily intake of genistein is 3 $mg{\cdot}day^{-1}$. In dietary supplements and extracts, the content of isoflavone aglycones as a percentage of total isoflavones can range between 15% to 85% (Setchell *et al* 2001). Furthermore the rising inclusion of soy in processed foods represents the new primary source of isoflavones in UK diets (Boker *et al* 2002; Clarke *et al* 2004).

Flavonoids such as genistein reportedly exert numerous beneficial physiological effects (Havsteen 2002; Middleton & Kandaswami 1994; Hodek *et al* 2002). For example, genistein can lower blood pressure by up-regulating nitric oxide (NO) synthesis in the vascular endothelium (Si & Liu 2008; Liu *et al* 2004). Furthermore, genistein inhibits cell growth and MAP kinase activity in aortic smooth muscle (Dubey *et al* 1999), increases TGF-β secretion (Kim *et al* 2001), and is implicated in antioxidant protection of DNA and low-density lipoprotein, and systemic processes such as the modulation of inflammation, inhibition of platelet aggregation, and modulation of adhesion receptor expression (Morton *et al* 2000; Birt *et al* 2001; Nijveldt *et al* 2001; Duthie *et al* 2000).

5.1.2. Genistein as a 17β-Oestradiol Analogue

The diverse biological activity of genistein is generally assumed to be a consequence of its chemical structure (Figure 5-1:B) which resembles 17β-oestradiol (Figure 5-1:C). As such genistein can bind to oestrogen receptors (ERs) *in vivo* and is able to exert modest oestrogenic effects (Zhang *et al* 1999) by inducing ER dimerisation and subsequent DNA binding at oestrogen response elements (EREs), similar to 17β-oestradiol (Kostelac *et al* 2003). Genistein has been shown to be effective at activating oestrogen receptors *in vitro* at 1 mM (Liu *et al* 2004) with the oestrogenic potency of genistein on ERα and and ERβ being 0.025% and 0.8% of that of 17β-oestradiol respectively (Kuiper *et al* 1998).

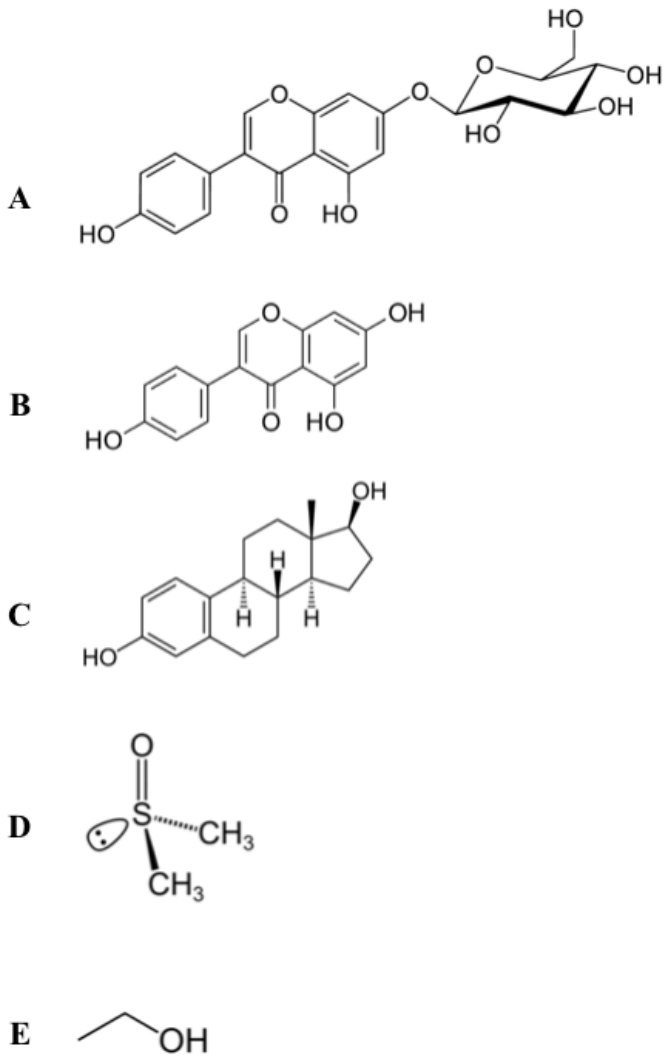

Figure 5-1 — The molecular structures of **A**: Genistein-7-Glucoside (Genistin), **B**: Genistein, **C**: 17β-oestradiol, **D**: Dimethyl Sulfoxide (DMSO), and **E**: Ethanol.

5.1.3. Reproductive Significance

As discussed in Section 1.2.3. the reproductive roles and significance of 17β-oestradiol are well known. In contrast, effects of genistein on reproductive physiology are less well understood. One important study reported that mice treated neonatally with genistein *in vivo* developed uterine adenocarcinoma (Newbold *et al* 2001). In this study normal female cesarian derived (CD1) mice were treated with oestrogen equivalent doses of genistein on days 1-5 with a separate cohort analogously treated with the a positive control diethylstilbestrol

(DES) — a known carcinogen and oestrogen analogue. At 18 months the incidences of uterine adenocarcinomas for the genistein treated mice was in fact higher (35%) than the DES treatment group (31%).

Since then the same research group has also shown in neonatal female CD1 mice that subcutaneous injections of genistein (equivalent to oral administration of genistein-7-glucoside) disrupted the oestrous cycle and gave rise to multi-oocyte follicles (Jefferson *et al* 2007). The same study moreover showed that increasing doses of genistein cumulatively perturbed the implantation process — as demonstrated visually by 47% of the genistein treated mice showing signs of pregnancy compared to 93% of the controls. This finding was later confirmed by Singh & Lata (2014). Furthermore the mice exposed to the higher doses of genistein did not deliver any live pups (Jefferson *et al* 2007).

Jefferson *et al* (2009) later performed an elaborate series of experiments to examine the developmental competence of oocytes in addition to the timing of embryo loss from mice neonatally treated with genistein. One key finding from this study was that when oocytes were fertilised *in vivo* and retrieved at the pronucleus stage prior to *in vitro* culture the percentage of embryos from genistein-treated mice reaching blastocyst stage was significantly reduced. Therefore although it seemed as though genistein disrupted embryonic cleavage in the oviduct (Jefferson *et al* 2009), in a similar study Jefferson *et al* (2012) found that genistein perturbed the expression of several immune response genes in CD1 mouse oviduct epithelium — a consequence of which was in fact an increase in embryo cleavage and a decreased ratio of trophectoderm to inner cell mass cells in the developing offspring *in vivo*. Although this did not affect full term development after embryo transfer, these findings highlight the potential of genistein to compromise offspring health via oviduct epithelial cell physiology (Jefferson *et al* 2012).

Other studies have less convincingly investigated the effects of genistein on reproduction. For instance Ryokkynen *et al* (2005) fed human concentrations of genistein to pregnant minks and at 21 days post-parturition observed that the genistein exposed offspring were lighter than the controls. Ryokkynen *et al* (2006) later performed a similar experiment in mice and again found that the genistein exposed offspring were lighter at 21 days post-parturition and moreover had heavier prostates and seminal vesicles.

Nagao *et al* (2001) investigated the reproductive function of post-pubescent rats following postnatal exposure to genistein and found that genistein exposed females had oestrous cycle irregularities, disrupted fertility, and histopathological changes in their ovaries and uteri whereas genistein did not appear to alter male rat physiology. Despite these findings, the presence of genistein in the female reproductive tract has yet to be confirmed.

5.1.4. Aims and Objectives

Building on these findings, the *in vitro* oviduct model was used to investigate whether effects of genistein on the developing conceptus could be direct and/or indirect. Direct action implies that genistein is capable of transversing the oviduct epithelium thereby being able to effect the embryo itself whereas indirect action would be the capacity to influence the broader embryonic microenvironment. Thus the research questions were (***a***) does genistein traverse the oviduct epithelium, thereby permitting a direct effect on the embryo (Figure 5-2:A), and/ or (***b***) does genistein supplementation impact on the amino acid composition of *iv*DOF, thus potentially affecting the embryo indirectly (Figure 5-2:B)? A subsequent research question became: does BOEC treatment with genistein result in a similar amino acid profile as BOECs treated with 17β-oestradiol?

The first respective hypothesis was that (***i***) genistein would cross the oviduct epithelium. Despite the reproductively adverse effects of genistein, from a chemical perspective it was supposed that genistein (a small hydrophobic molecule) would transverse the oviduct epithelial barrier — particularly as it has been shown to cross other *in vitro* cell types such as the human epithelial colorectal adenocarcinoma 2 (Caco-2) cell line and the blood brain barrier (BBB) (Yang *et al* 2014). Moreover although oviduct epithelia stain positive for efflux transporters such as the multi drug resistance 1 (MDR1) protein (Wijnholds *et al* 2000) and express numerous adenosine triphosphate binding cassette (ABC) genes (Dixon *et al* 2011b), the lack of any evidence for genistein efflux by these proteins further supported the hypothesis that genistein would transverse the BOEC monolayer. In line with the second aim it was expected that (***ii***) genistein would yield an amino acid *iv*DOF profile similar to that of 17β-oestradiol as the oestrogenic effects of genistein have been well documented in the literature (Section 5.1.2.).

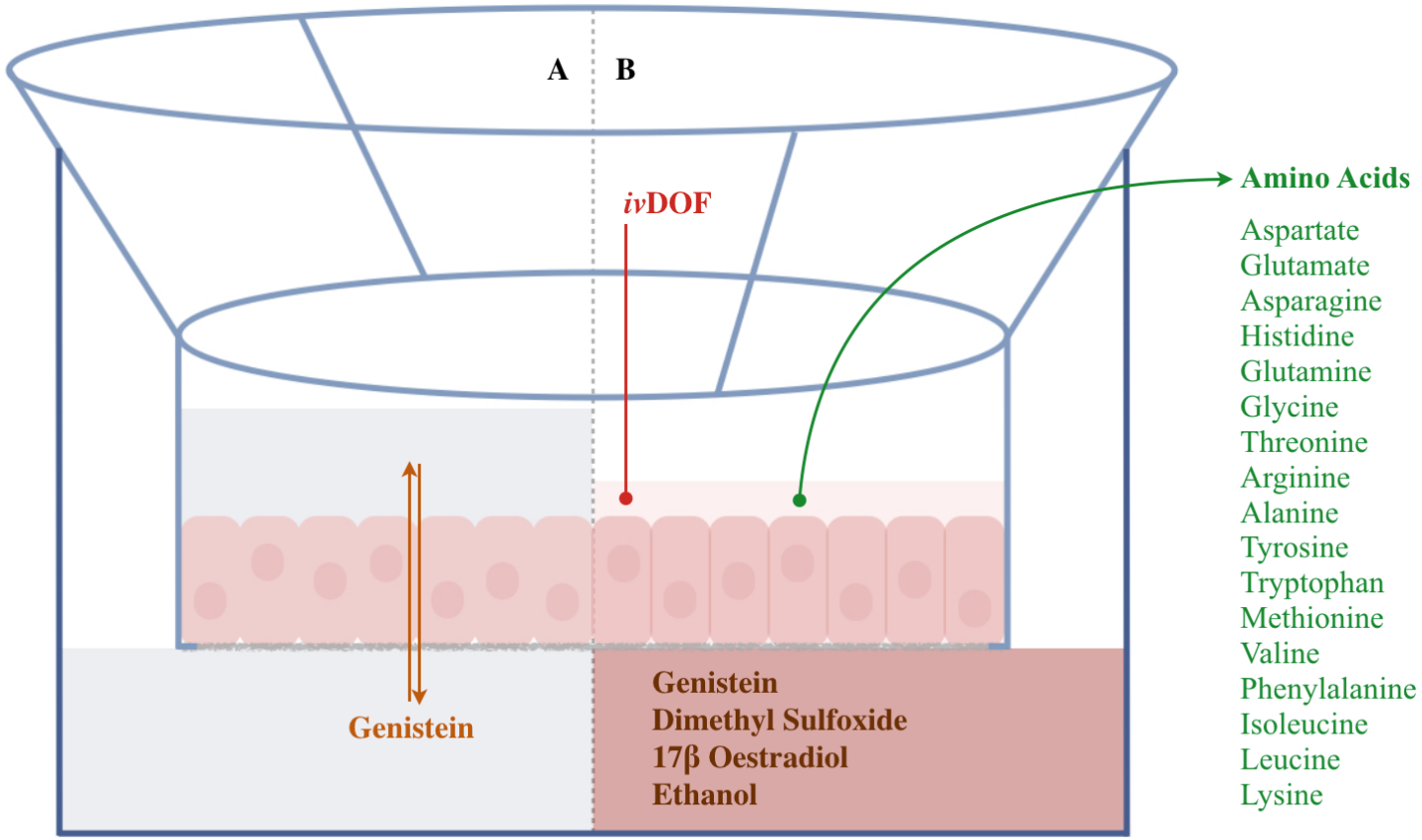

Figure 5-2 — Schematic diagram of the basic experimental premise of this chapter interested in **A**: the transport kinetics of genistein across the bovine oviduct epithelium at four concentrations, between 2ml Krebs Ringer medium apically and basally; and **B**: An analogous schematic comparing the impact of genistein and 17β-oestradiol on *iv*DOF composition in conjunction with associated vehicular controls: DMSO and ethanol.

5.2. Materials and Methods

5.2.1. Genistein Transport Studies

BOECs were cultured as described in Section 2.3.4. but at confluence were incubated for 20 minutes at 39°C in 5% CO_2 in pre-equilibrated normal Krebs Ringer medium; 2 ml were added basally and a further 2 ml apically. After incubation, genistein transport experiments were conducted by supplementing the basal chamber with genistein for 150 minutes at physiological and supraphysiological concentrations: 50 µM 100 µM, 150 µM or 180 µM. More specifically 50 µM and 100 µM were selected as physiological values (Konstantakopoulos *et al* 2006; Rajah *et al* 2012) whereas 150 µM and 180 µM were chosen to investigate the impact of supraphysiological concentrations of genistein on the oviduct as a proof of principle.

Krebs Ringer medium was sampled (30 µl) apically and basally at 15 minute intervals. TER values remained in excess of 700 $\Omega \cdot cm^{-2}$ throughout all experiments conducted. Unless otherwise stated transport experiments were done at 39ºC with the exception of sampling (~ 30 second duration) being performed at room temperature (~ 21ºC). Medium sampled for analysis was not replaced in order to avoid unnecessary dilution. The apparent permeability coefficient ($\mathbf{P}_{app}$) of genistein transport was also determined and is defined as:

Equation 5-1: $\mathbf{P}_{app} = \mathbf{dQ} \times \mathbf{dt}^{-1} \times (\mathbf{AC}_0)^{-1}$

where dQ/dt is the rate of genistein appearance ($\mu M \cdot min^{-1}$), C_0 is the initial concentration of genistein (µM), and A is the surface area of the monolayer (cm^2). P_{app} is therefore expressed in units of $cm^2 \cdot min^{-1}$ — adapted from Khan *et al* (2011).

5.2.2. Genistein Quantification

Samples collected from transport experiments were analysed individually using an Agilent 1100 HPLC coupled with an Agilent Zorbax™ C-18 silica based column. Genistein eluted from the column with a buffer consisting of 99.5% methanol + 0.5% formic acid and detected at 288 nm. A representative standard curve is provided in Figure 5-3.

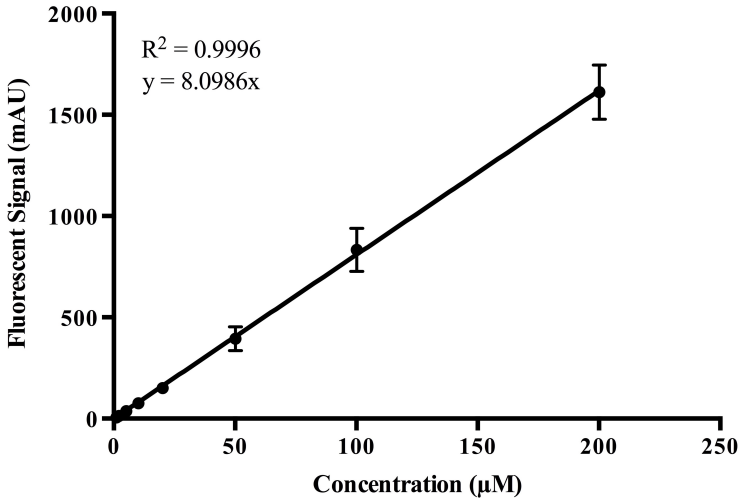

Figure 5-3 — Representative eight point standard curve depicting the strong positive linear relationship between the concentration of genistein (µM) and fluorescent signal detected by HPLC (mAU) with concentrations between 1 µM - 200 µM (n=3 ± SD).

5.2.3. Effect of Genistein on *iv*DOF

Upon reaching confluence (>700 $\Omega \cdot cm^{-2}$) cells were incubated in an air:liquid interface as previously described in Section 2.3.5. for 24 hours at 39°C in 5% CO_2 in culture medium basally supplemented with either 100 µM genistein. The *iv*DOF accumulated was subsequently stored at -20ºC until amino acid composition analysis was conducted by HPLC (Section 2.4.2.). TER values remained in excess of 700 $\Omega \cdot cm^{-2}$ throughout. The addition of 17β-oestradiol was conducted as described in Section 3.2.5. E2 was dissolved in ethanol whereas genistein was dissolved in dimethyl sulfoxide (DMSO).

5.3. Results

5.3.1. Transport Kinetics

At all four concentrations measured, genistein crossed the *in vitro* bovine oviduct epithelial monolayer in a basal to apical direction in accordance with a broadly polynomial (non-linear) kinetic profile (Figure 5-4). Moreover, genistein transport by BOECs was greater than the linear rate of spontaneous diffusion across a blank membrane (negative control).

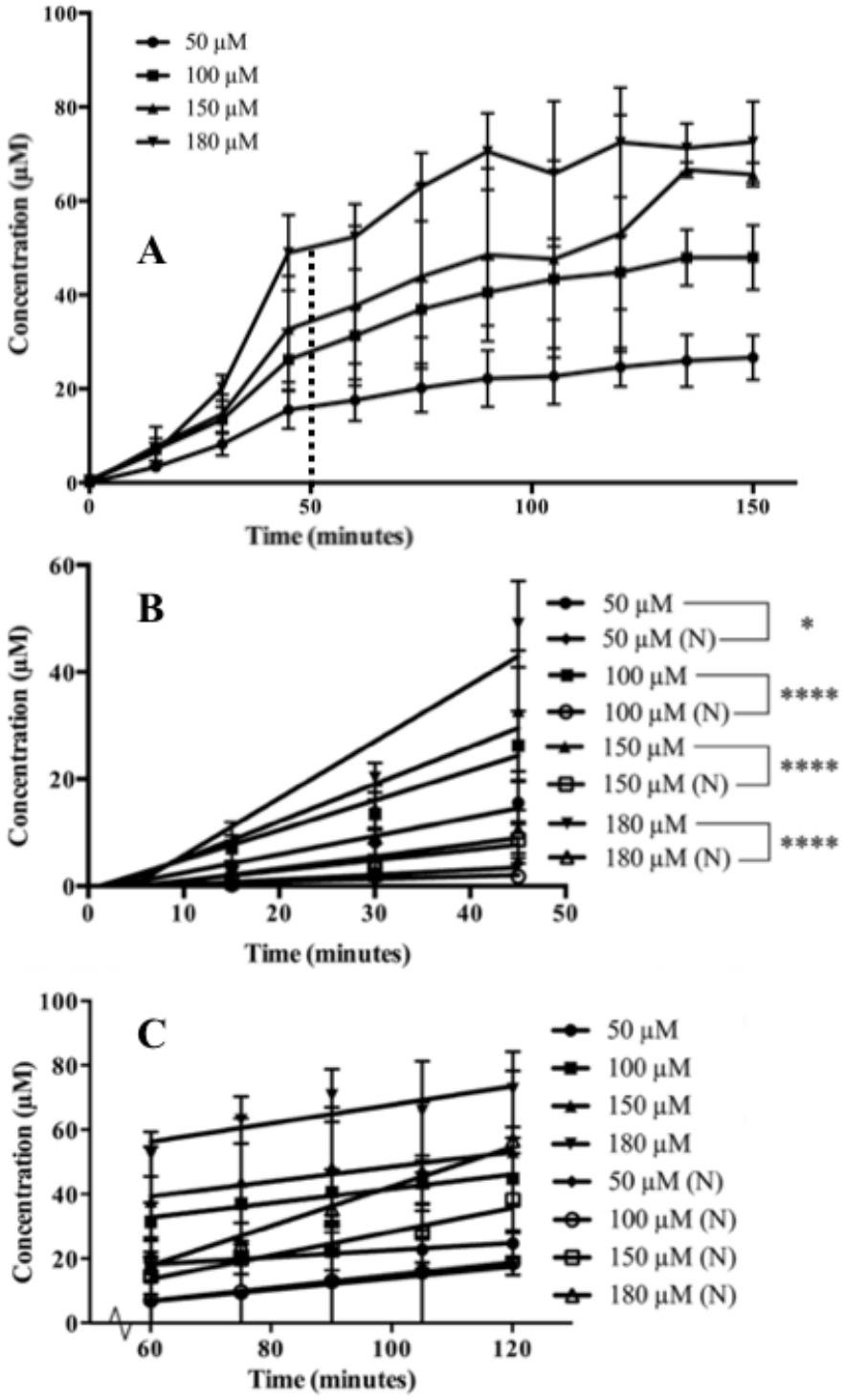

Figure 5-4 — **A**: Apical accumulation of genistein across bovine oviduct epithelial cell monolayers at 50 µM, 100 µM, 150 µM and 180 µM over two hours (n=3 ± SD). **B** & **C**: Dissected view of genistein flux across BOEC monolayers and genistein movement across empty, or blank, Transwell™ membranes (negative control) during the first 50 minutes (**B**) of flux and thereafter (**C**). Statistically significant differences were determined by two-way ANOVA followed by a Holm-Sidak *post hoc* analysis, where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Upon closer examination of the polynomial nature of Figure 5-4:A it became apparent that the movement of genistein across the oviduct epithelium was biphasic. Specifically, Figure 5-4:B reveals that genistein flux at all four concentrations measured occurred by two independent phases of transport; an initial concentration-dependent *burst* phase (Figure 5-4:B), followed by a slower concentration independent *plateau* phase (Figure 5-4:C) following 50 minutes of transport.

As a first approach to discover whether the *bust* and/or *plateau* phases of transport observed in Figure 5-5 were facilitated, the experiment was conducted at laboratory temperature (~21 ºC) in addition to the physiologically relevant incubator temperature of 39 ºC. Figure 5-5:A shows that this temperature reduction significantly reduced genistein flux at 20 mins.

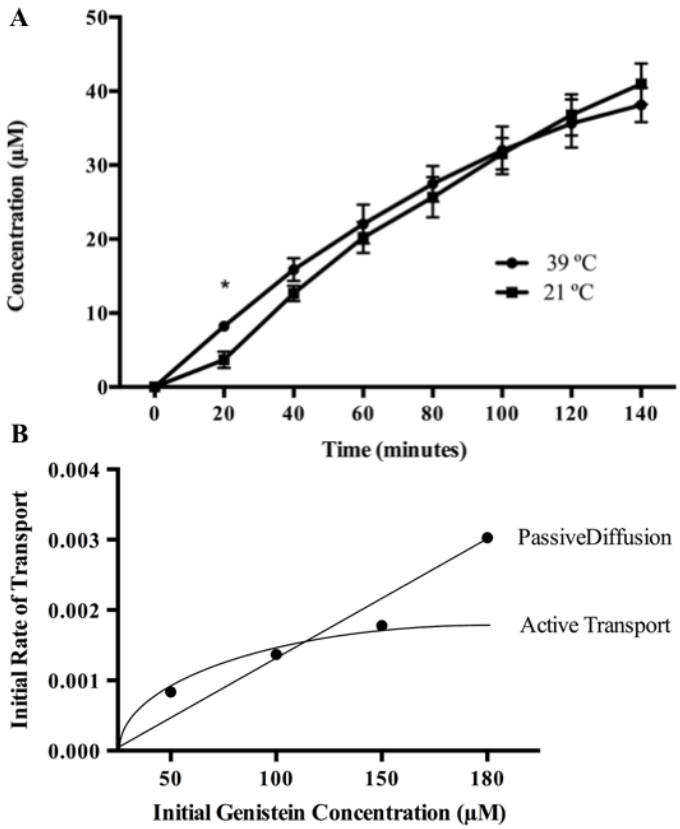

Figure 5-5 — **A**: The apical accumulation of 100 µM genistein across the bovine oviduct epithelium at physiological (39 ºC) and room (21 ºC) temperature (n=3 ± SD). One statistically significant difference was determined by unpaired t-test (p= 0.00226286). **B**: Genistein transport data plotted as the initial rate of transport ($\mu M \cdot min^{-1}$) *vs* the initial genistein concentration $[genistein]_0$ (µM), qualitatively showing a good fit to both passive ($R^2 = 0.868$) and facilitated ($R^2 = 0.770$) kinetic profiles (n=3).

Taking this observation further and plotting the data from Figure 5-4:A as the initial rate of genistein transport and the initial concentration of substrate (Figure 5-5:B) shows that the data fit both classic facilitated and passive kinetic profiles.

To determine whether there was a difference in the directionality of transport, 100 µM genistein was added to the apical chamber and its depletion measured in comparison to that from the basal to apical direction (Figure 5-6:A). The accumulation of genistein in the respective chambers was also measured (as in Figure 5-4) but shown as apparent permeability coefficients (P_{app}) (Section 5.2.1.). The difference between the P_{app} of the bovine oviduct epithelium to genistein flux

from apical to basal *vs* basal to apical (Figure 5-6:B) was negligible and not statistically significant (1.71 x 10^{-4} $cm^2 \cdot min^{-1}$). Thus there was no difference in directionality.

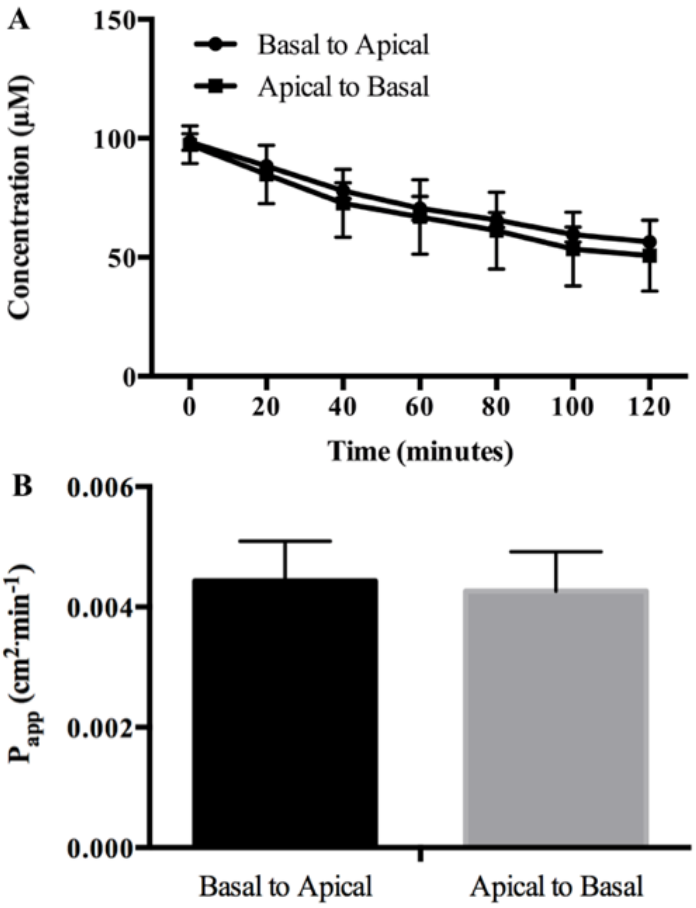

Figure 5-6 — **A**: The depletion of genistein from apical and basal compartments plotted as concentration (µM) *vs* time (minutes) (n=4 ± SD). **B**: The respective P_{app} value of the bovine oviduct epithelium to genistein accumulation in the respective chambers was 4.43 x 10^{-3} $cm^2 \cdot min^{-1}$ (basal to apical; n=7 ± SEM) and 4.26 x 10^{-3} $cm^2 \cdot min^{-1}$ (apical to basal; n=7 ± SEM) with a genistein concentration of 100 µM.

There was no difference in directionality when genistein was added at equilibrium (Figure 5-7:A). However when this experiment was conducted it became apparent that the total (sum) of genistein present from both chambers decreased over time. Figure 5-7:B is a condensed re-plot of the concentration of genistein internalised and/or degraded *vs* time.

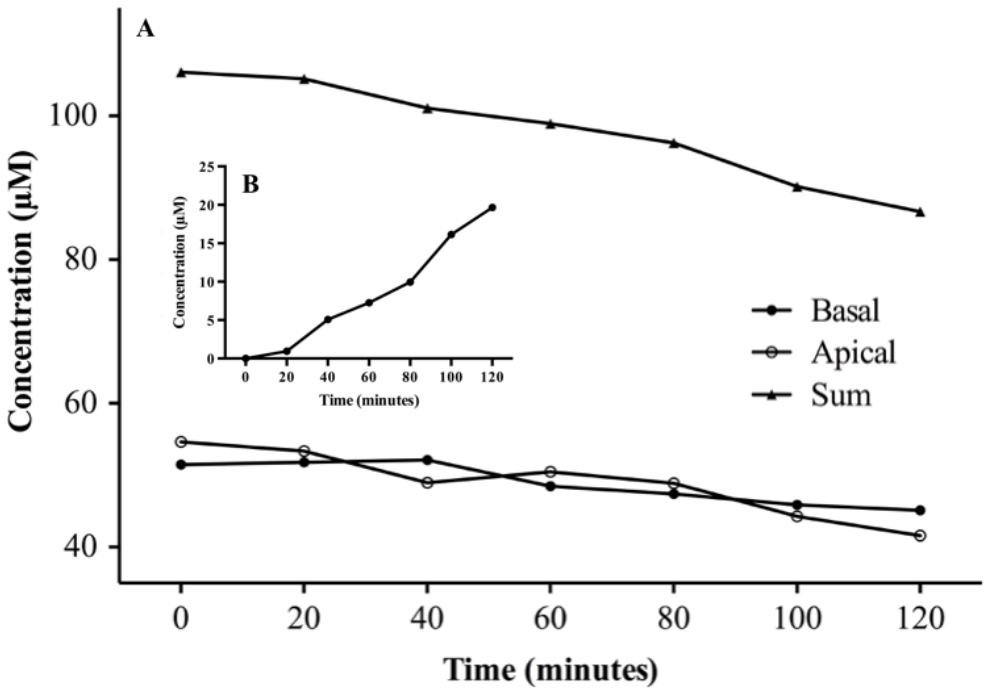

Figure 5-7 — **A**: The concentration of apical and basal genistein *vs* time when added in equilibrium at 50 µM·chamber^{-1} and **B**: The calculated (not experimentally determined) addition of apical and basal genistein concentrations (sum).

5.3.2. Effects on *iv*DOF

In order to investigate the impact of genistein on *in vitro* oviduct secretion, BOECs were cultured as described in Section 2.3. Once confluence was confirmed by a TER in excess of 700 Ω·cm^{-2} the basal chambers were supplemented with 100 µM genistein. The apical medium was subsequently removed and *iv*DOF allowed to accumulate for 24 hours (Section 2.3.5.). Figure 5-8 shows that genistein significantly modified the secretion of 12 of the 18 amino acids measured relative to native. However Figure 5-8 also shows that the vehicle DMSO also influenced the amino acid composition of *iv*DOF. From Figure 5-8 therefore it seems that genistein does act independently of DMSO, but not as an oestrogen analogue.

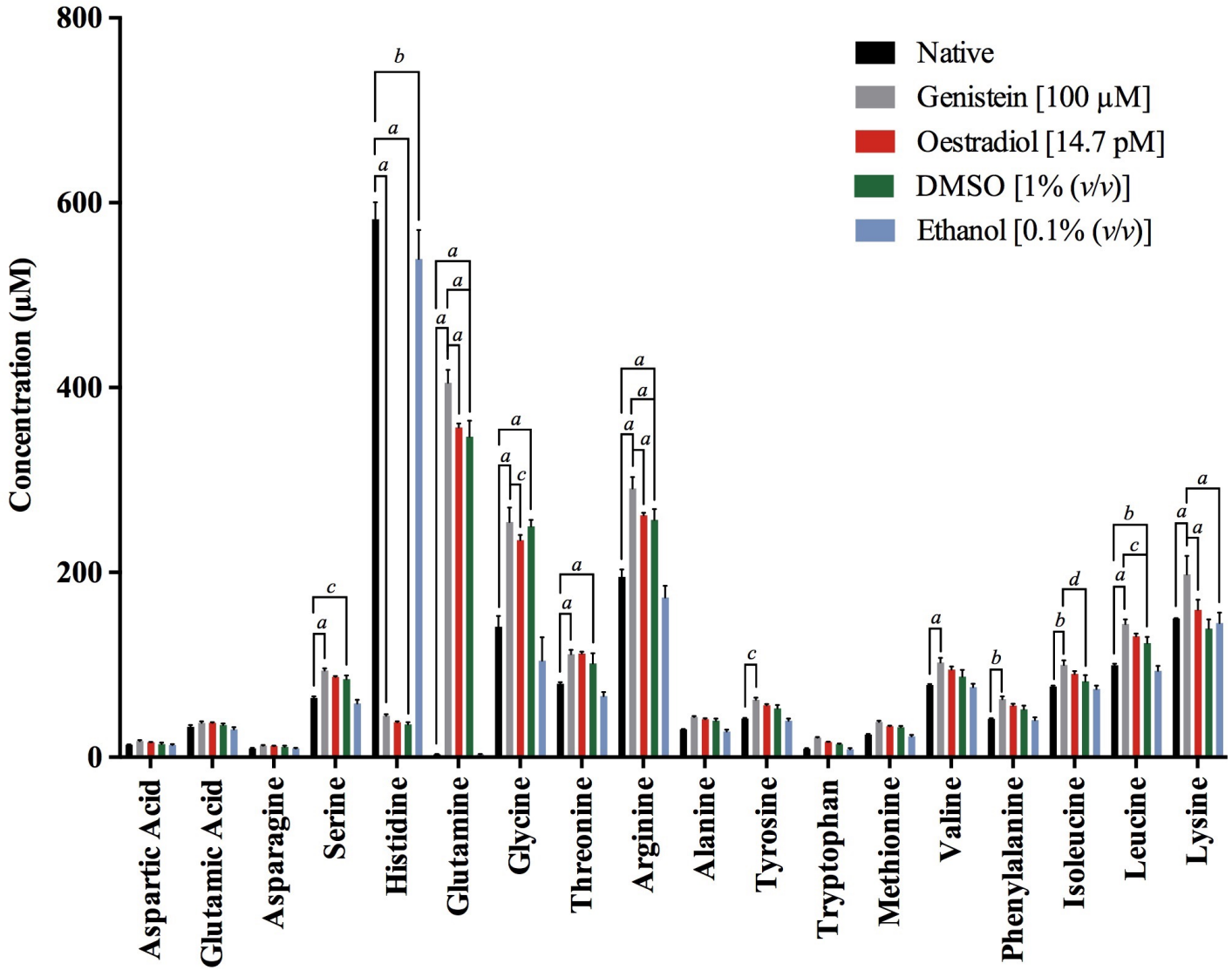

Figure 5-8 — The amino acid composition of *iv*DOF accumulated apically from native (untreated) BOECs (n=3 ± SD) (black) *vs* that from BOECs basally treated with 100 µM genistein (n=6 ± SD) (grey) *vs iv*DOF derived from oviduct epithelia exposed to 14.7 pM 17β-oestradiol (n=3 ± SD) (red) *vs iv*DOF derived from BOECs supplemented with 1% *v/v* dimethyl sulfoxide (DMSO) - the genistein vehicle control (n=3 ± SD) (green) *vs iv*DOF derived from BOECs supplemented with 0.1% *v/v* ethanol – the 17β-oestradiol vehicle control (n=3 ± SD). *iv*DOF accumulated over 24 hours. Statistically significant differences were determined by two-way ANOVA followed by a Holm-Sidak *post hoc* analysis, where a represents $p \leq 0.0001$, *b* represents $p \leq 0.001$, *c* represents $p \leq 0.01$, and *d* represents $p \leq 0.05$.

Ethanol addition had little impact on the amino acid composition of *iv*DOF. Moreover, although not an original aim of this study, the amino acid profiles of *iv*DOF derived from BOECs treated with DMSO and 17β-oestradiol were similar.

5.4. Discussion

The experiments in this chapter have been designed to test the application of the model described in Chapter 3; specifically to determine whether the dietary derived isoflavone and embryo-toxin genistein (**a**) transverses the oviduct epithelial monolayer, and/or (**b**) significantly alters the amino acid composition of *iv*DOF.

5.4.1. Transport Kinetics

In line with the hypothesis (Section 5.1.4.), genistein crossed the oviduct epithelial membrane, and moreover appeared to do so in accordance with a biphasic (*burst* and *plateau* phase) kinetic profile (Figure 5-4). The initial *burst* phase of genistein flux occurred at a rate significantly higher than that of spontaneous diffusion across a Transwell™ membrane free of cells (Figure 5-4:B), whereas the subsequent *plateau* phase did not show a rate higher than simple diffusion (Figure 5-4:C).

A secondary aim was to determine the potential mechanism(s) of apical genistein accumulation. From an energetic perspective, the fact that genistein initially transversed the oviduct epithelium faster than the rate of diffusion (Figure 5-4) indicates that genistein flux into the oviduct lumen *in vitro* is highly likely to be driven by facilitated diffusion, although primary active (ATP-dependent) transport is possible.

Active transport generally involves the movement of compounds against a concentration gradient. Nonetheless rare exceptions have been identified, such as the active flux of ranitidine across Madin-Darby canine kidney (MDCK) cell monolayers despite an energetically favourable ranitidine concentration gradient (Cruciani *et al* 2000).

It is well established that active transport is temperature dependent (Neuhoff *et al* 2005). Specifically, the rate of active transport decreases as the temperature deviates from physiological (Hidalgo & Borchardt 1990) — temperature increases induce protein denaturation whereas temperature decreases reduce cellular metabolism and ATP availability (Wishart 1984; Soini et al 2005; Amato & Christner 2009).

To test whether either phase (*burst* or *plateau*) of genistein flux might be active, the transport experiment was repeated at laboratory temperature (~21 ºC). Figure 5-5:A shows that the ~18 ºC temperature reduction significantly impaired genistein movement at 20 mins, suggesting that the initial *burst phase* observed could be partly actively mediated. However when genistein was added to both apical and basal compartments of the Transwell™ membranes in equilibrium (Figure 5-6), there was no transport of genistein against a concentration gradient, and therefore no active movement. Hence the most likely mechanism underlying the initial *burst phase* of genistein flux is facilitated diffusion, with the decreased transport at 20 minutes at 21 ºC, perhaps attributable to a decrease in Brownian motion (Mori 1965) of genistein at 21 ºC and therefore impairing flux.

With regard to the *plateau* phase, transport remaining unaffected by a reduction in temperature (Figure 5-5:A) and a flux rate comparable to spontaneous genistein diffusion (Figure 5-4:C), both implied that the *plateau* phase of flux occurs by passive diffusion as genistein approaches equilibrium (Peterson *et al* 2007).

In addition, passive transport is kinetically characterised as the positive linear relationship between the initial rate of transport and the initial concentration of substrate, whereas facilitated transport is kinetically characterised as a positive hyperbolic relationship between the initial rate of transport and the initial concentration of substrate (Turner 2000). With this in mind, the experimentally determined initial rates of genistein transport relative to the initial concentration of genistein present were plotted (Figure 5-5:B) and revealed that genistein permeation of the *in vitro* oviduct epithelium fitted both passive and facilitated curves with regression coefficients (R^2) of 0.868 and 0.770 respectively.

Spatially, three potential routes for genistein transport exist: (***i***) paracellular flux, (***ii***) intracellular movement or (***iii***) a combination of both routes (Figure 1-9). Paracellular flux is a passive (ATP-independent) process whilst intracellular flux is generally an active (ATP-dependent) process (Aronson 1981). Given the lack of evidence for any actively mediated flux, it is most likely that genistein transverses the *in vitro* oviduct paracellularly. To this end, an explanation for the facilitated *burst phase* observed could be the bovine oviduct epithelial phospholipid exterior serving as a surface catalyst, thereby accelerating of transport of this small hydrophobic molecule until close to equilibrium.

The directionality of genistein transport was also examined. Neither the depletion of genistein from either chamber (Figure 5-6:A) nor the apparent permeability coefficient of genistein accumulation in the apical or basal chambers (Figure 5-6:B) differed. Therefore no difference in directionality was observed. This was also the case upon genistein addition in equilibrium (Figure 5-7:A). Moreover, from Figure 5-7:B it is apparent that there was some genistein disappearance over time. Whether this was degradation or cellular internalisation is unknown, but could be a subject for future research.

Although genistein has never been shown to cross the oviduct epithelium before, it is known to transverse other cellular monolayers *in vitro* including rat immortalised small intestinal epithelial (IEC-18) (Steensma *et al* 2004), human corneal epithelial cells (HCEC) (Steensma *et al* 2004), rat brain microvascular (BBB) endothelial cells (Yang *et al* 2014), and human colorectal carcinoma epithelial (Caco-2) cells (Oitate *et al* 2001; Murota *et al* 2002; Steensma *et al* 1999; Yang *et al* 2014).

Yang *et al* (2014) reported a P_{app} of genistein flux across Caco-2 monolayers of 16.23 x 10^{-6} $cm^2 \cdot sec^{-1}$ which equates to 0.974 x 10^{-3} $cm^2 \cdot min^{-1}$ determined using 20 μM genistein. Assuming linearity between P_{app} and genistein concentration (Norris & Powell 1990) their P_{app} value would be 4.74 x 10^{-3} $cm^2 \cdot min^{-1}$ for 100 μM. The analogous value obtained in BBB cells was 4.03 x 10^{-6} $cm^2 \cdot min^{-1}$ (Yang *et al* 2014). These findings are similar to the value obtained across the oviduct epithelium: 4.43 x 10^{-3} $cm^2 \cdot min^{-1}$ (Figure 5-6:B). From this it can determined that the oviduct epithelium is inherently less permeable to genistein than BBB cells but more so than Caco-2 cells, although these values are all broadly comparable.

The slight differences observed are interesting and tentatively point to the observation that that permeability to genistein may be proportional to TER; specifically cellular confluence is established at >500 $\Omega \cdot cm^{-2}$ in BBB cells (Yang *et al* 2014), >700 $\Omega \cdot cm^{-2}$ in bovine oviduct epithelia (Simintiras *et al* 2012), and >900 $\Omega \cdot cm^{-2}$ in Caco-2 cells (Ciarlet *et al* 2001). This observation supports the notion that genistein flux across cellular monolayers *in vitro* is paracellular.

Regardless of the exact mechanism of transport, these data suggest that gametes and early embryos could be directly exposed to genistein *in vivo*. To evaluate whether genistein might pose indirect effects, the amino acid composition of *iv*DOF from BOECs treated with genistein was investigated.

5.4.2. Effects of Genistein Supplementation on *iv*DOF

Figure 5-8 suggests that 100 μM genistein significantly influenced the secretion of 12 of 18 amino acids relative to native fluid. However after conducting the vehicle control experiment for genistein it was apparent that DMSO was also having an effect on the amino acid composition of *iv*DOF. The effect of genistein appeared to be independent of DMSO with regard to the secretion of 5 amino acids measured; specifically, the transport of glutamine, glycine, arginine, leucine, lysine and isoleucine was significantly different between DMSO *vs* genistein treated groups. It is difficult to explain this pattern since these 5 amino acids do not share common transporters or chemical characteristics (Figure 3-1). Nonetheless, although it is difficult to evaluate any specific effect of genistein on the *in vitro* oviduct beyond that of DMSO, it appears that genistein does alter the amino acid composition of *iv*DOF. In light of this, the aim became to evaluate whether genistein acts on the *in vitro* oviduct epithelium as a 17β-oestradiol analogue.

The affinity of genistein for ERα and and ERβ is 0.7% and 13% respectively of that for the endogenous ligand 17β-oestradiol (Kuiper *et al* 1998). Although not a high percentage, the corresponding dissociation constants can be calculated to be as low as 7 nM for ERα and 0.6 nM for ERβ. Given these high affinities of genistein for the oestrogen receptors, it is unsurprising that genistein exerts oestrogenic effects by activating oestrogen response element (ERE) regulated genes in several mouse tissues, as determined by ERE-luciferase linked reporter assays (Montani *et al* 2008), activating ERE regulated genes in human breast cancer cells as determined by qRT-PCR mRNA quantification (Wang *et al* 1996), and by means of modulating rat behaviour (Pisani *et al* 2012).

To evaluate whether genistein might be acting on the *in vitro* oviduct as a 17β-

oestradiol mimic, BOECs were treated with 17β-oestradiol at a physiological concentration of 14.7 pM. Although it was predicted that the amino acid profiles of *iv*DOF obtained from genistein and 17β-oestradiol would be similar, it was observed that 7 of the 18 amino acids measured were significantly different (Figure 5-8). The data therefore suggest that genistein does change the composition of oviduct secretions *in vitro* but not as a 17β-oestradiol mimic. Furthermore, with the exception of histidine, the 17β-oestradiol vehicle control (ethanol) had no effect on *iv*DOF composition.

In spite of this, an interesting observation was revealed — there are no differences between the amino acid profiles of *iv*DOF derived from 17β-oestradiol and DMSO treated cells. This raised the question of whether DMSO could be acting as a 17β-oestradiol mimic (Chapter 7).

5.4.3. Summary

Numerous studies have been conducted on the bioavailability of isoflavones in adults (Wantanabe *et al* 1998; Rowland *et al* 2000; Setchell *et al* 2003a, 2003b; Markiewicz *et al* 1993) but little is known about the possible delivery and effects of flavonoid conjugates on specific tissues, including the oviduct. The data presented show that (***a***) the oviduct epithelium is permeable to genistein, and facilitates transport into the lumen in accordance with a biphasic kinetic profile. and (***b***) that genistein presence impacts the amino acid composition of *iv*DOF.

Supported by the data presented it is proposed that genistein transverses the bovine oviduct epithelium paracellularly, initially by facilitated diffusion followed by passive diffusion. Further work is required to confirm the exact mechanism(s) and the potential relative contribution to genistein flux of each (*burst* or *plateau*) phase.

Within the wider context of biology the results demonstrate the potential use of this *in vitro* model in characterising the transport or barrier properties of the oviduct towards a range of circulating xenobiotics. The broader impact of these findings is discussed in Chapter 6.

Chapter 6

Uterine Luminal Fluid Composition Assessment *in vivo*

Chapter 6 — Uterine Luminal Fluid Composition Assessment *in vivo*

6.1. Introduction

6.1.1. Conceptus-Maternal Interactions

As with the oviduct, early conceptus-maternal interactions involve the secretion of key nutrients and molecules by the bovine reproductive tract; specifically the bovine uterine epithelial cells (BUECs) to aid conceptus development (Forde *et al* 2012; Dorniak *et al* 2012; Bazer *et al* 2012; Bazer 1975). Conceptus-maternal interactions are critical to successful implantation/attachment as in the bovine, embryonic loss usually occurs before pregnancy recognition, at approximately day 16 of pregnancy (Diskin & Morris 2008; Betteridge *et al* 1980).

This need for uterine-derived secretions and transported nutrients has been well established in the sheep uterine gland knockout model wherein the conceptus fails to elongate (Gray *et al* 2002). Furthermore, uterine luminal fluid (ULF) *in vivo* is a prerequisite for embryo development beyond the hatched blastocyst stage since attempts to induce elongation of bovine conceptuses *in vitro* have been unsuccessful (Alexopoulos *et al* 2005; Brandao *et al* 2004) although Shahbazi *et al* (2016) and Deglincerti *et al* (2016) have recently achieved this in the human.

Much like oviduct fluid, the ULF of ruminants comprises selectively transported molecules including glucose (Gao *et al* 2009), ions (Gao *et al* 2009), fatty acids (Meier *et al* 2011), amino acids (Gao *et al* 2009; Groebner *et al* 2011) and various secreted proteins (Berendt *et al* 2005). Moreover, the expression of amino acid transporters is spatiotemporally dynamic in both the endometrium and conceptus of sheep during early pregnancy (Gao *et al* 2009) and is modulated *in vivo* by P4, interferon tau (IFNτ), prostaglandins, and cortisol (Dorniak *et al* 2011; 2012).

However in spite of the importance of uterine secretions, and knowledge that a

major component, amino acids, are utilised by the early embryo both *in vitro* and *in vivo* (Morris *et al* 2002; Leese 2012; Wale & Gardner 2010; Sturmey *et al* 2010), little is known about the requirements for conceptus elongation and successful pregnancy recognition in cattle. For this reason the first aim of this chapter was to analyse the amino acid content of ULF during the oestrus cycle and peri-implantation period.

6.1.2. Sex Linked Conceptus-Maternal Interactions

Uterine endometrial responses to a conceptus can be detected as early as day 13 (Forde *et al* 2012) however, initial pregnancy recognition occurs on day 16 when IFNτ is released from the trophectoderm of the conceptus and causes the endometrium to undergo significant transcriptomic changes (Forde *et al* 2011).

Interestingly, transcriptomic profiles of the endometrium at day 20 are predictive of the type of conceptus (*in vivo* or *in vitro* derived or cloned) present (Mansouri-Attia *et al* 2009; Bauersachs *et al* 2009). Consequently, it was decided to explore whether the same was the case for male *vs.* female conceptuses, and to examine whether any transcriptomic changes were reflected in the amino acid composition of the respective ULF.

6.1.3. Effects of Lactation on Pregnancy

A significant challenge facing the dairy industry is dairy cow subfertility in arising from negative energy balance, *i.e.* the deficit between energy intake and expenditure. This leads to elevated non-esterified fatty acid (NEFA) and beta hydroxy butyrate (BHBA) levels in addition to decreased circulating insulin, insulin-like growth factor 1 (IGF-1), and glucose (Rizos *et al* 2010; Forde *et al* 2015).

While the effects of such a physiological challenge on the follicular environment are well known (Leroy *et al* 2015) additional evidence suggests that the reproductive tract of the lactating dairy cow may be compromised in response to negative energy balance in its ability to support early development compared to nulliparous heifers (Rizos *et al* 2010) and postpartum non-lactating cows (Forde *et al* 2015). Furthermore gene expression analyses have shown that a negative energy balance alters oviduct (Fenwick *et al* 2004) and endometrial gene expression (Cerri *et al* 2012). It is unclear however whether global conceptus gene expression is affected by lactation status.

6.1.4. Aims and Objectives

The previous chapters have involved the characterisation of the *in vitro* oviduct preparation (Chapter 3) and its application in studying the impact of hyperandrogenaemia and insulin (Chapter 4), and dietary and pharmacological manipulation (Chapter 5) on bovine oviduct epithelial cell luminal fluid formation and physiology. In parallel to these experiments, various aspects of the *in vivo* bovine ULF were examined. Particular emphasis was placed on how the composition of ULF collected *in situ* is influenced by (***a***) early pregnancy (specifically the presence of male *vs* female embryos) and (***b***) the physiological status of the animal (lactating *vs* dry cows *vs* heifers). The aims of this chapter were thus to (***i***) discover whether the amino acid content of bovine ULF varies during the oestrus cycle and early pregnancy, (***ii***) determine whether conceptus sex impacts on ULF amino acid composition at day 19, and (***iii***) examine whether the amino acid and carbohydrate composition of ULF differs between lactating *vs* dry cows *vs* heifers.

6.2. Materials and Methods

HPLC and carbohydrate analyses were conducted as described in Sections 2.4.2. and 2.4.9. respectively. The data presented in this chapter formed part of a collaborative link with Professor Patrick Lonergan and Dr Niamh Forde at University College Dublin (UCD), who developed the overall experimental design and performed the experiments. All work involving animals was reviewed by UCD institutional ethical review panels and performed in accordance with local regulations.

6.2.1. Conceptus-Maternal Interactions

The oestrous cycles of 100 cross-bred beef heifers were synchronised and those in standing heat (day 0) were randomly assigned to either an inseminated group (n = 59) or a non-inseminated cyclic control group (n = 24). Inseminated heifers were slaughtered on day 7, 10, 13, 16 or 19 of pregnancy whereas cyclic heifers were slaughtered on day 7, 10, 13, or 16 of their cycle — timed to correspond with the various stages of blastocyst development. At slaughter the uterine horn ipsilateral to the corpus luteum was flushed with 20 ml of 10 mM Tris at pH 7.2 whereas for the inseminated group, only flushings containing an appropriately developed conceptus were further processed. Recovered ULF was centrifuged at 1000 x *g* for 15 minutes and the supernatant was immediately snap frozen in liquid nitrogen and stored at -80 °C prior to amino acid analysis.

6.2.2. Sex Linked Conceptus-Maternal Interactions

Cross bred heifers were synchronised as described above, with those subsequently in standing oestrus inseminated with semen from a proven sire (n=30). Heifers were slaughtered on day 19 following oestrus to correspond with the initiation of

conceptus implantation. Each uterine horn was flushed with 10 ml PBS at 30 minutes post-slaughter and only flushings containing an appropriately developed conceptus were further processed (n=24). All samples were snap frozen in liquid nitrogen and stored at -80 °C prior to amino acid analysis.

6.2.3. Effects of Lactation on Pregnancy

In-calf dairy heifers (n=40) and maiden dairy heifers (n=20) were enrolled into the study and following calving the cows were randomly assigned to a lactating or non-lactating group. Animals in the lactating group were milked twice a day whereas those of the non-lactating group were never milked. In total there were three treatment groups — (***i***) lactating cows (n=20), (***ii***) non-lactating (dry) cows (n=20), and (***iii***) maiden heifers (n=20). The animals were synchronised as described above following insemination and slaughter in accordance to the experimental outline depicted by Figure 6-1. The uteri were flushed within 20 minutes of slaughter as previously described.

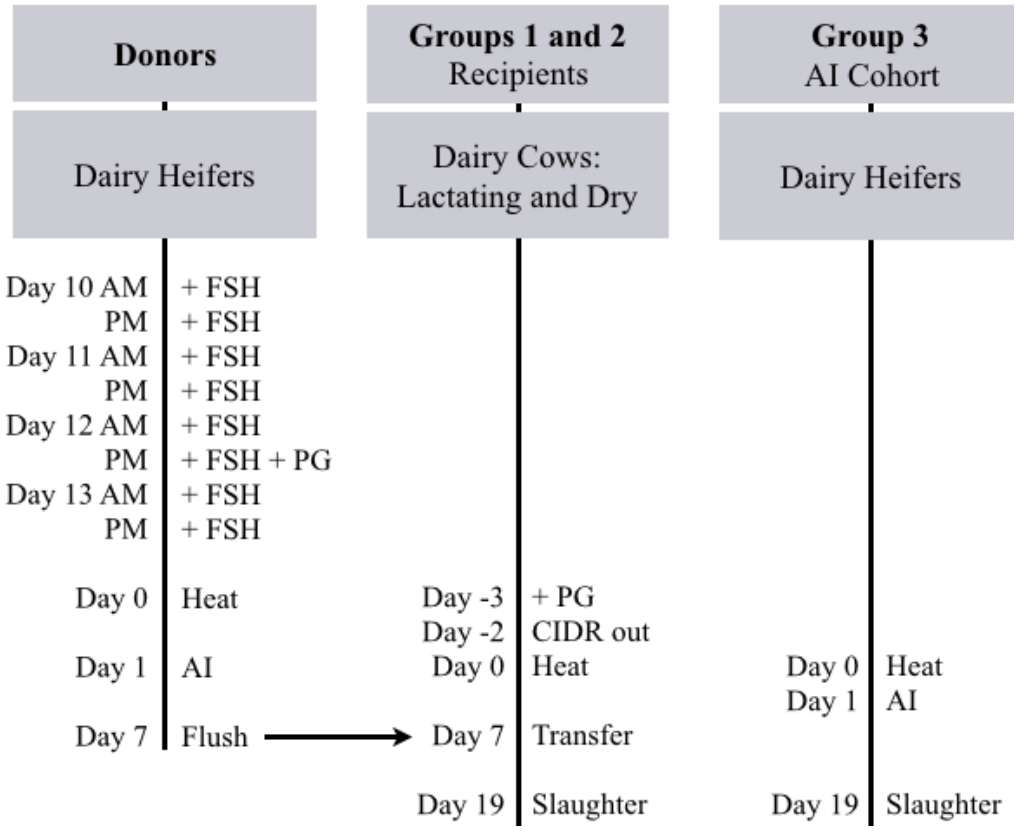

Figure 6-1 — Schematic depiction of the synchronisation protocol for animals from which uterine luminal fluid was collected. Abbreviations are: follicle stimulating hormone (FSH), prostaglandin F2α (PG), artificial insemination (AI), and controlled intra-vaginal drug-releasing device (CIDR).

6.2.4. Statistical Analyses

For Figures 6-2, 6-3, and 6-5 the statistical analyses were performed using Prism Graphpad 6 software by two way analysis of variance (ANOVA) followed by a Holm-Sidak non-parametric post hoc analysis where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ as described in Section 2.6. In Figure 6-4 statistically significant difference were similarly determined but using the multiple (unpaired) t-test function. For Table 6-1 the statistics were performed by Dr Alan Kelly at University College Dublin using the SAS statistical package (SAS Institute Inc., Cary, NC, version 9.1.3) mixed procedure.

6.3. Results

In line with the first aim — to test whether the amino acid content of bovine ULF is temporally dynamic during the oestrus cycle and early pregnancy — Table 6-1 describes the impact of pregnancy on amino acid composition of bovine ULF. In pregnant animals, arginine, glutamine, and methionine were more abundant in ULF compared to that from non-pregnant cycling animals. Furthermore, the arginine, aspartate, asparagine, threonine, glutamine, methionine, and valine composition of ULF varied depending on the day of pregnancy. However most notably, when both pregnancy and day effects were considered, differences in threonine, glutamate, and valine flux in ULF were observed.

Amino Acid	Treatment	Day 7	Day 10	Day 13	Day 16	Day 19	Day Effect	Pregnancy Effect	Day x Pregnancy
Arginine	*Cyclic*	12.34 ± 2.77 *ax*	8.34 ± 2.48 *ax*	13.32 ± 2.48 *ax*	7.31 ± 2.77 *bx*	-	***	*	ns
	Pregnant	7.89 ± 2.26 *ay*	5.97 ± 2.26 *ay*	7.32 ± 2.26 *ay*	7.14 ± 2.77 *ax*	15.94 ± 7.15 *b*			
Histidine	*Cyclic*	5.67 ± 1.21 *ab*	4.23 ± 1.21 *ab*	5.79 ± 1.21 *a*	2.37 ± 1.21 *b*	-	ns	ns	ns
	Pregnant	3.69 ± 0.99 *a*	4.26 ± 0.99 *a*	5.01 ± 1.40 *ab*	4.33 ± 1.21 *a*	8.54 ± 2.88 *b*			
Lysine	*Cyclic*	16.38 ± 2.92 *a*	9.78 ± 2.90 *ab*	13.57 ± 2.61 *a*	5.58 ± 1.75 *b*	-	ns	ns	ns
	Pregnant	10.38 ± 2.95 *a*	7.23 ± 2.76 *a*	8.43 ± 1.77 *a*	9.55 ± 10.70 *a*	21.47 ± 8.51 *b*			
Aspartate	*Cyclic*	12.52 ± 2.92 *a*	12.62 ± 2.61 *a*	12.25 ± 2.61 *a*	4.50 ± 2.92 *b*	-	*	ns	ns
	Pregnant	9.08 ± 2.38 *a*	9.70 ± 2.38 *a*	8.93 ± 2.38 *a*	4.13 ± 2.92 *b*	9.76 ± 4.37 *a*			
Glutamate	*Cyclic*	47.11 ± 10.66 *a*	43.84 ± 9.54 *a*	43.54 ± 10.74 *a*	22.06 ± 16.60 *a*	-	ns	ns	ns
	Pregnant	38.58 ± 7.60 *a*	39.53 ± 4.72 *a*	40.40 ± 7.74 *a*	21.76 ± 2.42 *a*	69.43 ± 27.18 *b*			
Alanine	*Cyclic*	35.27 ± 8.09 *a*	39.35 ± 7.24 *a*	42.54 ± 7.24 *a*	23.90 ± 8.09 *a*	-	ns	ns	ns
	Pregnant	26.63 ± 6.61 *a*	27.54 ± 6.61 *a*	30.28 ± 6.61 *a*	30.59 ± 8.09 *a*	51.13 ± 17.99 *a*			
Asparagine	*Cyclic*	3.12 ± 0.35 *ax*	1.63 ± 0.35 *b*	1.44 ± 0.31 *b*	0.80 ± 0.40 *b*	-	***	ns	0.1
	Pregnant	2.14 ± 0.29 *ay*	1.33 ± 0.29 *b*	1.28 ± 0.40 *ab*	1.68 ± 0.35 *ab*	3.41 ± 1.47 *ab*			
Glycine	*Cyclic*	114.12 ± 42.78 *a*	150.84 ± 92.73 *a*	65.91 ± 28.07 *a*	52.35 ± 30.50 *a*	-	ns	ns	ns
	Pregnant	129.90 ± 57.83 *a*	133.98 ± 77.48 *a*	57.41 ± 36.66 *a*	30.41 ± 6.00 *a*	54.78 ± 22.77 *a*			
Serine	*Cyclic*	21.63 ± 3.94 *a*	20.42 ± 3.52 *a*	17.27 ± 4.55 *a*	14.89 ± 3.94 *a*	-	ns	ns	ns
	Pregnant	15.78 ± 3.22 *a*	13.13 ± 3.22 *a*	22.23 ± 3.52 *a*	22.90 ± 3.94 *a*	27.64 ± 9.26 *a*			
Threonine	*Cyclic*	177.03 ± 14.41 *ax*	268.62 ± 12.89 *bx*	213.52 ± 12.89 *ax*	181.33 ± 14.41 *ax*	-	***	0.06	***
	Pregnant	208.61 ± 11.77 *ax*	195.70 ± 12.89 *ay*	212.91 ± 11.77 *ax*	151.07 ± 14.41 *bx*	124.53 ± 5.96 *b*			
Glutamine	*Cyclic*	24.37 ± 4.25 *ax*	17.08 ± 4.45 *abx*	24.18 ± 4.17 *ax*	12.74 ± 3.74 *bx*	-	***	***	***
	Pregnant	14.60 ± 7.07 *ax*	16.47 ± 7.45 *ax*	21.97 ± 8.20 *ax*	18.17 ± 1.62 *by*	36.79 ± 11.89 *c*			
Isoleucine	*Cyclic*	8.81 ± 1.58 *a*	5.87 ± 1.41 *ab*	7.36 ± 1.41 *ab*	3.27 ± 1.58 *b*	-	ns	ns	ns
	Pregnant	5.79 ± 1.29 *a*	4.45 ± 1.29 *a*	4.39 ± 1.29 *a*	5.15 ± 1.58 *a*	9.78 ± 3.82 *b*			
Leucine	*Cyclic*	17.60 ± 3.26 *a*	12.27 ± 2.91 *ab*	15.73 ± 2.91 *a*	6.57 ± 3.26 *b*	-	ns	ns	ns
	Pregnant	11.78 ± 2.66 *a*	9.18 ± 2.66 *a*	9.73 ± 2.66 *a*	10.45 ± 3.26 *a*	20.28 ± 9.32 *b*			
Methionine	*Cyclic*	10.98 ± 1.68 *ax*	11.83 ± 1.94 *ax*	5.92 ± 1.50 *bx*	2.85 ± 1.68 *bx*	-	***	*	ns
	Pregnant	7.83 ± 1.37 *ax*	6.97 ± 1.37 *aby*	4.48 ± 1.37 *abx*	3.43 ± 1.68 *bx*	7.66 ± 3.39 *a*			
Phenylalanine	*Cyclic*	7.46 ± 1.22 *a*	5.84 ± 1.22 *ab*	6.14 ± 1.09 *a*	2.77 ± 1.22 *b*	-	ns	0.07	ns
	Pregnant	5.14 ± 1.00 *a*	3.69 ± 1.00 *a*	3.59 ± 1.00 *a*	4.59 ± 1.22 *a*	9.48 ± 3.82 *b*			
Tryptophan	*Cyclic*	3.46 ± 0.83 *a*	3.42 ± 0.83 *a*	2.89 ± 0.96 *a*	1.23 ± 1.18 *a*	-	ns	ns	ns
	Pregnant	2.03 ± 0.68 *a*	2.65 ± 0.68 *a*	2.03 ± 0.96 *a*	2.43 ± 0.96 *a*	5.49 ± 2.64 *b*			
Tyrosine	*Cyclic*	7.55 ± 1.45 *a*	6.06 ± 1.45 *ab*	6.15 ± 1.30 *ab*	2.66 ± 1.45 *b*	-	ns	ns	ns
	Pregnant	4.82 ± 1.19 *a*	5.41 ± 1.19 *a*	3.66 ± 1.19 *a*	4.43 ± 1.45 *a*	8.45 ± 3.14 *b*			
Valine	*Cyclic*	15.57 ± 3.90 *ax*	10.07 ± 3.81 *ax*	13.15 ± 2.97 *ax*	6.33 ± 0.00 *bx*	-	***	ns	***
	Pregnant	10.88 ± 1.08 *ax*	8.81 ± 0.66 *bx*	7.89 ± 0.53 *bx*	9.31 ± 0.00 *cy*	17.10 ± 6.31 *d*			

Table 6-1 — The effect of day and pregnancy status on the abundance of amino acids (μM) in bovine uterine luminal fluid during the peri-implantation period of pregnancy on Days 7, 10, 13, 16 for cyclic heifers and Days 7, 10, 13, 16 and 19 for pregnant heifers (n=5 per treatment per time-point). Significance of day, pregnancy status, and their interactions are noted by an asterisk (*). Temporal differences are indicated by different suffixes – letters *a*, *b*, *c*. For instance significant differences in amino acid abundance between sequential days of the oestrus cycle or early pregnancy when $p \leq 0.05$. Differences between pregnant and cyclic heifers on a given day are denoted by x,y when $p \leq 0.05$.

In line with the second aim — to determine whether conceptus sex impacts ULF amino acid composition at Day 19 — Figure 6-1 shows that the composition of ULF differed depending on whether the conceptus was XX or XY. Specifically, asparagine, glutamine, arginine, tryptophan, methionine, phenylalanine,

isoleucine, and lysine were present at higher levels in the ULF of XY positive uteri *vs* XX containing uteri.

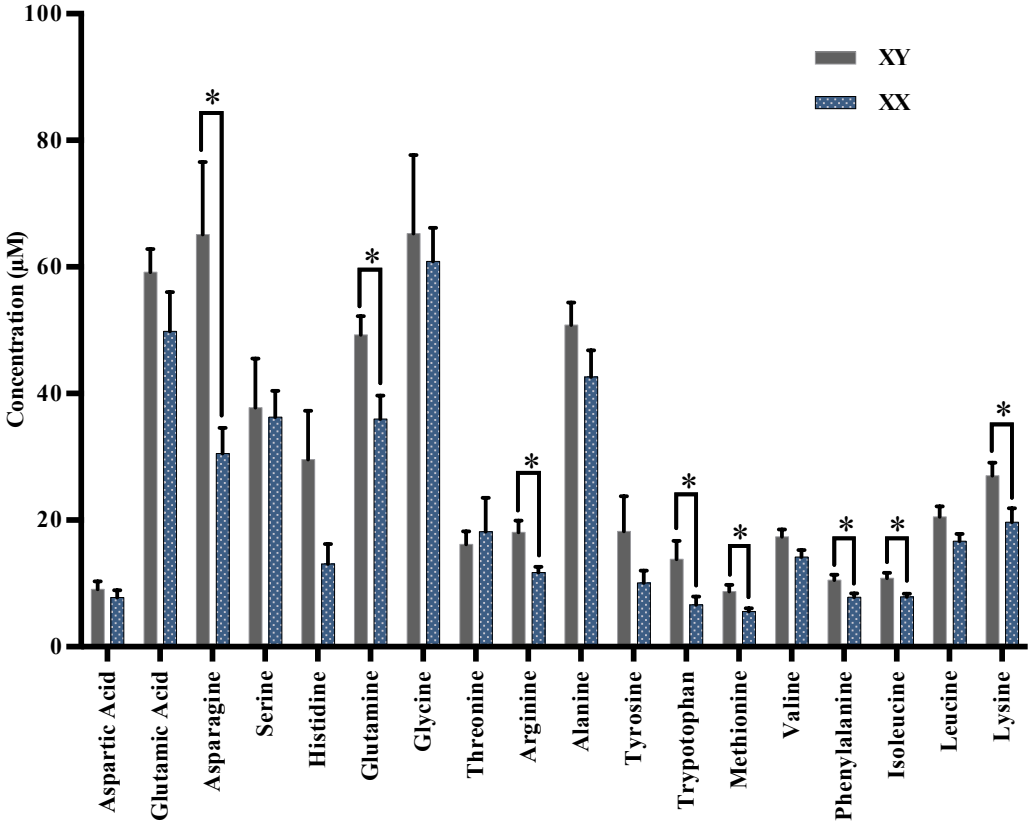

Figure 6-2 — Concentrations (µM ± SEM) of amino acids in the ULF recovered on day 19 of pregnancy from heifers with a male (black bars) or female (open bars) conceptus present. Significant differences in amino acid concentrations from the uterus containing a male versus a female conceptus are noted with an asterisk (*) when $p < 0.05$.

In line with the third aim — to investigate whether the lactation status of the animal influenced ULF composition — the amino acid (Figure 6-3) and carbohydrate (Figure 6-4) content of ULF from Lactating *vs* Dry cows *vs* Heifers was compared.

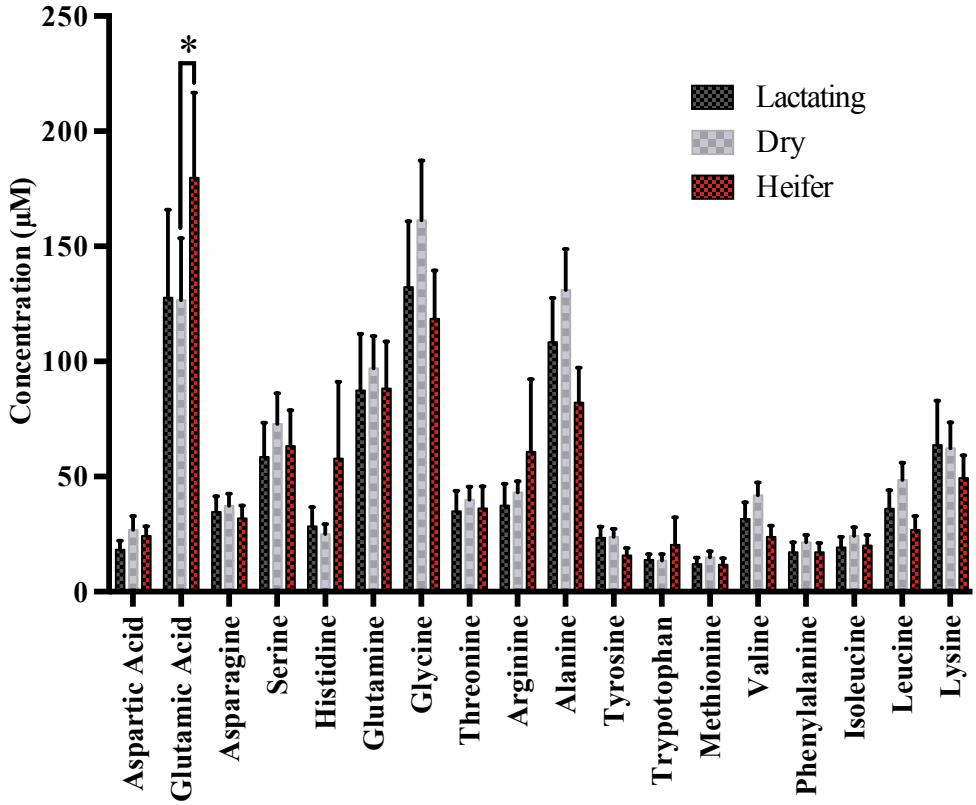

Figure 6-3 — Concentration (µM) of amino acids in the ULF from confirmed pregnant heifers (n=4), lactating cows (n=5), and non-lactating cows (n=5) on Day 19 of pregnancy following AI (heifers) or embryo transfer on Day 7 (lactating and non-lactating cows). One significant difference between groups was observed ($p < 0.05$).

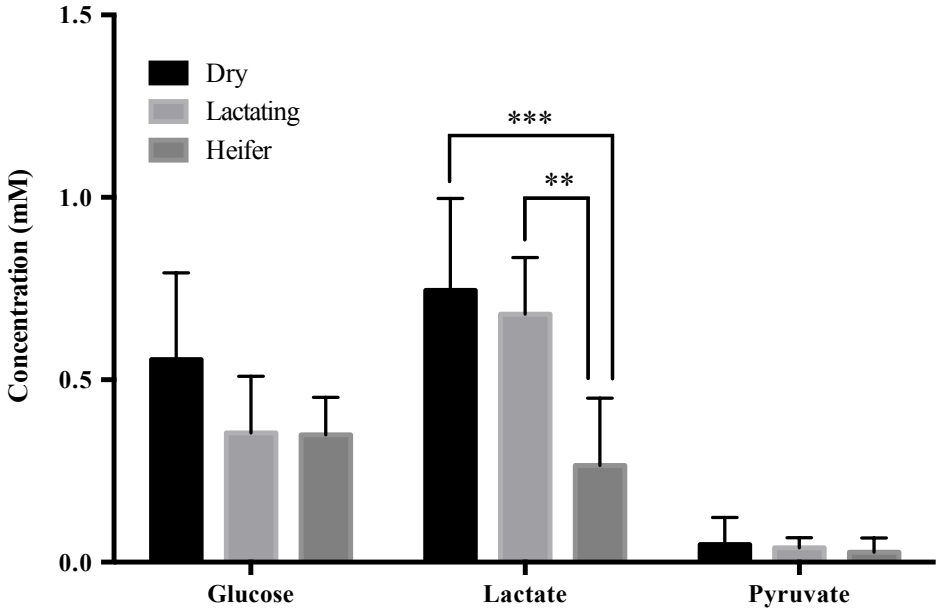

Figure 6-4 — Average concentrations of glucose (mM ± SD) in the ULF of lactating cows (n=5), dry cows (n=6), and maiden heifers (n=4) confirmed pregnant on Day 19 of pregnancy (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

The only differentially transported amino acid was glutamate (Figure 6-3) which was lower in lactating cows relative to heifers but not dry cows. Conversely, lactate was in the ULF of lactating animals in higher concentrations than that of heifers but not dry cows (Figure 6-4) whereas glucose and pyruvate remained unchanged across independent variables.

Next, the amino acid composition of ULF from the ipsilateral and contralateral uterine horns (Figure 6-5) were compared. These data show that glycine is present in lower concentrations in the ULF from contralateral uterine horns.

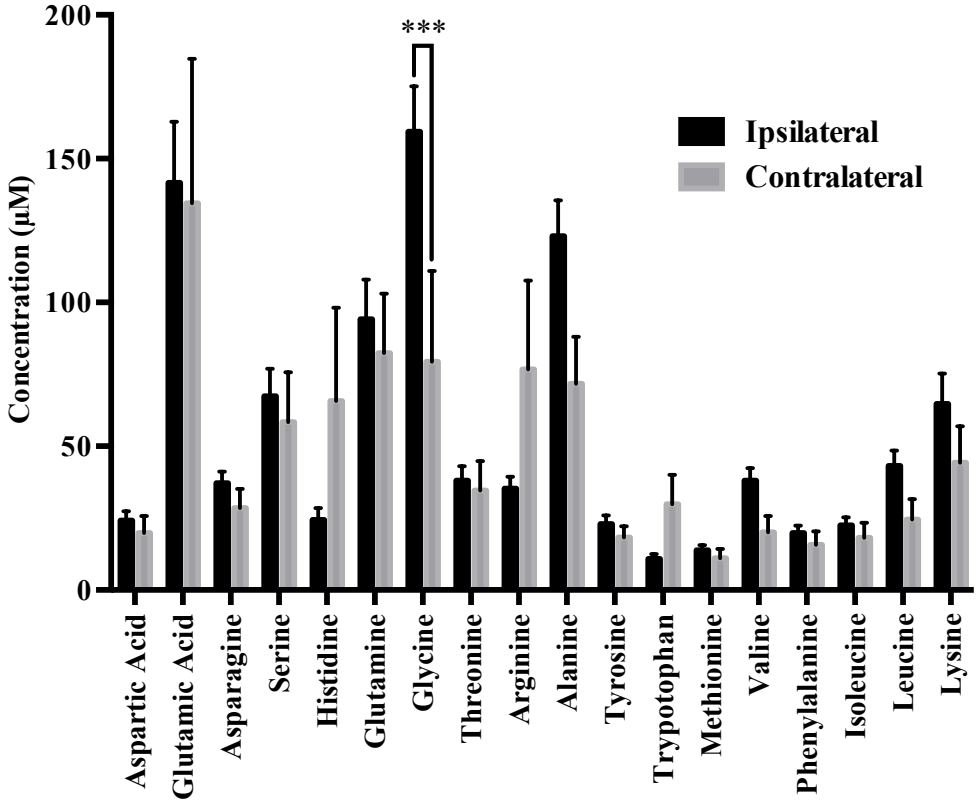

Figure 6-5 — The ULF amino acid composition from the ipsilateral, or gravid (n=22 ± SD) *vs* contralateral (n=7 ± SD) uterine horns (*** $p \leq 0.001$) on day 19 of pregnant cows.

6.4. Discussion

This chapter presents novel insights into maternal-conceptus interactions of the bovine under different physiological states.

6.4.1. Conceptus-Maternal Interactions

This study has measured temporal variations in bovine ULF amino acid content during the oestrous cycle and during the pre- and peri-implantation stages of pregnancy (Table 6-1). The results showed that threonine and glycine were consistently the most abundant amino acids in bovine ULF. Hugentobler *et al* (2007) similarly reported that glycine was present in ULF in the highest concentrations on all 3 days (6, 8, and 14) of the oestrous cycle measured. Glycine was also the most abundant amino acid detected in ULF in the sheep (Gao *et al* 2009). The observed high concentrations of threonine where unexpected, since previous studies have not reported such high levels of this amino acid. Moreover, threonine levels decreased on days 16 and 19 of pregnancy in the bovine (Table 6-1) but increased between days 12 and 15 in the sheep (Gao *et al* 2009) and increased from day 6 to 14 in the Hugentobler *et al* (2007) study.

From a broader perspective, the concentration of most amino acids increased following pregnancy recognition in the sheep (Gao *et al* 2009) similarly to Table 6-1 which shows that the amino acid content of ULF increased as the blastocyst progressed to an elongated filamentous conceptus. This increase in amino acid content may occur in order to accommodate for the greater metabolic demands of the developing offspring (de Souza *et al* 2015).

Interestingly, previous work has shown that blastocysts derived *in vitro* exhibit a higher amino acid turnover than those derived *in vivo* and expanded blastocysts

deplete more amino acids than those that do not undergo expansion (Sturmey *et al* 2010). Moreover, the concentration of amino acids in ULF was lower in subfertile animals (Meier *et al* 2014) and in cows pregnant with developmentally compromised (cloned) embryos (Groebner *et al* 2011). This is consistent with the data in Table 6-1 which demonstrates an increase in the amino acid content in ULF on day 19 of pregnancy indicating that increasing levels of amino acids are important for successful pregnancy establishment.

6.4.2. Sex Linked Conceptus-Maternal Interactions

It has been hypothesised that the endometrium is able to sense the quality and competency of the embryo (Mansouri-Attia *et al* 2009; Bauersachs *et al* 2009) although the mechanism of this sensing remains unclear. In line with this idea the next aim was to investigate whether there is a link between the sex of the embryo and the amino acid content of the uterine lumen on day 19; consistent with the notion that the uterine endometrium is a biosensor of the developmental competency of the developing offspring (Sandra *et al* 2012) and the finding that one-third of genes expressed in the bovine endometrium at the blastocyst stage of development are regulated by the sex of the conceptus (Bermejo-Alvarez *et al* 2010).

The data in Figure 6-2 shows that 8/18 amino acids present in lower concentrations in the ULF of uteri with female (XX) conceptuses compared to male (XY). There are two possible explanations for this finding: (***i***) that the uterus senses and caters for the different metabolic needs of each and/or (***ii***) amino acid consumption of XX *vs* XY conceptuses is different whilst ULF secretions are constant.

In support of the latter idea, Sturmey *et al* (2010) found that bovine XY

blastocysts depleted fewer amino acids and moreover exhibited a lower amino acid turnover relative to XX blastocysts. Moreover, in work accompanying this research (Forde *et al* 2016) it was identified that the differential amino acid composition in ULF is not attributable to altered endometrial transcription of amino acid transporters. Hence overall the data suggest that XX and XY embryos could differentially utilise similar ULF environments in a sex-dependent manner *in vivo* on day 19 and/or the differences are observed due to differential transport, regulated post-transcriptionally.

6.4.3. Effects of Lactation on Pregnancy

Figure 6-4 suggests that, on balance, the lactation state of the animal does not impact on ULF amino acid composition since 17 of the 18 measured amino acids remained unchanged regardless of the day of lactation. The exception is glutamate which was present in the ULF of heifers in higher concentrations than dry and lactating cows. In contrast to glutamate, lactate was present in lactating and dry animal ULF in higher amounts than in heifer ULF (Figure 6-5). The reasons for these differences are unclear and deviate from the hypothesis that the ULF of lactating cows differs from that for dry animals and heifers. However one explanation is that given the heifer group was artificially inseminated at oestrous whereas the lactating and dry cows had embryos transferred it could be the case that conceptus origin (AI *vs* transfer) has a greater impact on the composition of ULF than maternal physiology — specifically whether the mother is lactating or dry. This could be because the levels of IFNτ would be higher in the AI subjects thereby eliciting a response from the uterus that would be absent or diminished in embryo transfer.

6.4.4. Ipsilateral *vs.* Contralateral Effects

A supplementary observation was that the ULF from uterine horns ipsilateral to the corpus luteum contained more glycine than those contralateral on day 19 (Figure 6-5). In the bovine, Groebner *et al* (2011) reported substantial increases in the abundance of amino acids in the gravid (ipsilateral) uterine horn on day 18 of pregnancy, suggestive that conceptus presence stimulates differential amino acid flux into the lumen. Another hypothesis is that the ipsilateral endometrium is exposed to higher concentrations of P4 released by the large luteal cells of the corpus luteum which signals greater luminal glycine secretion — as discussed in Section 3.4.4.3. glycine is an important amino acid for embryo development, and P4 moreover increased glycine flux in the *in vitro* oviduct (Figure 3-10). Such a mechanism of differential signalling and subsequent glycine secretion by uterine P4 threshold sensing is worthy of further investigation and encourages the prospect of adapting the *in vitro* oviduct model for uterine epithelial cells and investigating its endocrine sensitivity.

6.4.5. Summary

An interesting observation from the studies presented in this chapter and the thesis prior is the amino acid glycine whose flux in ULF was not affected by conceptus presence (Table 6-1), or XX *vs.* XY embryo presence (Figure 6-2), or maternal lactation status (Figure 6-3). Moreover in the *in vitro* oviduct model glycine flux did not change between apical native *iv*DOF and basal culture medium (Figure 3-10) or following dual culture with fibroblasts (Figure 4-17). Glycine flux however was always hormonally responsive [increased flux following E2 and P4 supplementation (Figure 3-10), decreased transport in response to T (Figure 3-10)] and furthermore reduced in contralateral uterine horn luminal fluid (Figure 6-5) — the side likely to be exposed to less E2 and P4. These data therefore suggest

that the movement of glycine, an important embryonic osmolyte (Steeves *et al* 2003), within the bovine reproductive tract is regulated by the endocrine system as opposed to via apical conceptus or basal fibroblast signalling.

In summary this data combined illustrate the degree to which the uterine luminal composition can be influenced by a variety of factors including the day of pregnancy (Table 1), conceptus sex (Figure 6-2), maternal lactation physiology (Figures 6-3 and 6-4), and even ipsilateral *vs* contralateral uterine horn variations (Figure 6-5). The subsequent general discussion contextualises these findings within the broader context of this thesis.

Chapter 7

Discussion and Conclusions

Chapter 7 — Discussion and Conclusions

Until now, oviduct fluid composition has been analysed using 'decentralised' or 'retrospective' approaches — *i.e.* fluid has been isolated from various species using *in situ* and *ex vivo* techniques such as oviduct flushes from anaesthetised or slaughtered animals. As discussed in Section 1.8. these methods pose major limitations and offer very narrow scope for experimental exploration (Leese *et al* 2008b).

This study has circumvented these problems by devising a functional *in vitro* model of the bovine oviduct epithelium, enabling detailed *real-time* multivariate examinations of *in vitro* Derived Oviduct Fluid (*iv*DOF) formation within a controlled laboratory environment. This thesis has shown that the amino acid and glucose composition of *iv*DOF can be modulated by physiological (Chapter 3) and pathophysiological endocrine stimulation, metformin, fibroblast dual culture (Chapter 4), and genistein supplementation (Chapter 5). The present work moreover provides mechanistic insight into oviduct fluid formation in the form of solute carrier protein gene expression analyses (Chapters 3 and 4) and transepithelial monolayer resistance measurements (Chapter 4). The transport properties of the *in vitro* oviduct epithelium to carnitine (Chapter 4) and genistein (Chapter 5) have also been investigated and finally ULF from cows has been analysed to investigate how ULF composition changes under a variety of stimuli *in vivo* (Chapter 6). Moreover Figure 9-1 shows embryos being cultured in *iv*DOF (n=1) — data obtained during the resubmission phase of this thesis. The core experimental elements of this thesis are summarised by Figure 7-1 whilst the major findings of this project are elaborated upon below.

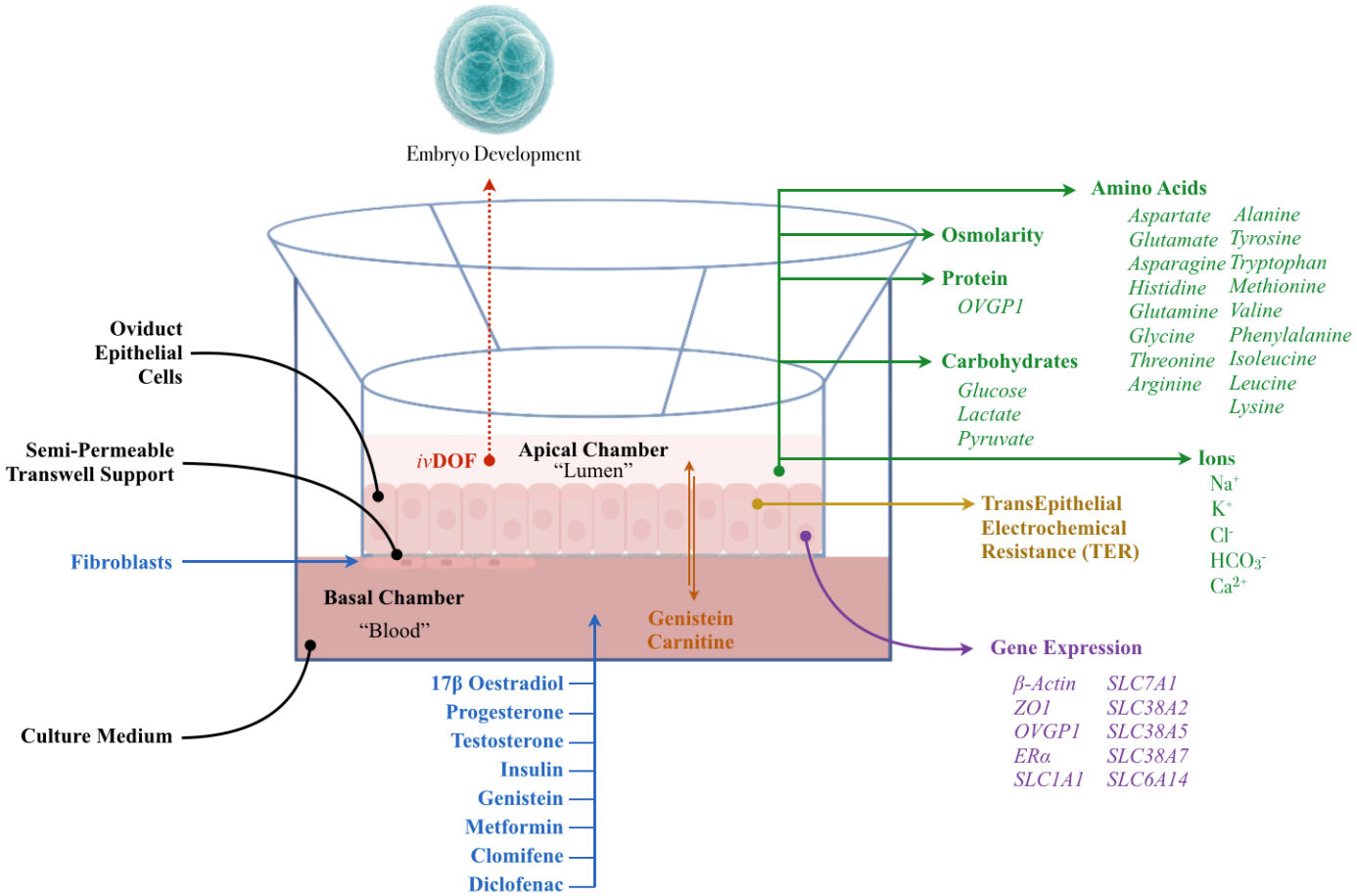

Figure 7-1 — A schematic depiction of the core experiments conducted and presented in this thesis. Parameters in blue represent independent variables of the *in vitro* model whilst those in black show constant parameters. Outputs are shown in green, orange, yellow and purple to highlight the dependent variables measured as a function of the oviduct epithelium. Red highlights the fact that *iv*DOF supports early embryo development.

7.1. Characterising the *in vitro* Oviduct

Owing to the novelty of the *in vitro* preparation to study the mechanisms underlying oviduct fluid formation in health and disease, it was important to characterise both the *in vitro* oviduct epithelium and *iv*DOF for meaningful conclusions to be drawn from subsequent experiments. Initial analyses confirmed that the cell harvest method from primary tissue yielded a very high purity of epithelia (Figure 3-4). These cells furthermore show oviduct epithelial cell characteristics such as secretory vesicles (Figure 3-5) and morphologically appear epithelial also (Figures 3-6 and 3-7). Further support for the model was gained by observing that the carbohydrate (Figure 3-9), ionic (Table 3-5), and amino acid (Figure 3-10), composition of *iv*DOF was comparable to previously reported *in vivo* data (Hugentobler *et al* 2007b). Moreover the amino acid composition of *iv*DOF was distinct to that of the culture medium provided basally (Figure 3-10).

Other features such as confirming *ESR1* and *OVGP1* gene expression (Figure3-11) were crucial to the characterisation, however strong support for the efficacy of the model came from the discovery of OVGP1 protein in *iv*DOF (Figure 3-8) and more recently the observation that *iv*DOF supports early embryo development (Figure 9-1).

This comprehensive characterisation provided justification to perform basic functional experiments including investigating the influence of individual hormones on aspects of the secretome and gene expression of BOECs *in vitro*. It is well established that the physiology of the oviduct *in vivo* changes throughout the oestrus cycle as a result of hormonal stimulation (Section 1.2.1.) (Aguilar & Reyley 2005), and thus it was reassuring to see that the *in vitro* preparation was similarly responsive to hormonal stimulation (Sections 3.3.3. and 3.3.4.). Another interesting finding was that insulin supplementation hindered all fluid formation;

the potential mechanism of which is provided in Figure 3-16.

In summary, Chapter 3 investigated the impact of E2, P4 and T on *iv*DOF composition. Relative to native *iv*DOF, supplementation with E2 decreased the flux of five amino acids and increased that of two, P4 supplementation led to an increase in the secretion of five amino acids and a decrease in two (Figure 3-10). This finding was interesting because E2 is the the dominant hormone during the proliferative phase (Murray 1997) whereas P4 is the dominant hormone during the secretory phase of the oestrus cycle and pregnancy (Abe 1996). It was therefore encouraging that E2 reduced amino acid flux whilst progesterone primarily enhanced secretions of key amino acids such as glutamine — a primary embryo metabolite shown to improve preimplantation mouse embryo development *in vitro* (Rezk *et al* 2004; Smith & Sturmey 2013; Rieger *et al* 1992; Mathew *et al* 1993) (Section 3.4.4.1.).

The gene expression of several amino acid transporters was also investigated (Figure 3-11) with the conclusion that hormonal stimulation regulates amino acid flux, but not at a transcriptional level in the *in vitro* oviduct (Section 3.4.5.). A further finding in Chapter 3 was that supplementation with T, the dominant androgen whose increase in circulation in humans *in vivo* has been linked with reduced fertility (Speranda & Papic 2004), decreased the flux of ten amino acids. This finding led to an investigation into the effects of androgenic extremes on the *in vitro* oviduct epithelium (Chapter 4).

7.2. Expanding the Model

The first aim of this chapter was to mimic the pathological states of hypo and hyper androgenism, common to women with FADS and PCOS respectively; the latter of whom are commonly subfertile (Section 4.1.1.) (Cho *et al* 2008). In

reference to Figure 7-1, the independent experimental variable was the widening and narrowing of the T to E2 ratio (Table 4-1). Corresponding dependent variables, or outputs, were *iv*DOF volume (Figure 4-6), *iv*DOF carbohydrate content (Figure 4-7), the amino acid composition of *iv*DOF (Figure 4-8), TER measurements (Figure 4-10), and gene expression profiles (Figure 4-9). The first significant observation was that P hormone supplementation restored histidine, alanine, lysine, and glycine concentrations to levels more in line with what has been previously reported *in vivo* (Hugentobler *et al* 2007b; Tay *et al* 1997) whilst glutamine (NE) and arginine (CE) were significantly elevated relative to both native and *in vivo* (Figure 4-8) presumably owing to their importance in early embryo development (Section 4.4.2.). Another interesting observation was the *leaky oviduct* phenotype wherein epithelia treated with a hyperandrogenaemic hormonal profile displayed a significant reduction in TER (Figure 4-10) and a corresponding increase in *iv*DOF volume — mechanistically supported by decreased *ZO1* expression (Figure 4-9). The question of whether this could be an aetiology of the perceived subfertility in hyperandrogenic women is worthy of further investigation however measuring TER *in vivo* is currently not possible.

The model was further expanded and the impact of pharmacological intervention on the amino acid composition of *iv*DOF was assessed (Section 4.3.4. and 4.4.7.). Of the there drugs tested only metformin impacted *iv*DOF composition (Figure 4-11). However owing to low replicate values and the numerous cell signalling effects of metformin it is difficult to speculate as to the mechanism underlying the observed effects. However this study shows that the *in vitro* oviduct preparation offers the capacity to test the effects of drugs on early pregnancy. On this *proof of principle* theme the barrier properties of the oviduct were also tested using carnitine. The data (Figure 4-15) show that ~ 25% of carnitine initially supplied basally accumulated in the apical chamber over 90 minutes with ~ 37.5% still present in the basal compartment and the remaining ~ 62.5% not detected —

presumably metabolised by the epithelial cells (Section 4.4.8.). This data formed the conceptual basis for chapter 5.

Oviduct epithelial culture in the presence of fibroblasts was also examined (Section 4.2.6.), with the results showing that such dual culture significantly influenced the accumulation of 7/18 of the amino acids measured (Figure 4-17) relative to native control *iv*DOF. Although dual *vs* native culture are not directly comparable — since dual culture involves an increase in the total cell population, which will affect amino acid composition — the data do suggest that the presence of fibroblasts provides a preparation closer to the physiological situation. Within the wider field of biology, it would be of interest to use this dual culture preparation for the dual culture of stem cells with underlying fibroblast feeder layers. Stem cells are usually cultured with inactivated stromal feeder layers to prevent differentiation (Yamanaka 2007; Takahashi & Yamanaka 2006) however subsequent stem cell isolation from culture has proven challenging as fibroblast contamination is commonplace (Manello & Tonti 2007). To circumvent this problem Chen *et al* (2013b) cultured stem cells adjacent to fibroblasts, similar to the dual culture preparation presented in Chapter 4 using an elaborate polydimethylsiloxane (PDMS) porous membrane-assembled 3D-microdevice chips. Although their method is comparable to the dual culture preparation, it is considerably more expensive (£16 per Transwell™ *vs* £350 per chip) and yields a mouse embryonic stem cell (mES) purity of 89.2% compared with the 99% purity achievable (Figure 4-17) using the dual culture apparatus presented in Section 4.2.6.

Building on the characterisation of the *in vitro* oviduct (Chapter 3) and its expansion (Chapter 4), the aim of Chapter 5 was to investigate the transport of the dietary derived compound genistein across the oviduct cells and its influence on the secretion of amino acids in *iv*DOF.

7.3. Case Study of the Impact of Genistein on the *in vitro* Oviduct Epithelium

One of the most interesting findings from Chapter 5 was that the amino acid profile of *iv*DOF from BOECs treated with the genistein vehicle control (DMSO) was similar to the amino acid profile of *iv*DOF derived from E2 treated BOECs (Figure 5-12:B). Only one publication has previously suggested that DMSO might act as a E2 analogue (Mortensen & Arukwe 2006) and although this project does not provide direct proof of this, it is tempting to suggest that DMSO acts as a E2 mimic on bovine oviduct epithelial cells. Addressing this theory in oviduct epithelia in addition to other oestrogen responsive cell types across species would be of great interest owing to the widespread use of DMSO within the scientific community.

Chapter 5 additionally showed for the first time that genistein, an embryo-toxic phytoestrogen (Newbold *et al* 2001; Jefferson *et al* 2007, 2009, 2012) (Section 5.1.3.) traverses the *in vitro* oviduct epithelium faster than the rate expected if spontaneous diffusion were the sole mechanism, and in accordance with a biphasic kinetic profile (Figure 5-4). Genistein moreover altered the amino acid composition of *iv*DOF (Figure 5-8), but not as an E2 mimic as presumed.

These insights provide strong support for the use of the *in vitro* oviduct model to investigate the impact of small dietary molecules on the secretion and transport properties of the oviduct. In future it could be used to study the passage of pharmacological compounds across the oviduct and help clarify the concentration they might achieve in the environment of the early embryo. Further down the line, this model could be used to provide dietary advice to females wishing to conceive or already pregnant. However this model is neither a substitute for *in vivo* animal trials nor without limitations, as discussed in Section 7.5.

7.4. Uterine Luminal Fluid Composition Assessment *in vivo*

This chapter took a step back from the relatively isolationist *in vitro* studies to look at bovine reproductive fluid composition *in vivo*. Moreover the fluid analysed in this chapter was uterine — the natural extension of the oviduct. It is worth noting that this chapter is an amalgamation of four independent albeit related collaborative projects with the objectives being to better understand how the composition of ULF is influenced by the day of pregnancy, the presence of male *vs* female embryos, and the physiological status of the animal — specifically lactating *vs* dry cows *vs* heifers.

Intuitively the total amino acid content secreted increased with day of pregnancy — as the blastocyst progressed to an elongated filamentous conceptus (Table 6-1). Furthermore the ULF from XX inseminated conceptuses contained 8 amino acids which were present in significantly higher concentrations in XY subjects (Figure 6-2). The complementary gene expression studies to this (Appendix) show that whilst female conceptuses exhibited a generally higher expression of amino acid transporters, the maternal endometrial transcriptome did not differ between male *vs* female gestating cattle. This therefore strongly suggests that embryo amino metabolism is sex dependent.

Moreover pregnant lactating *vs* dry cows did not exhibit any differences in ULF amino acid (Figure 6-3) or carbohydrate (Figure 6-4) composition however both differed from heifers in glutamate and lactate ULF content suggestive that pregnancy status has an impact on ULF composition but lactation physiology does not. Finally the ULF from ipsilateral and contralateral uterine horns was compared (Figure 6-5) with the only difference being higher glycine presence in the ipsilateral. This was interesting given the parallel that glycine flux increased in the

in vitro oviduct following E2 and P4 supplementation (Figure 3-10) and was elevated in the uterine horn most likely to be exposed to greater levels of E2 and P4.

7.5. Limitations of the Preparation

In spite of the characterisation presented in Chapter 3 a variety of further approaches could be applied to further characterise the system, for example further imaging by confocal microscopy with staining for markers such as tight junction proteins for epithelial monolayer integrity confirmation, cytokeratins to confirm the epithelial nature of cells harvested (Moll *et al* 1983), vimentin to visually identify fibroblast contamination (Goodpaster *et al* 2008; Rottmayer *et al* 2006), and phospholipids to confirm the columnar cellular morphology of oviduct epithelia (Nioi *et al* 2007).

Moreover, further work is needed to investigate the variability of the data from experiments conducted under the same conditions. This phenomenon was prominent throughout the project. For instance in Figures 3-10, 4-8, and 5-8 native *iv*DOF is dominated by histidine but in Figure 4-14 the histidine composition of native *iv*DOF is greatly reduced and glutamine is highest. In Figure 4-14 glycine is present in the greatest amounts in native *iv*DOF with arginine around the mean whereas in Figures 4-13 and 4-14 arginine is present in native *iv*DOF in very low levels. Moreover the concentrations of amino acid in native *iv*DOF in Chapter 5 were generally lower than observed in Chapters 3 and 4. Regardless of these inconsistencies it was felt important to present the data in its respective cohort as opposed to averaging all native amino acid values to provide a single comparator across chapters, particularly owing to the novelty of this method and hence lack of comparable data from other laboratories. Similarly the volumes of native *iv*DOF obtained were highly inconsistent. From the

experiments conducted in Chapter 3 *iv*DOF collected was in the 10 µl range whereas when native *iv*DOF was quantified in Chapter 4 the volumes obtained were over double at ~ 25 µl.

A likely explanation for the variation in native *iv*DOF volume and composition is the lack of selectivity regarding tract ovarian staging, *i.e.* throughout this project tissue was not assigned according to the relative stage in the oestrous cycle at time of slaughter, owing to the often low abundance of abattoir derived material. Although there is value in examining oviduct epithelial behaviour holistically (*i.e.* by pooling tracts of all stages), in future it would be worth comparing and contrasting *iv*DOF from BOECs derived from tracts at different stages of oestrus.

Another limitation which ought to be noted is the fact that gene expression studies were conducted in plastic flasks as opposed to Transwell^{TM} membranes and are therefore not strictly directly comparable to the secretion data presented. Moreover there are some figures and tables in which biological replicates are too low for definitive conclusions to be drawn.

Another potential limitation in investigating the *in vitro* oviduct response to E2 was that cells were cultured in a medium largely comprised of DMEM containing phenol red. In addition to being a useful pH indicator for cell culture, phenol red is known to exert mild oestrogenic effects as a weak E2 analogue (Berthois *et al* 1986). Thus it is possible that minor oestrogenic effects have been inadvertently present from this source throughout this thesis. Although, at a concentration of 0.0159 $g \cdot L^{-1}$ phenol red this is unlikely, and it is moreover highly improbable that experiments involving pure E2 supplementation would be perturbed.

On this note of hormonal impact another inherent limitation is in the use of a bovine model to study hypo and hyper androgenism. Hyperandrogenism has been

previously investigated in bovine cell cultures (Comim *et al* 2013; Glister *et al* 2013) despite the limitation that it is a pathology not comparable in cows. There are however advantages to this. The experimental ideal of obtaining human tissue from healthy donors is impossible and using murine tissue is unfeasible owing to the extremely low amount of material obtained. Thereafter using tissue from hysterectomised women presents the challenge of controlling for underlying pathology. Thus using the bovine as a model provides consistent healthy tissue in sufficient volumes in addition to being a *blank canvas* on which the effects of hyperandrogenaemia can be investigated without individual predispositions. The reproductive similarities between the bovine and the human are discussed in Section 1.2.

7.6. Future Work

In addition to addressing the aforementioned limitations, this project has unearthed some key areas for future work, the obvious being the need to refine the *in vitro* model in terms of 'inputs' in addition to measuring more 'outputs' such that a more informed picture of oviduct fluid formation in health and disease can be established. This project consistently examined *iv*DOF accumulated over 24 hours. Significantly shorter incubation intervals are not viable owing to the experimentally restrictive volume of *iv*DOF obtained. However more chronic exposures particularly under hyperandrogenaemic-like states would be interesting and more indicative of the situation *in vivo*. Similarly it would be of great value to recapitulate the oestrus cycle *in vitro* and the menstrual cycle in human oviduct epithelia, to gain a better understanding of oviduct fluid formation across species, over a prolonged time period, and under more physiological conditions. This could involve daily apical *iv*DOF collections and basal media changes with a varying hormonal supplementations.

From a wider reproductive biology perspective, statistically significant differences, and lack thereof, have been provided throughout the data presented. This thesis however has not evaluated any 'biological significance' such as evaluating the impact of elevated histidine on preimplantation embryo development. This research initiative is important, both for improving existing IVF culture techniques and probing into the molecular aetiologies of subfertility, discussed below.

7.7. Subfertility

Subfertility is an intriguing concept and a complex challenge to modern medicine; it is neither a syndrome nor a disease nor illness. Unlike infertility it is neither a disability. Subfertility is a condition wherein those affected are less likely (not necessarily less able) to conceive. The complexity is accentuated by the fact that an average monthly fecundity rate of 20% renders humans intrinsically not particularly fertile (Evers 2002). Moreover, despite the global population rise, in excess of 60% of females are moderately subfertile. Subfertility has long been broadly defined as *any form of reduced fertility with prolonged time of unwanted non-conception* (Gnoth *et al* 2005). However from clinical and research perspectives, this definition fails to inform the patient what they did not already know, and from a research perspective is too loose to insight targeted experimental emphasis.

Habbema *et al* (2004) put forward a proposal *towards less confusing terminology in reproductive medicine* addressing the problem of benign lexicon, such as subfertility, in the field. However nomenclature and terminology refinement cannot explain the inherently undefined. Specifically, until the issue of subfertility is firmly structured by means of a clear hypotheses, as the basic scientific method dictates, we find ourselves akin to the mathematician: a blind man in a dark room

looking for a black cat which is not there. In an attempt to bridge this gap a theoretical interpretation of subfertility is presented below.

7.7.1. GOPEX

GOPEX is a model derived from a preposition that there are five primary components capable of and responsible for causing and influencing subfertility. These are: maternal genetics (G), oviduct biochemistry (O), maternal physiology and/or endocrinology (P), poor embryo quality (E), and external parameters (X). The model further supposes that each of these five parameter is related and can be modelled by a symmetrical Branko Grünbaum Venn diagram (Figure 7-2), which assumes that the overlap of all five variables (GOPEX) represents a central unison of infertility. Similarly the outermost universal set (F) represents full biological fertility, whilst the 30 sub-divisions represent subfertility.

The model presumes that the 5 divisions are concomitantly capable of imposing 30 defined and finite (albeit not mutually exclusive) subfertility domains. More specifically, the GOPEX model states that the segment labelled O represents a single category (or diagnosis) of subfertility that deviates from the physiological (F ideal) by virtue of defects in oviduct epithelium biochemistry. Similarly X subfertility arises from externally influenced parameters. X subfertility differs from PX subfertility in so much as the latter is moreover attributable to maternal physiology. Similarly OEPG subfertility is caused by failings in oviduct biochemistry, embryo quality, maternal physiology and maternal genetics.

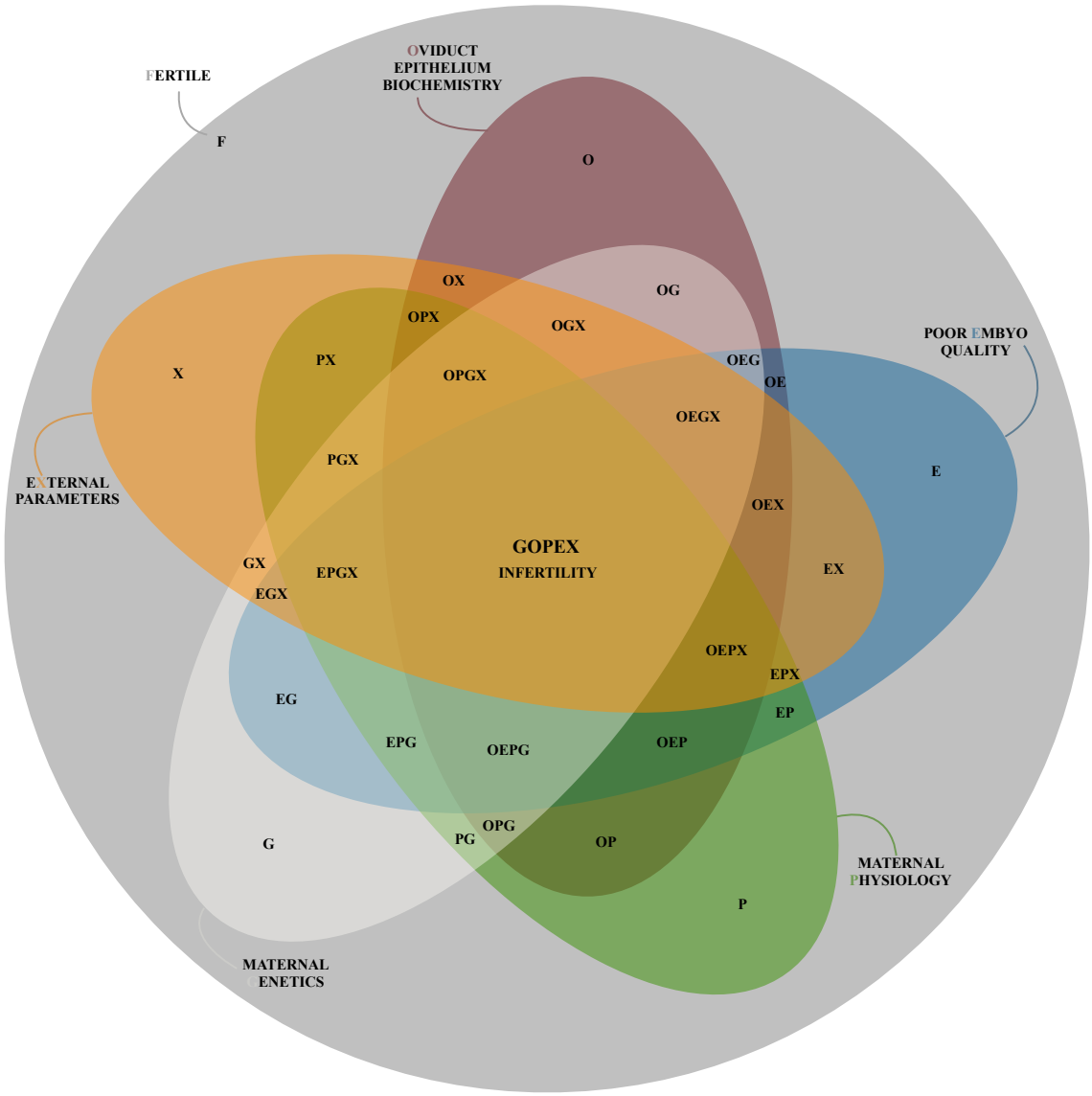

Figure 7-2 — The GOPEX model skeleton depicting the 5 presumed divisions of female subfertility in addition to 30 sub-divisions, considered *subfertility domains*.

From an applied perspective O subfertility may be attributable to ciliary dyskinesia whilst X subfertility could manifest from maternal drug abuse or exposure to radiation. A diet rich in genistein (Chapter 5) for instance may cause subfertility, and would be classified under OEX since it is a dietary derived disruptor (E) which impairs oviduct fluid secretion composition (O) and is known to perturb early embryo development (E). Figure 7-3 provides a non-exhaustive clinical interpretation of GOPEX.

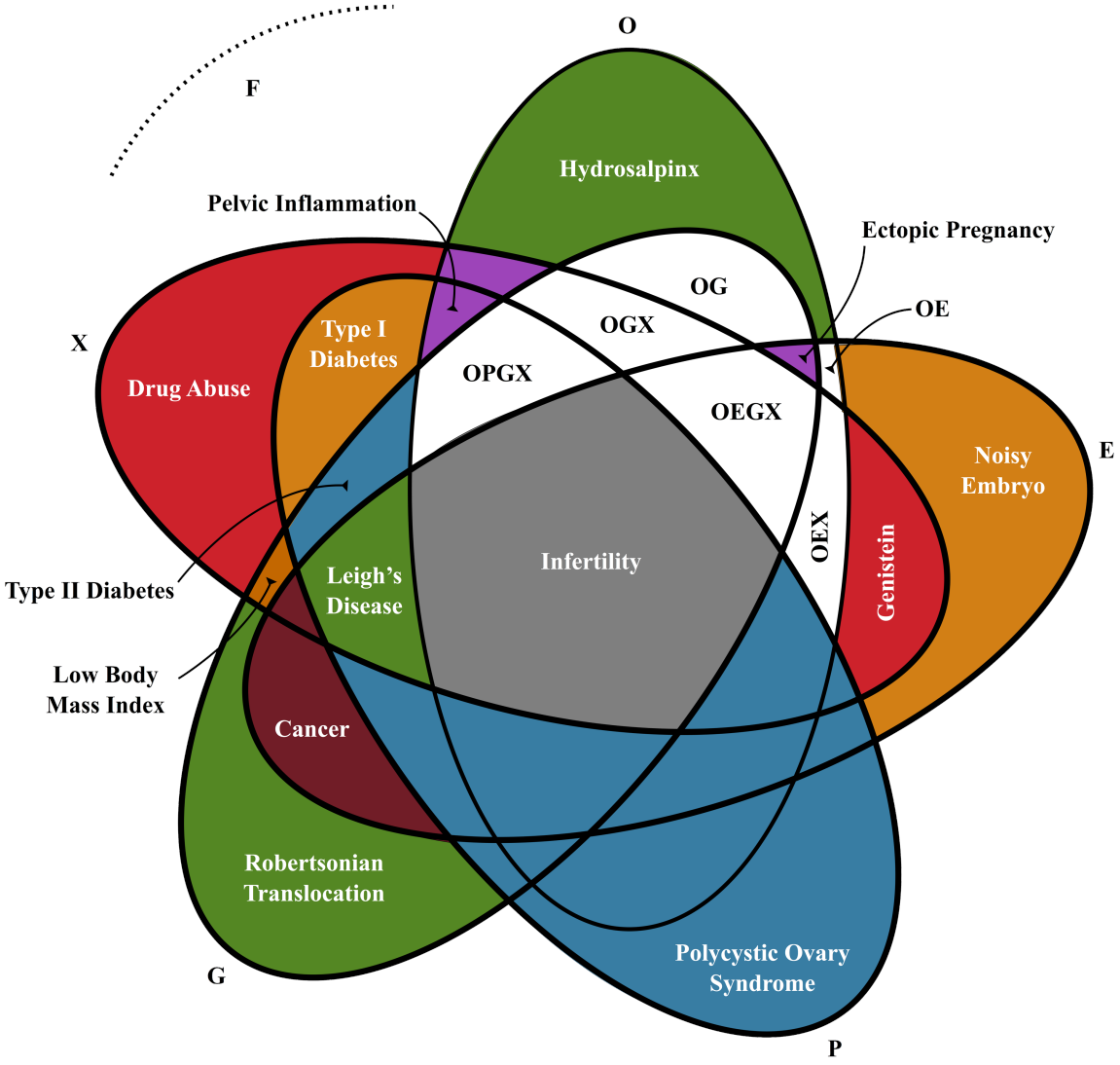

Figure 7-3 — Potential causes or diagnoses of subfertility aligned with the GOPEX model.

To the best of my knowledge, there are currently six undiagnosed areas of subfertility (Figure 7-2) — OG, OE, OGX, OEX, OPGX, and OEGX. This could be a result of faults in the model, or currently idiopathic aetiologies of subfertility. Interestingly all six unidentified domains have the oviduct in common. Also, the largest single overlap identified is PCOS.

7.7.2. Polycystic Ovary Syndrome

As discussed in Chapter 4, the wide-ranging symptoms of PCOS, encompassing symptoms such as ovulatory dysfunction, polycystic ovaries, hirsutism, hyperandrogenaemia, abnormal gonadotrophin levels, and insulin resistance (Azziz 2004), is reflected in the broad span of the PCOS locus in the GOPEX model. Figure 7-2 speculates that PCOS is predominantly determined by (and always attributed in part to) maternal physiology and/or endocrinology (P). The second most abundant cause of subfertility in PCOS is embryo quality (E) whilst impaired oviduct epithelium biochemistry (O) is hypothetically liable for half of PCOS related subfertility. A scientific description of OP subfertility within the vast PCOS domain could be a *leaky oviduct* (Chapter 4).

The point of the GOPEX model is to better conceptualise and define subfertility from a holistic perspective such that gaps in our knowledge pertinent to this complex reproductive disorder can be more effectively targeted. In other words the aim of the GOPEX model is to shed light onto currently unidentified areas of subfertility following optimisation. In order to achieve this the GOPEX model needs to be verified by real data. For instance — although the model in its current state is entirely theoretical and qualitative — the PCOS framework of the GOPEX model suggests that 18.52% (5 of 27 principal components) of PCOS-linked subfertility results from oviduct epithelium biochemistry defects. There is an 18.5% occurrence of the variant-Luteinizing Hormone (a known inducer of PCOS) amongst female subjects with PCOS (Tapanainen *et al* 1999). Although this is coincidental it is hoped that the model can be adjusted and tailored around a plethora of such data to accurately represent subfertility and provide experimental targets for elucidating the causes underlying idiopathic aetiologies of subfertility.

7.8. General Conclusion and Evaluation

This study has presented a functional *in vitro* model of the bovine oviduct epithelium, enabling detailed *real-time* multivariate examinations of *in vitro* Derived Oviduct Fluid (*iv*DOF) formation within a controlled laboratory environment and has shown that the amino acid and carbohydrate composition of *iv*DOF is a function of physiological (Chapter 3) and pathophysiological (Chapter 4) endocrine stimulation, fibroblast dual culture (Chapter 4), and can be influenced by pharmacological intervention and genistein supplementation (Chapter 5). Mechanistic insights into oviduct fluid formation in the form of solute carrier protein gene expression analyses (Chapters 3 and 4) and transepithelial monolayer resistance measurements (Chapter 4) have also been provided, whilst the transport properties of the *in vitro* oviduct epithelium to carnitine (Chapter 4) and genistein (Chapter 5) have also been investigated. In addition a plethora of future research initiatives, and a theoretical model of subfertility have been offered, all in an attempt to make the oviduct a less *neglected* organ (Leese *et al* 2001; Menezo *et al* 2015).

Chapter 8

References

Chapter 8 — References

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Chapter 9

Appendix

9.1. DMEM medium composition

$CaCl_2$	0.20000 $g{\cdot}L^{-1}$
$Fe(NO_3)_3 \cdot 9H_2O$	0.00010 $g{\cdot}L^{-1}$
$MgSO_4$	0.09767 $g{\cdot}L^{-1}$
KCl	0.40000 $g{\cdot}L^{-1}$
$NaHCO_3$	3.70000 $g{\cdot}L^{-1}$
NaCl	6.40000 $g{\cdot}L^{-1}$
NaH_2PO_4	0.10900 $g{\cdot}L^{-1}$
L-Arginine $\cdot$ HCl	0.08400 $g{\cdot}L^{-1}$
L-Cysteine $\cdot$ 2HCl	0.06260 $g{\cdot}L^{-1}$
Glycine	0.03000 $g{\cdot}L^{-1}$
L-Histidine $\cdot$ HCl $\cdot$ H_2O	0.04200 $g{\cdot}L^{-1}$
L-Isoleucine	0.10500 $g{\cdot}L^{-1}$
L-Leucine	0.10500 $g{\cdot}L^{-1}$
L-Lysine $\cdot$ HCl	0.14600 $g{\cdot}L^{-1}$
L-Methionine	0.03000 $g{\cdot}L^{-1}$
L-Phenylalanine	0.06600 $g{\cdot}L^{-1}$
L-Serine	0.04200 $g{\cdot}L^{-1}$
L-Threonine	0.09500 $g{\cdot}L^{-1}$
L-Tryptophan	0.01600 $g{\cdot}L^{-1}$
L-Tyrosine $\cdot$ $2Na^+ \cdot 2H_2O$	0.10379 $g{\cdot}L^{-1}$
L-Valine	0.09400 $g{\cdot}L^{-1}$
Choline Chloride	0.00400 $g{\cdot}L^{-1}$
Folic Acid	0.00400 $g{\cdot}L^{-1}$
Myo-inositol	0.00720 $g{\cdot}L^{-1}$
Niacinamide	0.00400 $g{\cdot}L^{-1}$
D-Panthothenic Acid $\cdot$ 0.5 Ca	0.00400 $g{\cdot}L^{-1}$
Pyridoxine $\cdot$ HCl	0.00404 $g{\cdot}L^{-1}$
Riboflavin	0.00040 $g{\cdot}L^{-1}$
Thiamine $\cdot$ HCl	0.00400 $g{\cdot}L^{-1}$
D-Glucose	1.00000 $g{\cdot}L^{-1}$
Phenol Red $\cdot$ Na^+	0.01590 $g{\cdot}L^{-1}$
Pyruvic Acid $\cdot$ Na^+	0.11000 $g{\cdot}L^{-1}$

Table 9-1 — Composition of Dulbecco's Modified Eagle's Medium (DMEM) according to the manufacturer's product information sheet — specifically Sigma Aldrich D5546.

9.2. F12 medium composition

$CaCl_2 \cdot 2H_2O$	0.0441000 $g \cdot L^{-1}$
$CuSO_4 \cdot 5H_2O$	0.0000025 $g \cdot L^{-1}$
$FeSO_4 \cdot 7H_2O$	0.0008340 $g \cdot L^{-1}$
$MgCl \cdot 6H_2O$	0.1230000 $g \cdot L^{-1}$
KCl	0.2240000 $g \cdot L^{-1}$
$NaHCO_3$	1.1760000 $g \cdot L^{-1}$
NaCl	7.1000000 $g \cdot L^{-1}$
Na_2HPO_4	0.1420400 $g \cdot L^{-1}$
$ZnSO_4 \cdot 7H_2O$	0.0008630 $g \cdot L^{-1}$
L-Alanine	0.0090000 $g \cdot L^{-1}$
L-Arginine · HCl	0.2110000 $g \cdot L^{-1}$
L-Asparagine · H_2O	0.0150100 $g \cdot L^{-1}$
L-Aspartic Acid	0.0133000 $g \cdot L^{-1}$
L-Cysteine · HCl · H_2O	0.0350000 $g \cdot L^{-1}$
L-Glutamic Acid	0.0147000 $g \cdot L^{-1}$
Glycine	0.0075100 $g \cdot L^{-1}$
L-Histidine · 3HCl · H_2O	0.0209600 $g \cdot L^{-1}$
L-Isoleucine	0.0034900 $g \cdot L^{-1}$
L-Leucine	0.0131000 $g \cdot L^{-1}$
L-Lysine · HCl	0.0365000 $g \cdot L^{-1}$
L-Methionine	0.0044800 $g \cdot L^{-1}$
L-Phenylalanine	0.0049600 $g \cdot L^{-1}$
L-Proline	0.0345000 $g \cdot L^{-1}$
L-Serine	0.0105000 $g \cdot L^{-1}$
L-Threonine	0.0119000 $g \cdot L^{-1}$
L-Tryptophan	0.0020400 $g \cdot L^{-1}$
L-Tyrosine · $2Na^+ \cdot 2H_2O$	0.0077800 $g \cdot L^{-1}$
L-Valine	0.0117000 $g \cdot L^{-1}$
D-Biotin	0.0000073 $g \cdot L^{-1}$
Choline Chloride	0.0139600 $g \cdot L^{-1}$
Folic Acid	0.0013200 $g \cdot L^{-1}$
Myo-inositol	0.0180000 $g \cdot L^{-1}$
Niacinamide	0.0000370 $g \cdot L^{-1}$
D-Panthothenic Acid · 0.5 Ca	0.0002380 $g \cdot L^{-1}$
Pyridoxine · HCl	0.0000620 $g \cdot L^{-1}$
Riboflavin	0.0000380 $g \cdot L^{-1}$
Thiamine · HCl	0.0003400 $g \cdot L^{-1}$
Vitamin B-12	0.0013600 $g \cdot L^{-1}$
D-Glucose	1.8020000 $g \cdot L^{-1}$
HEPES	5.9580000 $g \cdot L^{-1}$
Hypoxanthine	0.0040800 $g \cdot L^{-1}$
Linoleic Acid	0.0000840 $g \cdot L^{-1}$
Phenol Red · Na^+	0.0013000 $g \cdot L^{-1}$
Putrescine · HCl	0.0001610 $g \cdot L^{-1}$
Pyruvic Acid · Na^+	0.1100000 $g \cdot L^{-1}$
Thioctic Acid	0.0002100 $g \cdot L^{-1}$
Thymidine	0.0007300 $g \cdot L^{-1}$
L-Glutamine	0.1460000 $g \cdot L^{-1}$

Table 9-2 — Composition of Ham's F12 nutrient mixture according to the manufacturer's product information sheet — specifically Sigma Aldrich N8641.

9.3. Embryo development in *iv*DOF

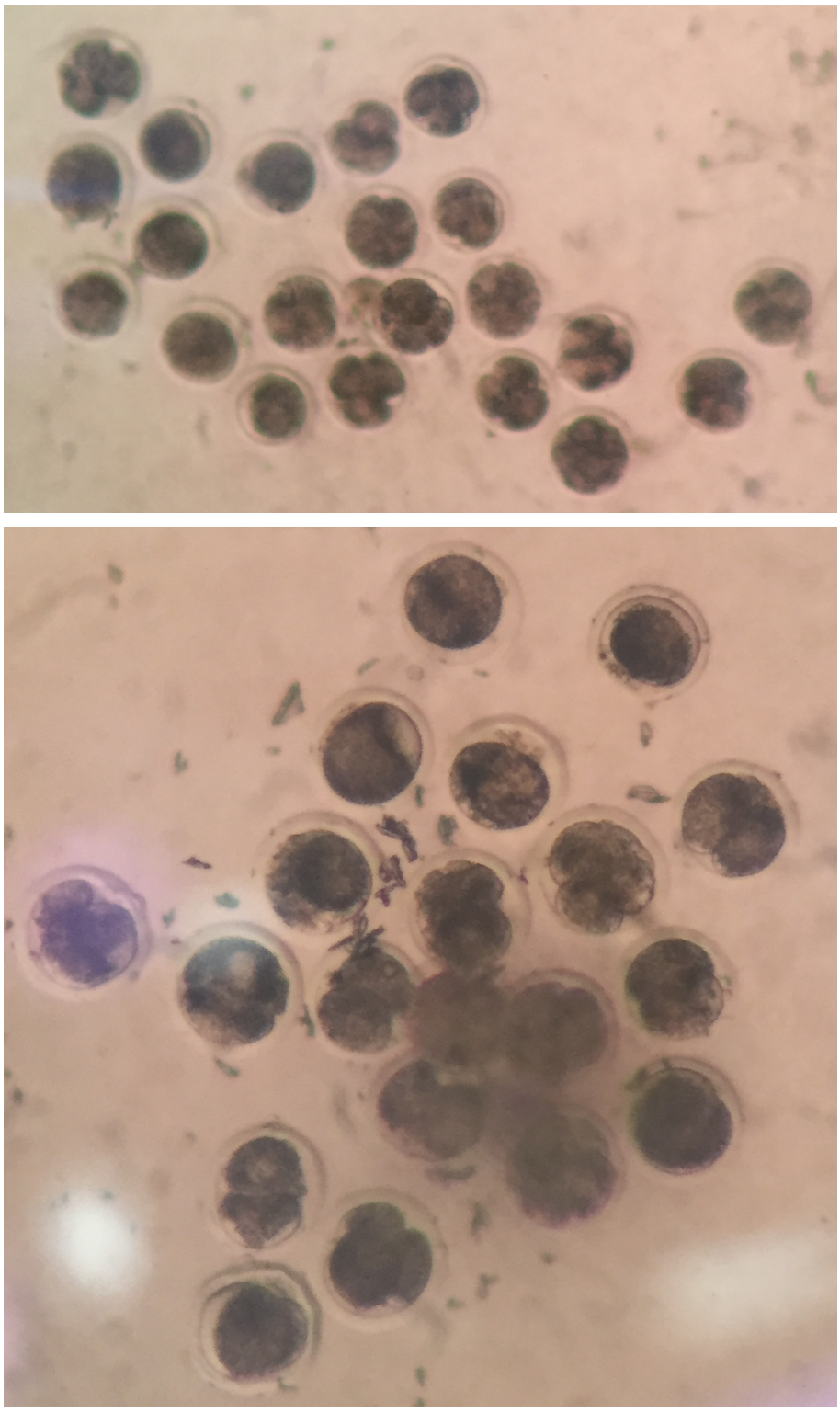

Figure 9-1 — Bovine embryo development in *iv*DOF (n=1).

9.4. Publication in *Reproduction*

Reproduction Advance Publication first posted on 13 October 2016 as Manuscript REP-15-0508

1 **Modelling oviduct fluid formation *in vitro***

2 **Short Title**
3 *In vitro* Derived Oviduct Fluid

4 **Keywords**
5 Oviduct
6 Fallopian tube
7 *In vitro* Derived Oviduct Fluid (*iv*DOF)
8 Dual culture
9 Hyperandrogenism
10 Hypoandrogenism

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31

Abstract

Oviduct fluid is the microenvironment that supports early reproductive processes including fertilisation, embryo cleavage, and genome activation. However, the composition and regulation of this critical environment remains rather poorly defined. This study uses an *in vitro* preparation of the bovine oviduct epithelium, to investigate the formation and composition of *in vitro* derived oviduct fluid (*iv*DOF) within a controlled environment. We confirm the presence of oviduct specific glycoprotein 1 in *iv*DOF and show that the amino acid and carbohydrate content resembles that of previously reported *in vivo* data. In parallel, using a different culture system, a panel of oviduct epithelial solute carrier genes, and the corresponding flux of amino acids within *iv*DOF in response to steroid hormones were investigated. We next incorporated fibroblasts directly beneath the epithelium. This dual culture arrangement represents more faithfully the *in vivo* environment and impacts on *iv*DOF composition. Lastly, physiological and pathophysiological endocrine states were modelled and their impact on the *in vitro* oviduct preparation evaluated. These experiments help clarify the dynamic function of the oviduct *in vitro* and suggest a number of future research avenues, such as investigating epithelial-fibroblast interactions, probing the molecular aetiologies of subfertility, and optimising embryo culture media.

Introduction

The lumen of the mammalian oviduct can be considered an optimal environment for reproductive processes including fertilisation and early embryo development (Coy *et al* 2012). During this time, critical developmental events occur, including activation of the embryonic genome and fate-decisions of the blastomeres (Gonzáles *et al* 2011). In the bovine, the early embryo spends approximately 4 days in the oviduct before moving into the uterus (Hackett *et al* 1993). Insights into the dynamic composition, formation, and regulation of oviduct fluid are therefore crucial to our understanding of the early events of mammalian reproduction.

Until now, descriptions of the composition of oviduct fluid have been based on analyses from samples isolated from various species using *in situ* and *ex vivo* techniques (Aguilar & Reyley 2005). These have included oviduct flushes from anaesthetised or slaughtered animals. As discussed by Leese *et al* (2008), these methods are limited and offer narrow scope for experimental exploration. Thus, there is a need for a robust method of studying oviduct fluid within a controlled laboratory environment.

A single layer of epithelial cells provides the limiting barrier between the maternal circulation and the oviduct lumen. In order to examine oviduct fluid formation in detail it is therefore necessary to isolate the oviduct epithelial cells and culture them in a system that maintains their proper spatial relationship as a polarised confluent layer. One method to achieve this is using the Transwell^{TM} system which enables the culture of oviduct epithelia in chambers which allow access to the apical and basal compartments (Walter 1995). This system allows the bidirectional movement of compounds across the oviduct epithelium to be examined. Using such as system, Dickens *et al* (1993) and Cox & Leese (1995) reported that a chloride secreting epithelium sensitive to purinergic agents lined rabbit and bovine oviducts. These findings have been followed up in detail by Keating & Quinlan (2008; 2012). Moreover, the culture of bovine oviduct epithelia on Transwell^{TM} inserts has allowed the basal to apical, and reverse, movement of nutrients across the oviduct epithelium to be examined (Simintiras *et al* 2012).

Building on these early studies, Levanon *et al* (2010) demonstrated that oviduct epithelia could be cultured at an apical-basal air-liquid interface in which the apical chamber was comprised of moist air. Under air-liquid interface conditions, oviduct epithelia resemble the *in vivo* state more closely and can be cultured in this manner long term (Gualtieri *et al* 2012). Interestingly, patches of oviduct epithelial cells maintained at an air-liquid interface for over two weeks post-confluence regained ciliation (Gualtieri *et al* 2013) despite a lack of estradiol supplementation, which is normally required for re-ciliation *in vitro* (Comer *et al* 1998; Ulbrich *et al* 2003). Chen *et al* (2013a) cultured porcine oviduct epithelial cells for more than 10 days at an air-liquid interface together with steroid hormones and found they were morphologically closer to *in vivo* controls. This interesting approach results in a system in which *in vitro* oviduct epithelial cell cultures mimic *in vivo* behaviour more closely.

In spite of these advances, there is only partial knowledge of the mechanisms underlying the formation and regulation of oviduct fluid, especially when compared with epithelia lining tissues such as the gut and the airways. This can be attributed to (a) ethical and technical limitations surrounding the study of oviduct fluid *in vivo*, and (b) the lack of a robust *in vitro* model enabling the exploration of the formation of oviduct fluid, and how the process responds to stimuli under controlled experimental conditions.

We now present a preparation of bovine oviduct epithelial monolayer to perform real time experiments on oviduct-derived fluid formation *in vitro*. With this system, we have confirmed the secretion of OVGP1 protein into the luminal compartment, which comprises a mixture of amino acids whose composition differs from that in the basal compartment. This apical cell-derived fluid is modified following basal supplementation with estradiol, progesterone and testosterone at physiological and pathophysiological concentrations. Furthermore, using a parallel culture system, we have correlated the expression of bovine oviduct epithelial cell (BOEC) solute carrier genes, with the flux of amino acids in *iv*DOF, following hormonal supplementation.

Materials & Methods

Unless stated otherwise, all reagents were sourced from Sigma Aldrich (Dorset, UK).

Bovine Oviduct Epithelial Cell Harvest

Primarily stage II (mid-luteal phase) abattoir-derived bovine reproductive tracts were transported to the laboratory at room temperature in Hank's Buffered Salt Solution (HBSS) (without $CaCl_2$ and $MgCl_2$) (Invitrogen), 10 mM HEPES, and 1 µM Aprotonin – although tracts were not staged for experimentation. Tracts reached the laboratory within 90 minutes of slaughter. Cells from isthmus to infundibulum were harvested similarly to Dickens *et al* (1993) and in accordance with the UK Animal and Plant Health Agency (APHA) regulations.

Bovine oviduct epithelial cells (BOECs) and bovine oviduct fibroblast cells (BOFCs) were subsequently isolated based on their differential adhesion times — cells were initially seeded together in T75 flasks (Sarstedt) and following 18 hours of culture, un-adhered BOECs were removed (Cronin *et al* 2012) and re-cultured. Culture medium consisted of 1:1 DMEM and F12; supplemented with 265 $U{\cdot}ml^{-1}$ PenStrep, 20 $\mu g{\cdot}ml^{-1}$ Amphotericin B, 2 mM L-Glutamine, 2.5% *v/v* NCS, 2.5% *v/v* FBS, and 0.75% *w/v* BSA.

Bovine Oviduct Epithelial Cell Transwell™ Culture

BOECs were seeded directly onto the apical surface of 24 mm Corning Transwell™ 0.4 µm pore cell culture inserts coated with 10 µg/ml laminin at a density of 10^6 cells/ml/insert. BOECs were subsequently maintained between apical and basal culture medium-filled chambers, at 39°C in 5% CO_2, 95% air. Apical and basal media were replaced every 48 hours.

Transepithelial Electrochemical Resistance (TEER)

BOEC confluence was determined by Transepithelial Electrochemical Resistance (TEER) measured using an Evom voltmeter fitted with handheld chopstick electrodes

(World Precision Instruments). From cell seeding to reaching full confluence, TEER rose from 250 $\Omega \cdot cm^{-2}$ to ~ 800 $\Omega \cdot cm^{-2}$ in the course of ~ 10 days. In addition to assessing monolayer confluence prior to experimentation, TEER was also used as a measure of post-treatment cellular integrity. Unless used as a dependent independent variable, data from BOECs whose TEER fell below 700 $\Omega \cdot cm^{-2}$ were excluded from analysis (Simintiras *et al* 2012).

In vitro Derived Oviduct Fluid (ivDOF)

Once confluent, BOECs were cultured in an apical-basal air-liquid interface (Levanon *et al* 2010) — the basal medium comprised 2 ml of culture medium while the apical compartment comprised moist air in 5% CO_2. After 24 hours, a thin film of fluid formed in the apical chamber — termed *in vitro* Derived Oviduct Fluid (*iv*DOF) (Figure 1A).

Dual Culture

Bovine oviduct fibroblast cells were harvested by trypsinisation from tissue culture flasks after 5 days in culture. $1x10^6$ fibroblast cells were added to the basal surfaces of Transwell™ semi-permeable supports (Figure 1B). Fibroblasts were maintained in this manner for approximately 5 days at which point Transwell™ inserts were reorientated and BOECs introduced to the apical surfaces.

Hormonal Supplementation

Hormone stocks were prepared in ethanol prior to supplementation to the basal Transwell™ chamber. Singular steroid hormone concentrations were based on peripheral plasma levels in the bovine throughout the oestrous cycle as previously reported (Kanchev *et al* 1976). Combinatorial stocks to determine the effects of a physiologically relevant range of hormone concentrations on the *in vitro* model were similarly prepared to represent a minimum, mean and maximum pathophysiological endocrine profile (Kanchev *et al* 1976; Pastor *et al* 1998; Balen 2004; Di Sarra *et al* 2013; O'Reilly *et al* 2014). The maximum solvent (ethanol) contribution was <1% (*v*/*v*) similar to Bromberg & Klibanov (1995) and showed no effect throughout (Table 1).

Fluorescence Activated Cell Sorting (FACS)

BOECs and BOFCs were identified based on positive staining for cytokeratin-18 (CK18) and vimentin primary antibodies (Abcam, Cambridge, UK), respectively (Rottmayer *et al* 2006; Goodpaster *et al* 2008). Samples were analysed on FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) running CELLQuest software and >10,000 events were counted, similarly to Vince *et al* (2011).

Haematoxylin and Eosin Staining

Confluent BOECs cultured on Transwell™ inserts were manually isolated using a blade. The supports were rinsed three times in pre-equilibrated PBS prior to 5 minute incubation at room temperature in 100% haematoxylin. Cells were then rinsed three times in 18.2 milliQ water and incubated for 5 minutes with 1% eosin. Following further washes, cells were supplemented with Hydromount™ (Natural Diagnostics), placed onto microscope slides, and imaged on a Zeiss ApoTome 2 Observer Z1 microscope with a x20 objective lens and an Axiom 506 mono imager coupled with ZEN imaging software.

Transmission Electron Microscopy (TEM)

BOECs fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in the same buffer, were stained en-bloc in 1% uranyl acetate (aq) then serially dehydrated in ethanol before being embedded in Epon-Aradite resin. (All chemicals from Agar Scientific, Stansted, Essex). Subsequently 50nm sections were cut using a diamond knife on a Leica UC6 Ultramicrotome and collected on carbon-coated copper grids (EM Resolutions, Saffron Walden). Images were obtained using an Ultrascan 4000 digital camera (Gatan Inc, Pleasanton, Ca. USA) attached to a Jeol 2011 Transmission Electron Microscope (Jeol UK Ltd, Welwyn Garden City) running at 120 kV.

Generation of anti-Oviduct Specific Glycoprotein (OVGP1) Antibodies

The peptide KMTVTPDGRAETLERRL corresponding to amino acids 521–537

of bovine OVGP1 (UniProtKB - Q28042) was synthesized with a 433A Peptide Synthesizer (Applied Biosystems, Waltham, MA, USA) using Fmoc chemistry (FastMoc Ω previous peak method, as suggested by the manufacturer) and TentaGel SRAM (RAPP Polymere, Tübingen, Germany) resin. To further increase immunogenicity, a proprietary peptide carrier was C-terminally coupled. Peptide cleavage and deprotection was performed by incubation in 92.5% trifluoroacetic acid, 5% triisopropylsilane, and 2.5% water for 1.5 h. The peptide was precipitated and washed with cool tert-butyl methyl ether. Peptides were further purified using reversed-phase chromatography and the correctness of the peptide was confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (4800 series; Applied Biosystems). Murine anti-OVGP1 sera were generated by immunization of female BALB/c mice in time intervals of 3 wk with 100 µg peptide applied subcutaneously. For the first injection, complete Freund's adjuvant and for the following three injections, incomplete Freund's adjuvant was used. Bleeding was performed 10 d after the fourth injection.

Western Blotting

OVGP1 from both abattoir-derived oviduct fluid and *iv*DOF was qualitatively identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated by 10%-18% gradient SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked for 24 hours with 10% milk dissolved in Tris-buffered saline-Tween (0.1%), then incubated at 4°C with the custom mouse anti-OVGP1 primary antibody described above (1:1000) for 24 hours, washed, and subsequently incubated with an anti-mouse horseradish peroxidase (HRP) linked antibody (1:10000) (Cell Signaling Technologies, USA) for 1hr at room temperature. Bands were visualised by enhanced chemiluminescent (ECL) detection.

Osmolarity and Fluorometric Assays

Osmolarity was measured using an Osmomat 030 Osmometer (Gonotec GmbH, Berlin, Germany). Glucose, lactate, and pyruvate were quantified indirectly using enzyme-linked fluorometric assays as described in Leese (1983), Leese & Barton

(1984), Gardner & Leese (1988, 1990), and Guerif *et al* (2013).

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was used to measure 18 amino acids as described previously (Humpherson *et al* 2005).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

At confluence, BOECs from T25 flasks were subjected to hormonal supplementation (Table 1) for 24 hours prior to isolation using trypsin. BOECs were washed four times by centrifugation at 1000 x g for 5 mins at 4°C and resuspension in pre-chilled 1 ml phenol red free HBSS. Total RNA was extracted using Trizol reagnet and chlroform (Chomczynski & Sacchi 1987). Global cDNA was synthesised by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Fisher Scientific) in accordance to manufacturer instructions. The concentration (ng/µl) and purity (A260/A280) of cDNA generated was determined using a NanoDrop spectrophotometer. All cDNA was diluted to 1 µg/ml. Three technical PCR replicates were prepared per sample in optical 96 well plates and sealed before being loaded onto a Step-one Real-Time PCR machine (Applied Biosystems) for qPCR. The bovine specific exon spanning primers used are provided in Table 2. To ensure correct product length, melt curves were performed (Giglio *et al* 2003) whilst ΔΔCt method (Livak & Schmittgen 2001) was used to determine relative expression.

Experimental Design

Retrieved bovine oviduct epithelia were pooled, typically yielding sufficient viable cells to seed 6 Transwell^{TM} inserts. The standard experimental design was to assign 3 Transwell^{TM} membranes for treatment with the dependent experimental variable and the remaining 3 as negative controls. The *iv*DOF obtained from each group was pooled for subsequent analysis. This was defined as a single biological replicate (n=1). Unless otherwise stated n=3 indicates data from three independent abattoir collections and *iv*DOF isolations.

In this study, *in vitro* derived oviduct fluid (*iv*DOF; Figure 1A) from untreated (native) bovine oviduct epithelial cells was analysed (Figure 3: A-D) and compared with previously reported *in vivo* observations. The composition of *iv*DOF following singular cellular hormonal supplementation was analysed (Figure 3F), and the impact of dual culture (Figure 2B) was also examined (Figure 3G). These data are contrasted against native *iv*DOF. This system was subsequently expanded upon to investigate the impact of physiological *vs.* pathophysiological endocrine stimulation on fluid composition and cellular physiology (Figure 5: D-G). Cell culture flasks were seeded in parallel for gene expression studies to complement *iv*DOF findings (Figures 4 and 5A-C).

Statistical Analyses

Statistical analyses were performed using Prism Graphpad 6 software for Apple Macintosh. All statistical analysis were two way analysis of variance (ANOVA) followed by a Holm-Sidak non-parametric *post hoc* analysis.

Results

BOEC and BOFC Isolation

Figures 2A and 2B confirm the epithelial nature of cells in culture in our model. Additionally, over 95% of cells were positive for CK18, (Figure 2C) and over 99% of the BOFC population stained positive for vimentin (Figure 2D).

ivDOF Characterisation

The volume of *iv*DOF from untreated BOECS after a 24h period of culture was 25.2 ± 4.5 µl (Figure 3A) and the mean osmolarity was 297 ± 12 mOsm (Figure 3B). Untreated *iv*DOF contained 4.30 ± 1.18 mM glucose, 4.70 ± 0.68 mM lactate, and 0.83 ± 0.34 mM pyruvate (Figure 3C). Qualitative western blots for OVGP1 were performed on oviduct fluid derived from fresh abattoir tissue (Figure 3D) and compared with blots given by *iv*DOF (Figure 3E). These figures confirm OVGP1 presence in both oviduct fluids. However OVGP1 collected from abattoir derived *in vivo* oviduct fluid showed two prominent bands at 80 kDa and 90 kDa whereas OVGP1 identified in *iv*DOF was present at 60 kDa.

Figure 3F shows that the amino acid composition of *iv*DOF from untreated BOECs was distinct from that in the medium provided basally (C) with respect to 6/18 amino acids measured. When E2 was added to the basal compartment (Table 1), asparagine, histidine, glutamine, threonine and tyrosine secretion were decreased whereas the apical accumulation of serine and glycine were elevated compared to native *iv*DOF (Figure 3F). Similarly the addition of P4 (Table 1) increased the apical flux of glutamine, glycine, arginine, alanine, and lysine whilst decreasing histidine and tyrosine secretion (Figure 3F). Interestingly treatment with T (Table 1) significantly decreased the accumulation of 10 amino acids in *iv*DOF relative to native fluid (Figure 3F). Figure 3G shows that culturing BOECs in a dual culture configuration with basally adjacent BOFCs altered the secretion of 7/18 amino acids: asparagine, histidine, threonine, and tyrosine movement decreased while glutamine, arginine, and tryptophan flux increased.

BOEC Gene Expression

OVGP1 and *ESR1* were expressed in flask-cultured cells post harvest and increased following 24 hours of E2 exposure (Figure 4). In addition, a panel of solute carrier genes was analysed (Table 2). In brief, *SLC1A1* and *SLC6A14* were up-regulated in response to T, *SLC38A7* expression increased following E2 exposure, and *SLC7A1* and *SLC38A5* expression was elevated following P4 supplementation. The ethanol vehicle control showed no significant impact on gene expression.

Impact of Pathophysiological Endocrine Supplementation

To further explore the impact of endocrine action on oviduct epithelial cell secretions, and to test the capacity of the model for investigating disease, one physiological, and two pathophysiological ranges of hormones were added to the basal compartment (Table 1); the latter represented hypo- and hyper- androgenism. Figure 5 panels A-C show that hyperandrogenism (HYPER) increased the expression of *ESR1* in flask cultured BOECs whilst reducing *OVGP1* and *ZO1* expression whereas hypoandrogenism (HYPO) decreased the relative expression of all the genes investigated relative to physiological (PHYS). Hyperandrogenism also reduced BOEC TEER following 24 hours (Figure 5D) and caused an increase in the volume of *iv*DOF produced (Figure 5E). Figure 5F shows that hypo and hyper treatments had no significant impact on the carbohydrate composition of *iv*DOF. Lastly hypoandrogenism reduced histidine, glutamine, glycine, threonine, arginine, alanine, and lysine secretion whereas hyperandrogenism reduced histidine and arginine but elevated the apical accumulation of glycine (Figure 5G).

Discussion

We present a novel application for an existing bovine oviduct epithelial cell preparation, which can be used to examine the formation of oviduct fluid *in vitro* under a variety of conditions. A layer of BOECs were grown on Transwell™ membranes (Figure 1A) and were confirmed as confluent by TEER, expressed CK18 (Figure 2C), and displayed a number of morphological features typical of epithelial cells (Figures 2A and 2B). Following culture in an air-liquid interface for 24 hours after confirmation of confluence, a film of liquid appeared in the apical chamber, which contained OVGP1 protein (Figure 3E) and was biochemically distinct from the culture medium provided basally (Figure 3F). We therefore propose that this constitutes an *in vitro* Derived Oviduct Fluid (*iv*DOF). We furthermore present a method for achieving dual culture *in vitro* (Figure 1B) and show that incorporating basally adjacent fibroblasts into the model also impacts *iv*DOF amino acid composition (Figure 3G). In addition, parallel flask-cultured BOECs expressed the genes *ESR1* and *OVGP1* in an E2 responsive manner (Figure 4). The above were then expanded to test the capacity of this preparation to model pathophysiological endocrine states (Figure 5).

ivDOF Characterisation

The volume of native *iv*DOF produced in 24 hours was found to be 25.2 ± 11.0 µl (Figure 3A); a rate of formation less than the 1.505 ± 0.291 $\mu l \cdot min^{-1}$ previously reported *in vivo* by Hugentobler *et al* (2008). The osmolarity of native *iv*DOF however was 297 ± 12 mOsm (Figure 4B) which correlates well with both what has been observed *in vivo* 281.0 ± 2.56 mOsm (Paisley & Mickelsen 1979) and the 270 - 300 mOsm range of embryo culture media (Sirard & Coenen 2006). Similarly Hugentobler *et al* (2008) investigated the glucose, lactate and pyruvate composition of *in vivo* bovine oviduct. Multiple t-tests between these data and Figure 3C reveals no significant difference between the basic carbohydrate content of *iv*DOF *vs in vivo*.

OVGP1 in *iv*DOF was ~ 60 kDa (Figure 4E) suggestive of the de-glycosylated form, in contrast to the ~ 80-90 kDa product titrated from abattoir derived oviduct fluid and cell lysates (Figure 4F) (Boice *et al* 1990; Bauersachs *et al* 2004). Abe & Abe (1993)

and Sendai *et al* (1994) also reported two OVGP1 specific bands in the murine and bovine at 95 kDa and ~ 55 kDa respectively. This difference is likely due to a lack of post-translational glycosylation, which would impair electrophoretic mobility by up to 25.3 kDa (Unal *et al* 2008). We suspect this is because the culture medium provided is deficient in substrates such as n-acytlglucosamine, required for glycosylation.

The amino acid composition of *iv*DOF (Figure 4F) resembled data on cannulated oviducts of anaethetised heifers (Hugentobler *et al* 2007). However there were some notable differences between the amino acid content of *in vivo* and *in vitro* oviduct fluid. Histidine was significantly more abundant in *iv*DOF than previously recorded levels in the oviduct lumen (Hugentobler *et al* 2007; Guerin *et al* 1995). One possible explanation for this is that histidine, an imidazole, can act as a pH buffer. The *in situ* bovine oviduct pH is 7.6 (Hugentobler *et al* 2004) whereas *in vitro* BOECs were cultured at ~ pH 7.4. Although a small difference in pH the latter represents a 58.5% increase in free H^+ ions. It could therefore be the case that the native bovine oviduct epithelium secretes histidine to buffer free H^+ ions and balance *iv*DOF pH. Addition of E2 caused histidine in *iv*DOF to fall and P4 administration further decreased histidine to 159.3 µM; closer to the levels observed previously *in vivo* (Guerin *et al* 1995). The addition of T dramatically reduced histidine secretion from 1071.1 µM to 9.7 µM thus histidine transport appears to be subject to T regulation in addition to E2 and P4.

Glutamine was present in native *iv*DOF at levels very close to those reported *in vivo* (Guerin *et al* 1995 Hugentobler *et al* 2007), yet significantly lower than the concentration in the basal culture medium (Figure 4F). This is one example that the BOEC epithelium *in vitro* forms a highly selective barrier. E2 drastically reduced apical glutamine flux, from 170.0 µM to 5.3 µM whilst T had no impact and P4 markedly increased glutamine content in *iv*DOF to 953.5 µM. This might relate to the importance of glutamine in bovine embryo metabolism (Rieger *et al* 1992). Thus it is unsurprising that P4, the dominant circulatory hormone during pregnancy elevated oviduct glutamine output.

Next BOECs and BOFCs were simultaneously cultured on either side of the same membrane (Figure 1B) to provide a closer to physiological environment for modelling

the oviduct epithelium (Fazleabas *et al* 1997). In this dual culture system, the composition of *iv*DOF was modified; with increased appearance of 3 amino acids and a decrease in 4 (Figure 3G). Again histidine and glycine were brought to levels more comparable to *in vivo,* perhaps suggestive of a compensatory mechanism of oviduct fluid regulation.

Fibroblast-epithelial communication has been extensively studied in the cells of the airways in a variety of species (Parrinello *et al* 2005, Noble 2008, Woodward *et al* 1998, Srisuma *et al* 2010, Ohshima 2009, Chhetri *et al* 2012, Nishioka *et al* 2015, Knight 2001, Sakai & Tager 2013) but fibroblast-epithelial interactions have been investigated to a much lesser extent in the oviduct. However Chen *et al* (2013b) reported a highly differentiated porcine oviduct epithelial phenotype when cultured in fibroblast-conditioned medium.

BOEC Gene Expression

To further understand the amino acid transport the expression of a number of key amino acid transporters were investigated in BOECs cultured in plastic flasks.

Expression of *Slc1a1,* the high affinity L-aspartate excitatory amino acid co-transporter 3 (EAAC3), was increased in response to T (Figure 4C) in agreement with Franklin *et al* (2006). However *Slc1a1* expression did not respond to P4 *in vitro* corresponding to earlier reports that *Slc1a1* expression decreases in the bovine uterine endometrium during the progesterone dependent phase (day 16-20) of ruminant pregnancy (Forde *et al* 2014). Notably as *Slc1a1* expression rose in response to T aspartate transport fell (Figure 3F) suggesting that aspartate flux is not solely a function of *Slc1a1* gene expression.

Expression of *Slc7a1*, the arginine and lysine specific cationic amino acid transporter 1 (CAT1) (Broer 2008) increased in response to P4 supplementation (Figure 4D), as did the accumulation of arginine and lysine in *iv*DOF when BOECs were supplemented with P4 (Figure 3F). P4 similarly up-regulated *Slc38a5 in vitro* (Figure 4F) corresponding with an increase in alanine and glycine transport as expected (Figure 3F) and further suggesting that amino acid transport in the oviduct is

hormonally regulated.

Using this model, we confirm that BOECs *in vitro* express hormonally responsive genes, which correlate with previously reported *in vivo* findings. In most cases the secretion of amino acids in *iv*DOF correlated well with transporter expression.

The Impact of Pathophysiological Endocrine Supplementation

As a proof-of-principle sub-study, the efficacy of the aforementioned *in vitro* oviduct preparation was tested for studying the impact of disease states on the oviduct epithelium and fluid composition. The model was subjected to pathophysiological endocrine stimuli at either end of the androgenic spectrum, in addition to a physiological hormonal balance as a form of control (Table 1).

ESR1 expression (Figure 5A) in flask-cultured BOECs was surprising as it correlated negatively with E2 supplementation, but positively with T addition to culture (Table 1). This, however, could be explained by T having a low affinity for the oestrogen receptor *in vitro* (Rochefort & Garcia 1976). *OVGP1* expression was highest following physiological hormonal supplementation (Figure 5B) with hypo- and hyperandrogenic treatment similarly decreasing *ZO1* expression relative to physiological (Figure 5C). To investigate the latter from a functional perspective, using the *in vitro* oviduct model described, TEER measurements were taken, as epithelial resistance is proportional to *ZO1* expression (Sultana *et al* 2013). Figure 5D shows that a hyperandrogenic endocrine profile indeed reduced TEER and moreover increased the volume of *iv*DOF produced (Figure 5E). It is tempting to speculate that this *leaky oviduct* phenotype is driven by impaired ERα activity as *ZO1* expression is responsive to E2 (Zeng *et al* 2004) and ER activity (Weihua *et al* 2003), potentially via a miR-191/425 mediated mechanism (Di Leva *et al* 2013). Moreover Liu *et al* (1999) reported that T reduced TEER in the Caco-2 cell line.

Figure 5F shows that pathophysiological endocrine conditions did not affect glucose, lactate, and pyruvate secretion. Moreover these carbohydrate outputs did not differ from those from untreated cells (Figure 3C) despite the known effects of sex hormone mediated anabolism (Miers & Barrett 1998) and the associated heightened energetic

demands. In contrast hyperandrogenic treatment had a lesser impact on amino acid flux regulation than hypoandrogenism. A striking observation was the elevation of arginine following physiological hormonal supplementation (Figure 5G) compared to all other treatments. Given its role in reproduction (Wu *et al* 2009; Wang *et al* 2015) and early embryo metabolism (Sturmey *et al* 2010; Leary 2015), it is unsurprising that this amino acid would appear in *iv*DOF. Such high appearance could be explained by the fact that arginine can be readily synthesised from glutamate via ornithine (Wu 2010). Glycine was also interesting as it was elevated in *iv*DOF following hyperandrogenic incubation (Figure 5G) but reduced following singular T supplementation (Figure 3F), implying that the regulation of glycine flux is not solely T dependent.

Conclusions

We present a method for examining the formation of oviduct fluid under dual culture and a variety of singular, physiological, and pathophysiological endocrine conditions within a controlled environment. This development offers the prospect of modelling the influence of the oestrous cycle (in animals) and the menstrual cycle (in women) with the possibility of using the data on the *iv*DOF generated to optimise embryo culture media.

Declaration of Interests

The authors declare no conflict of interest.

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Figure and Table Legends

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Figure 5 — The effects of hypoandrogenic (HYPO), physiological (PHYS), and hyperandrogenic (HYPER) like endocrine supplementation on **(A)** *ESR1*, **(B)** *OVGP1*, and **(C)** *ZO1* gene expression (n=3 ± SEM). **(D)** TEER values from BOECs before and after HYPO, PHYS, and HYER exposure in addition to native (n=3 ± SD). One statistically significant difference was determined by paired t-test (p=0.0214). **(E)** Volumes of *iv*DOF from HYPO, PHYS, and HYER treated BOECs (n=3 ± SD). **(F)** The carbohydrate composition of *iv*DOF from BOECs subjected to HYPO, PHYS, and HYER exposure (n=3 ± SD). **(G)** The amino acid content of *iv*DOF obtained from HYPO, PHYS, and HYPER treated cells (n=3 ± SD). All treatment durations were 24 hours. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$.

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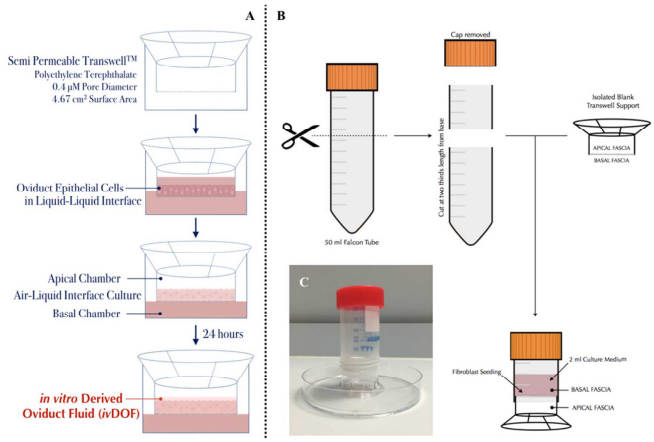

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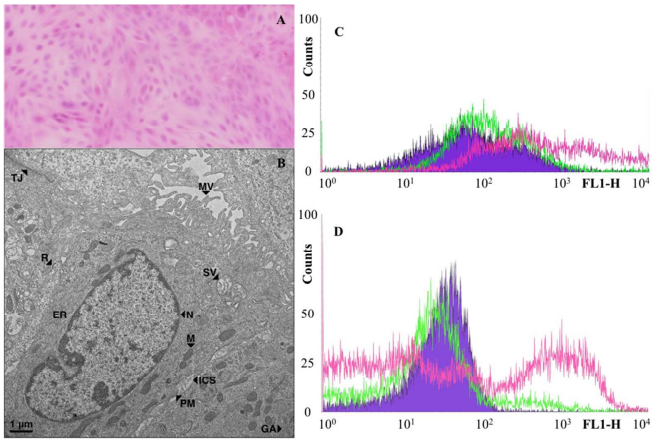

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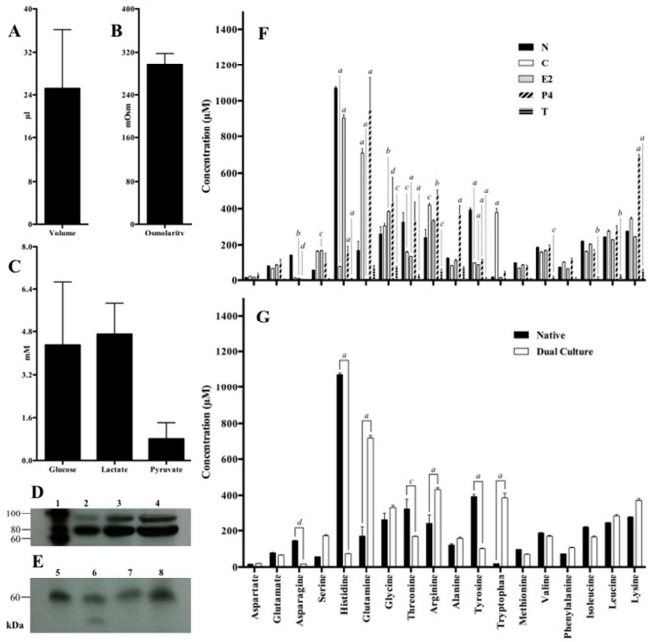
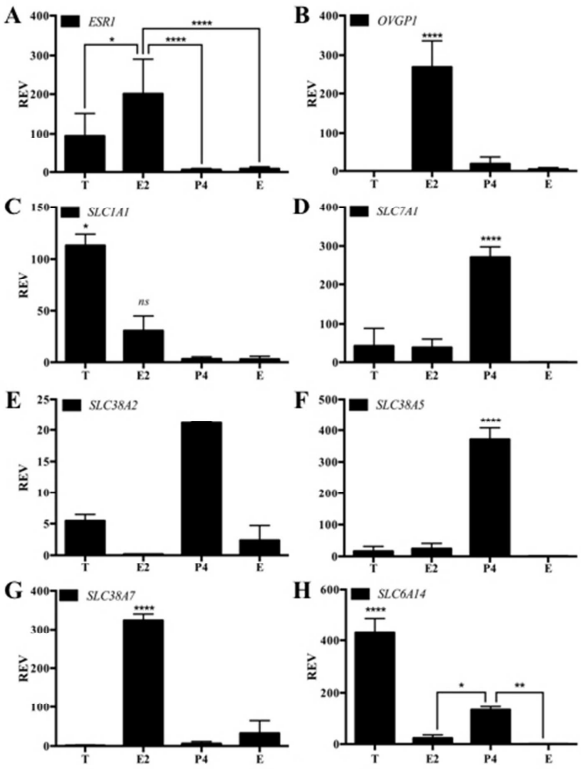


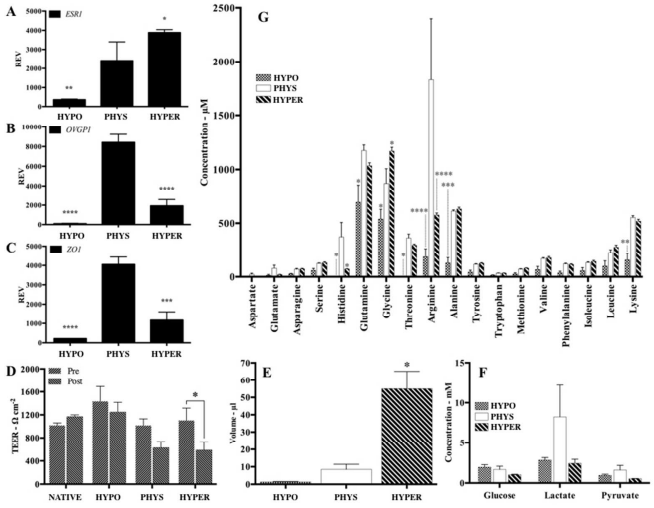
(A) The volume (n=6 ± SD), **(B)** osmolarity (n=3 ± SD), and **(C)** carbohydrate content (n=3 ± SD) of *iv*DOF obtained from native (untreated) epithelia. **(D-E)** Western (protein immuno) blots for OVGP1 from **(D)** *in vivo* derived oviduct fluid and cell lysates (n=1) and **(E)** native *iv*DOF (representative of n=4). Lane 1 was loaded with a staggered 200 kDa HRP-linked biotinylated protein ladder. Lane 2 with 10 mM (16.5 µl) total protein, lane 3 with 20 mM (33.3 µl) and lane 4 with 40 mM (66.7 µl). Lanes 5-8 were loaded with 40 µl (arbitrary concentrations) of native *iv*DOF. **(F)** The amino acid composition of *iv*DOF accumulated apically from native (N) BOECs (n=12 ± SD) *vs.* culture medium (C) supplied basally (n=3 ± SD) *vs. iv*DOF derived from BOECs basally supplemented with 29.37 pM 17β-oestradiol (E2; n=6 ± SD) *vs. iv*DOF from BOECs treated with 6.36 nM progesterone (P4; n=4 ± SD) *vs. iv*DOF from BOECs basally supplemented with 62.77 pM testosterone (T; n=3 ± SD). **(G)** The amino acid profile of native *iv*DOF (n=12 ± SD) *vs. iv*DOF from BOECs cultured with BOFCs basally adjacent in the dual culture arrangement (n=4 ± SD). All *iv*DOF accumulated over 24 hours and a = p ≤ 0.0001, b = p ≤ 0.001, c = p ≤ 0.01, and d = p ≤ 0.05.

Figure 3
250x247mm (72 x 72 DPI)



Gene expression profiles of **(A)** *ESR1*, **(B)** *Ovgp1*, **(C)** *Slc1a1*, **(D)** *Slc7a1*, **(E)** *Slc38a2*, **(F)** *Slc38a5*, **(G)** *Slc38a7* and **(H)** *Slc6a14* as determined by qRT-PCR (n=3 ± SEM). BOECs were subjected to 62.77 pM testosterone (T), 29.37 pM 17β-oestradiol (E2), 6.36 nM progesterone (P4), and 0.45% (*v/v*) ethanol (E) as vehicle control – all for 24 hours. Data were normalised to β-actin whilst the impact of treatment on gene expression was calculated relative to native BOECs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$.

Figure 4
170x223mm (72 x 72 DPI)



The effects of hypoandrogenic (HYPO), physiological (PHYS), and hyperandrogenic (HYPER) like endocrine supplementation on **(A)** *ESR1*, **(B)** *OVGP1*, and **(C)** *ZO1* gene expression (n=3 ± SEM). **(D)** TEER values from BOECs before and after HYPO, PHYS, and HYER exposure in addition to native (n=3 ± SD). One statistically significant difference was determined by paired t-test (p=0.0214). **(E)** Volumes of *iv*DOF from HYPO, PHYS, and HYER treated BOECs (n=3 ± SD). **(F)** The carbohydrate composition of ivDOF from BOECs subjected to HYPO, PHYS, and HYER exposure (n=3 ± SD). **(G)** The amino acid content of *iv*DOF obtained from HYPO, PHYS, and HYPER treated cells (n=3 ± SD). All treatment durations were 24 hours. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$.

Figure 5
316x245mm (72 x 72 DPI)

	17β-Oestradiol (E2)	Progesterone (P4)	Testosterone (T)
Native (N)	0 pM	0 pM	0 pM
17β-Oestradiol (E2)	29.37 pM	0 pM	0 pM
Progesterone (P4)	0 pM	6.36 nM	0 pM
Testosterone (T)	0 pM	0 pM	62.77 pM
Hypoandrogenic (HYPO)	29.37 pM	6.36 nM	2.43 pM
Physiological (PHYS)	29.37 pM	6.36 nM	208 pM
Hyperandrogenic (HYPER)	19.46 pM	6.36 nM	6.27 nM

Gene	Direction	Sequence	T_m (°C)	GC (%)
β-Actin	Forward (3' to 5')	TTCAACACCCCTGCCATG	59.64	56
	Reverse (5' to 3')	TCACCGGAGTCCATCACGAT	59.73	55
OVGP1 *	Forward (3' to 5')	CTGAGCTCCATCCCCACTTG	57.20	60
	Reverse (5' to 3')	GTTGCTCATCGAGGCAAAGG	57.10	55
ESR1 *	Forward (3' to 5')	AGGGAAGCTCCTATTTGCTCC	57.00	52
	Reverse (5' to 3')	CGGTGGATGTGGTCCTTCTCT	57.50	57
SLC1A1 †	Forward (3' to 5')	CACCGTCCTGAGTGGGCTTGC	61.30	67
	Reverse (5' to 3')	CAGAAGAGCCTGGGCCATTCCC	61.30	64
SLC38A2 †	Forward (3' to 5')	GAACCCAGACCACCAAGGCAG	58.10	62
	Reverse (5' to 3')	GTTGGGCAGCGGGAGGAATCG	61.80	67
SLC38A5 †	Forward (3' to 5')	TGGCCATCTCGTCTGCTGAGGG	63.20	64
	Reverse (5' to 3')	GCTCCTGCTCCACAGCATTCCC	62.00	64
SLC38A7 †	Forward (3' to 5')	CGGCAGCCCGAGGTGAAGAC	61.60	70
	Reverse (5' to 3')	GCCGCAGATACCTGTGCCCAT	60.90	62
SLC6A14 †	Forward (3' to 5')	TCGAGGGGCAACTCTGGAAGGT	60.80	59
	Reverse (5' to 3')	GGCAGCATCTTTCCAAACCTCAGCA	62.90	52
ZO1	Forward (3' to 5')	CTCTTCCTGCTTGACCTCCC	56.80	60
	Reverse (5' to 3')	TCCATAGGGAGATTCCTTCTCA	55.20	45

9.5. Publication in *Biology of Reproduction*

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Sexually dimorphic gene expression in bovine conceptuses at the initiation of implantation[1]

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ABSTRACT

In cattle, maternal recognition of pregnancy occurs on Day 16 via secretion of interferon tau (IFNT) by the conceptus. The endometrium can distinguish between embryos with different developmental competencies. In eutherian mammals, X-chromosome inactivation (XCI) is required to ensure an equal transcriptional level of most X-linked genes for both male and female embryos in adult tissues, but this process is markedly different in cattle than mice. We examined how sexual dimorphism affected conceptus transcript abundance and amino acid composition as well as the endometrial transcriptome during the peri-implantation period of pregnancy. Of the 5132 genes that were differentially expressed on Day 19 in male compared to female conceptuses, 2.7% were located on the X-chromosome. Concentrations of specific amino acids were higher in the uterine luminal fluid with male compared to female conceptuses, while female conceptuses had higher transcript abundance of specific amino acid transporters (*SLC6A19* and *SLC1A35*). Of note, the endometrial transcriptome was not different in cattle gestating a male or a female conceptus. These data support the hypothesis that, far from being a blastocyst specific phenomenon, XCI is incomplete before and during implantation in cattle. Despite differences in transcript abundance and amino acid utilization in male versus female conceptuses, the sex of the conceptus itself does not elicit a different transcriptomic response in the endometrium.

INTRODUCTION

In cattle, pregnancy recognition both at the physiological [1, 2] and transcriptomic level [3, 4] is initiated on day 16 in order to prevent release of luteolytic pulses of prostaglandin F2 alpha from the endometrium, corpus luteum regression, and subsequent return to cyclicity. Subtle responses of the endometrium to the conceptus can be detected as early as day 13 [5], but

the major transcriptomic response to the pregnancy recognition signal in cattle, interferon tau (IFNT) from the cells of the conceptus trophectoderm, occurs at day 16 [3]. Additional studies have demonstrated a high degree of similarity in the changes in the endometrial transcript abundance between pregnant and cyclic endometrium as pregnancy progresses from Day 15 through to Day 20 i.e. during the period of maternal recognition of pregnancy [3, 4, 6-9]. The endometrial transcriptomic response to early pregnancy is quite specific to the type of conceptus present, such that by Day 18 and 20 the response signature can distinguish between *in vivo*, *in vitro* and cloned embryos and thus the developmental outcome of the embryo [10, 11].

In eutherian mammals, X-chromosome inactivation (XCI) is required in females to ensure an equal transcriptional level of most X-linked genes for both males and females. In female (XX) preimplantation embryos, both X-chromosomes are transcriptionally active from embryonic genome activation (EGA) until a long non-coding RNA (X-inactive specific transcript: *XIST*) mediates the inactivation of one of them. Okamoto *et al*. [12] revealed substantial diversity in the timing and regulation of XCI initiation between mice, in which this phenomenon has been studied in most detail, and rabbits and humans. For example, *XIST* transcript abundance is not imprinted in rabbit and human embryos, and the choice of which X chromosome to inactivate seems to occur downstream of *XIST* upregulation and X-chromosome coating, which differs significantly from the processes in the mouse.

We found that the process of XCI in the bovine differs markedly from that of the mouse. Similar to the situation reported in humans, XCI in cattle is far from being accomplished at the blastocyst stage [13]. Furthermore, abundance of many X-linked transcripts which escaped XCI in the bovine blastocyst were effectively equalized among sexes in Day 14 elongated conceptuses [14]. This mirrors the situation in the rabbit late blastocyst, and suggests that a large component of XCI occurs after the differentiation of TE/ICM lineages, but before gastrulation, indicating a significant amount of discord between the mouse and many other mammalian species. In addition, we found sexually dimorphic differences exist between male and female embryos in terms of developmental rate [15], and Day 7 blastocyst transcriptome [13] as well as amino acid turnover [16], with as many as one third of all actively expressed transcripts in the blastocyst being determined by the sex of the embryo [13].

In this study, we hypothesized that sex-related differences in transcript abundance remain throughout conceptus elongation and that male and female embryos elicit a different response in the endometrium. Thus, the aims of this study were to: (i) examine the effect of conceptus sex on conceptus transcript abundance and amino acid utilization at Day 19; (ii) compare the temporal changes in conceptus transcript abundance between Day 7 and Day 19; and (iii) determine whether male and female embryos on Day 19 elicit a different response from the endometrium.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. Unless otherwise stated, all chemicals and reagents were sourced from Sigma (Dublin, Ireland).

Animal model and sample collection

The estrous cycles of crossbred beef heifers (n=30) were synchronized using an 8-day controlled internal drug release (CIDR) device (1.38 g P4; Pfizer Animal Health, Sandwich,

Kent, UK) placed intra-vaginally. One day prior to CIDR removal, a 2 ml intramuscular injection of a prostaglandin $F_{2\alpha}$ analogue (Estrumate, Intervet, Dublin, Ireland; equivalent to 0.5 mg cloprostenol) was administered. All heifers were then observed at 4 h intervals and only those observed in standing estrus (=Day 0) were inseminated with semen from a proven sire. All heifers were slaughtered on Day 19 following estrus corresponding to the initiation of implantation in cattle. Thirty minutes after slaughter each uterine horn was flushed with 10 ml of PBS and the presence of a conceptus was observed under a stereo-microscope. Only those heifers from which a conceptus was recovered were further processed for tissue collection (n=24). Each conceptus was dissected into 4 pieces, 3 containing only extra-embryonic tissue (EET) and one containing the embryonic disc along with associated trophectoderm cells, and immediately snap-frozen in liquid nitrogen. The uterine luminal flush samples were then placed into 1ml aliquots and snap frozen in liquid nitrogen prior to amino acid analysis. The uterine horn ipsilateral to the corpus luteum was opened longitudinally and intercaruncular endometrium from the mid-part of the horn was dissected away from the underlying myometrium and snap frozen in liquid nitrogen. All samples were stored at -80 °C prior to processing.

Conceptus sexing

DNA was extracted from a sample of EET cells from each conceptus with phenol/chloroform treatment and finally re-suspended in 200 µL of milliQ water. Two microliters of each sample were used to perform embryo sexing by PCR amplification of sex-specific polymorphic fragments in the amelogenin gene as previously described [17].

RNA extraction and microarray hybridization

Total RNA was extracted from the EET cells from confirmed female (n=5) and male (n=5) conceptuses as well as their corresponding intercaruncular endometrial tissue (100 mg), using Trizol reagent as per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Following on column DNase digestion and RNA clean up, (Qiagen, Crawley, West Sussex, UK) both the quality and quantity of the RNA was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop 1000 (Thermo Fischer Scientific Inc. Wilmington, DE, USA), respectively. A subset of samples (both conceptus and corresponding intercaruncular endometrial tissue) with an RNA Integrity Number of greater than 8.0, were randomly chosen for microarray analysis (n=5 per tissue type). Transcriptomic analysis was carried out using the Affymetrix GeneChip Bovine Genome Array. Two micrograms of total RNA were converted to cDNA via first and second strand synthesis using the GeneChip Expression 3'-Amplification One-Cycle cDNA Synthesis kit. Biotin-labeled cRNA was synthesized from double-stranded cDNA using the GeneChip Expression 3'-Amplification in vitro transcription (IVT) Labeling Kit. cRNA quality was assessed on the Agilent 2100 Bioanalyzer and 25 µg of cRNA was fragmented using 5X Fragmentation buffer in RNase-free water contained within the GeneChip Sample Cleanup Module at 94 °C for 35 min and quality accessed again on the Agilent 2100 Bioanalyzer. Fifteen µg of fragmented cRNA and hybridization cocktail were added to the GeneChip Bovine Genome Array and hybridized for 16 h at 45 °C. Each array was then washed and stained on the GeneChip fluidics station 450 using the appropriate fluidics script and once completed the array was inserted into the Affymetrix autoloader carousel and scanned using the GeneChip Scanner 3000.

The raw signal intensities were read into R and pre-processed using functions of both affy and GCRMA packages of the BioConductor project [18]. Hierarchical clustering analysis was

performed to determine the greatest source of variation in the tissue samples. Lists of differentially expressed genes (DEGs) were determined by the Limma package [19] employing linear modeling and an empirical Bayes framework to shrink the variance of measurements on each probe set. A modified t-test was then carried out and all *p* values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method.

Analysis of Interferon Tau (IFNT) in the uterine luminal fluid

Concentrations of IFNT in the ULF recovered from heifers with a male (n=11) or female (n=11) conceptus was carried out using a cytopathic antiviral assay [20, 21] as previously reported [22]. Samples were examined in duplicate by titrating in a 96-well plate (1:3 serial dilution). Madin-Darby Bovine Kidney (MDBK) cells were added and incubated in medium (DMEM containing 10% FBS) at 37 ºC in 5% CO_2 in humidified air. After 24 h, cells were challenged with vesicular stomatitis virus for 1 h. Thereafter virus was removed and cells were incubated with growth medium (DMEM containing 10% FBS) for 18 h. Cell viability was determined after fixation (75% ETOH) using 0.5% [w/v] Gentain-violet. The ability of samples to prevent lysis by 50% was compared with a recombinant human IFN alpha standard (EMD Biosciences; 3.8 x 10^8 IU/mg). Data are presented as IU of antiviral activity per ml of conditioned medium. Unconditioned medium (blanks) did not contain antiviral activity.

Analysis of amino acid content of uterine luminal fluid

The amino acid composition of uterine luminal fluid (n=11 samples hosting a male and n=11 samples hosting a female conceptus) was quantitatively analyzed by High Performance Liquid Chromatography (HPLC) as previously described [16]. Briefly, amino acids in ULF were derivatised with *O*-Phthaldialdehyde (OPA) reagent, supplemented with 1 mg/ml 2-mercaptoethanol. Derivitised samples were subjected to reverse phase chromatography using an Agilent 1100 Series HPLC system coupled with a Phenomenex HyperClone 5 mm C-18 ODS 250 x 4.6 mm column (Phenomenex, Macclesfield, UK). A gradient elution with two buffers: (A) 80% 83 mM sodium acetate, 19.5% methanol, 0.5% tetrahydrofuran, and (B) 80% methanol and 20% 83 mM sodium acetate was used to separate OPA-amino acid conjugates at 30 °C with a flow rate of 1.3 ml/min for 60 min per sample. Concentrations of amino acids in the ULF (μM) were determined by comparing sample peak areas to those from certified standards.

Quantitative real-time PCR (qRT-PCR) analysis

One thousand nanograms of total RNA from the conceptus and corresponding endometrial tissue of n=5 male and n=5 female conceptuses were subjected to reverse transcription reaction using Superscript III (Applied Biosystems, Foster City, CA, USA) and random hexamers as per manufacturer's instructions. Primers for microarray validation were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries where possible. Primers for amino acid transporters have been previously reported [23]. Each qRT-PCR reaction was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems) with 5 ng of cDNA, optimized primer concentrations (Supplemental Table S1), and 7.5 μl FAST Sybergreen mastermix (Applied Biosystems) in a final reaction volume of 15 μl. The cycling conditions for all qRT-PCR reactions were as 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All reactions were carried out in duplicate, with the inclusion of a dissociation curve to ensure specificity of amplification as well as no template controls. A standard curve was included for each gene of interest as well as for

the normalizer gene to obtain primer efficiencies. All raw cycle threshold values were then imported into qbase^{plus} software (Biogazelle, Zwijnaarde, Belgium) where data were calibrated, normalized and expression values for each gene were determined in arbitrary units (calibrated normalized relative quantities, CNRQ).

Overrepresented gene ontology terms, upstream regulators, and pathway analysis

To further interrogate the differences in transcript abundance associated with the sex of the conceptus, the list of DEGs was subjected to analysis using the functional annotation tool in DAVID (http://david.abcc.ncifcrf.gov/) to generate overrepresented biological processes and molecular functions. In addition, Ingenuity Pathway Analysis (IPA) was performed (http://www.ingenuity.com) to identify overrepresented pathways associated with the sex of the conceptus i.e. those with a larger number of differentially expressed genes that one would expect by chance. IPA was also used to assess whether or not the sex of the conceptus modified upstream regulators of the DEGs identified between male and female conceptuses. For all overrepresented gene ontologies, pathways and upstream regulators a p-value for a given function was calculated by considering the number of functional analysis molecules that participate in that function e.g. ligand, receptor etc. and the total number of molecules that are known to be associated with that function/pathway in the DAVID/Ingenuity Knowledge Base. GO terms, pathways and/or upstream regulators with p-value less than 0.05 were considered significant (i.e. more differentially expressed genes associated with these than would be expected by chance).

RESULTS

Correspondence analysis revealed segregation between the different tissue types (i.e. endometrial tissue data clustered together and conceptus tissue data clustered together) (Figure 1A). Conceptus sex did not affect the overall transcriptional profile of the endometrium, but did affect its global transcript abundance in the conceptus (Figure 1B).

Differential transcript abundance in Day 19 male and female conceptuses

In Day 19 conceptuses, the abundance of 5132 transcripts were significantly different between males and females ($P<0.05$); 2498 were increased in male compared to female conceptuses while 2634 genes were increased in female compared to male conceptuses. Full gene descriptions, associated p-values, and log2 fold change differences are given in Supplemental Table S2. Of the transcripts whose abundance was highest in male conceptuses to the greatest extent, zinc finger protein 665-like (2.63 log2 fold change increase in male), lumican (2.34), zinc finger protein 107 (2.33), mirror-image polydactyly gene 1 protein-like (2.20), microRNA mir-2284d (2.03), unknown gene (2.01), zinc finger protein 208 (1.95), microRNA mir-2399 (1.95), CDC-like kinase 1 (1.91) and zinc finger protein 91-like (1.87), none were located on the X chromosome. Transcripts whose abundance was increased in female conceptuses compared to male to the greatest extent included XIST (4.65 log2 fold increase in female compared to male conceptuses), immunoglobin light chain VJ region (2.44), intestine-specific transcript 1 protein (2.17), synapsin I (1.91), uncharacterized protein C12orf54 homolog (1.84), TIMP metallopeptidase inhibitor 1 (1.69), pancreatic progenitor cell differentiation and proliferation factor homolog (zebrafish) (1.5), KxDL motif containing 1 (1.48), histone cluster 1, H3a-like 23 (1.47) and mucin 15, cell surface associated (1.45). Of these, X (inactive)-specific transcript, synapsin I and TIMP metallopeptidase inhibitor 1 are located on the X chromosome.

Of the total number of DEGs identified between male and female conceptuses, 140 were located on the X chromosome and 78 were increased in the female conceptuses while 62 were increased in the male conceptus on Day 19 of pregnancy. No probes on the microarray were located on the Y chromosome.

Gene Ontology (GO) and Ingenuity pathway analysis of overrepresented biological processes, molecular functions, pathways, and upstream regulators of differentially abundant transcripts in male compared to female conceptuses on Day 19.

In total, sex of the conceptus significantly affected 102 biological processes more than would be expected by chance with 68 molecular functions associated with conceptus sex on Day 19. A full list of the overrepresented biological processes and molecular functions and their associated genes are given in Supplemental Tables S3 and S4. Ingenuity pathway analysis identified 407 pathways that were overrepresented in this list of differentially abundant transcripts, the details of which are available in Supplemental Table S5. Included in these pathways were those associated with cell cycle progression (Role of CHK Proteins in Cell Cycle Checkpoint Control 32 DEGs: Cell Cycle Control of Chromosomal Replication 14 DEGs: Cell Cycle: G2/M DNA Damage Checkpoint Regulation 22 DEGs), Embryonic cell lineage (Mouse Embryonic Stem Cell Pluripotency 36 DEGs: Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency 40 DEGs: Telomerase Signaling 35 DEGs: Human Embryonic Stem Cell Pluripotency 32 DEGs: DNA Methylation and Transcriptional Repression Signaling 8 DEGs) as well as pathways involved in conceptus-maternal interactions (Role of PKR in Interferon Induction and Antiviral Response 18 DEGs: GM-CSF Signaling 24 DEGs: Androgen Signaling 39 DEGs: Glucocorticoid Receptor Signaling 81 DEGs: HGF Signaling 37 DEGs: MIF Regulation of Innate Immunity 11 DEGs: MIF-mediated Glucocorticoid Regulation 8 DEGs).

Interestingly, 23 of these pathways were associated with amino acid degradation, biosynthesis and signaling. In total, 66 DEGs were involved in the mTOR signaling pathway, six were involved in Tryptophan Degradation X, five involved in Methionine Degradation I, five in the pathway of Phenylalanine Degradation IV, with seven, five and two DEGs associated with the pathways of Isoleucine Degradation I, Leucine Degradation I and Lysine Degradation II, respectively.

Analysis of upstream regulators of the DEGs in male compared to female conceptuses revealed that 55 of these DEGs were identified as significant upstream regulators of other DEGs due to sex of the conceptus (Supplemental Table S6). Moreover, a significant proportion of these were transcriptional regulators (16 in total). Additional molecules identified as upstream regulators of genes that were different by virtue of conceptus sex included the amino acids serine and glutamine as well as interferon alpha.

Differences in amino acid composition of uterine luminal fluid (ULF) recovered from uteri with male or female conceptuses

To assess whether conceptus sex affected the amino acid composition of ULF we examined the amino acid content of ULF recovered from heifers with either a male or a female conceptus on Day 19. Of the 18 amino acids analyzed in the ULF containing female or male conceptuses, the concentrations of arginine, asparagine, glutamine, histidine, isoleucine, lysine, methionine, and tryptophan were significantly higher ($P<0.05$: Figure 2) in the ULF containing a male compared to a female conceptus on Day 19. Interestingly, amino acid concentrations were consistently higher in the ULF hosting a male compared to a female conceptus on Day 19.

Quantitative real-time PCR validation of microarray data and expression of amino acid transporters in the endometrium and conceptus on Day 19

Consistent with the microarray analysis, there was no difference in the abundance of any of 26 amino acid transporters analyzed between endometria from cattle gestating a male compared to a female conceptus (P>0.05). Expression of *XIST* was higher (P<0.001) in the endometrium of all animals analyzed compared to conceptus expression, but did not differ between groups (P>0.05).

Comparison of transcript abundance between male and female conceptuses revealed a higher expression of *XIST* in female compared to male conceptuses (P=0.01) with a large range in expression values in female conceptuses (range 0.118-2.62 CNRQ) compared to males (range: 0.004-0.016 CNRQ) while abundance in the endometrial tissue was substantially higher (range: 12.35-33.37 CNRQ). The expression of the amino acid transporters *SLC6A19* and *SLC1A3* was significantly higher in male compared to female conceptuses on Day 19 (Figure 3).

Comparison of sexually dimorphic transcript abundance in Day 19 conceptuses with that in the Day 7 blastocyst

In order to identify the temporal changes in sexually dimorphic gene expression that occur between the blastocyst stage (Day 7) and implantation (Day 19), the same pre-processing and stringency measures were applied to previously published data from our group [13]. This resulted in the identification of 2,295 DEGs between male and female Day 7 blastocysts, 1,176 of which were higher in the male and 1,119 were higher in female blastocysts. Of these, 7.1 % of DEGs (163) in the blastocyst linked with sex were located on the X chromosome in comparison to the 2.7 % of X-chromosome associated genes on Day 19 of conceptus development (Figure 4). A similar number of DEGs were identified on each chromosome as a proportion of the total number of genes on each chromosome. A comparison of these DEGs revealed 1392 were only differentially abundant on Day 7 (659 increased in male, 904 increased in female embryos) while 4239 genes were altered on Day 19 (2,418 increased in male and 2,210 increased in female conceptuses). Only 862 genes were differentially abundant in the embryo/conceptus on both days 7 and 19 (Figure 5A). These transcripts separated into 4 main categories; 164 DEGs were affected in the same way i.e. increased in male embryo compared to the female embryo on both Day 7 and 19, while 203 were increased in the female embryo/conceptus on both days. In contrast, 161 transcripts were increased in female embryos on Day 7 and decreased on Day 19 compared to male embryos, while for 334 genes the expression pattern was opposite *i.e*. decreased in female embryos on Day 7 and increased on Day 19 compared to male embryos (Figure 5B: Full information on fold change and P-value are given in Supplemental Table S7). The location of these temporally altered transcripts was notable, as 21.2% of these DEGs that were increased in female compared to male embryos on Day 7 and 19 and 14.9% of these DEGs that were increased in female embryos on Day 7 and decreased in female embryos on Day 19 were located on the X chromosome (Supplemental Table S8).

Endometrial transcriptomic response to a male vs female conceptus

Despite major differences in day 19 conceptus gene expression, no differences in the endometrial transcriptomic response to a male or female conceptus were observed (p>0.05: data accessible in Gene Expression Omnibus (GEO) database GSE75754). Consistent with this finding, the amount of IFNT present in the uterine flush from which a male (335,687±68,118

AU) or female (279,413±55,797 AU) conceptus was recovered was not different (P>0.05).

DISCUSSION

This study builds on the knowledge that one-third of transcripts present at the blastocyst stage of development in cattle are regulated by the sex of the embryo [13] as well as the novel hypothesis that the endometrium is a biosensor of the developmental competency or origin of the embryo [24] by addressing whether this sexual dimorphism is maintained throughout the elongation period of conceptus development and/or whether the endometrium is sensitive to the sex of the developing conceptus on Day 19. Using large-scale transcriptomic analysis, we have determined that more transcripts are differentially abundant between male and female conceptuses on Day 19 than in blastocysts on Day 7, but some differences (approximately 7%) are maintained between these two distinct morphological stages of embryo/conceptus development. Proportionally, more transcripts on autosomal chromosomes are modulated by sex as opposed to just the X-chromosome on Day 19 compared to Day 7. Despite these differences, the endometrium does not respond differently to the presence of a male or a female conceptus, at least not at the transcriptome level after pregnancy recognition has occurred and production of IFNT is not different. However, sex of the conceptus does affect the availability of amino acids in the ULF, likely due to the different requirements of the conceptuses for amino acids in a sex-dependent manner.

The significant number of DEGs between male and female conceptuses was a surprising finding for a number of reasons. Firstly, a number of studies in the literature have found little differences in transcript abundance between male and female conceptuses. While the lack of a significant effect of sex on gene expression is somewhat at odds with the current study, the design of these studies was very different and the platforms used were not always comparable. The study design in the paper of Degrelle *et al*., (2012) [25] was radically different the present study. They compared Day 18 somatic cell nuclear transfer (SCNT)-derived conceptuses (n=30) to those derived by AI (n=10) or IVP (n=10). All of the SCNT conceptuses were female one-half of which showed signs of atypical elongation and gastrulation. To generate SCNT and IVP conceptuses, 5-6 blastocysts were transferred per recipient. For the AI controls, animals underwent a mild superovulation protocol (600 IU PMSG) and they also used a different microarray platform. The absence of a sex effect was based on comparison of conceptuses derived from AI (males versus females) as well as females only (AI-IVP-SCNT). Similarly, Betscha *et al*., (2013) [26] compared gene expression in Day 16 conceptuses derived by AI, SCNT or IVP bovine using the Affymetrix bovine genome array. In this case, all SCN embryos were male. While the authors failed to detect differential gene expression amongst the AI and IVP embryos this was based on analysis of only one female and two male embryos in each case. Valour *et al*., (2014) [27] investigated the effect of dam physiological status on embryo development and conceptus gene expression in growing heifers and postpartum cows using a homemade 22K bovine oligonucleotide probe array. Sex of the conceptus explained 3.1% of the variability observed in the overall gene expression pattern, respectively. Conversely, the physiological status of the dams represented 23.7% of the variability. However, in these studies both the experimental design and platform differed from this study.

Secondly, the X-chromosome that is silenced is always the paternal X-chromosome in extra-embryonic membranes i.e. in the EET (reviewed by [28]). There are substantial data in the literature demonstrating a sex-dependent rate of growth/development. However, these data refer to development to the blastocyst stage only, and to our knowledge cell number in elongated

conceptuses has not been quantified. In as much as was possible to assess, conceptus length was not different between male and female conceptuses in this study. This is difficult to measure accurately at Day 19 because of the fragile nature of the conceptus at this stage and the fact that the conceptuses are often tangled due to the flushing recovery technique. However, two points would suggest that male conceptuses were not advanced compared to females. Firstly, IFNT content of uterine lumen fluid, which is highly correlated with conceptus length and trophectoderm cell number [29] was not different between males and females. Secondly, the expression of a number of marker genes of gastrulation in the EET cells (as detailed in the study by Degrelle *et al*., [25] - specifically the genes *CPA3*, *CALM1*, and *HNRNPDL*) were not differentially expressed between male and female conceptuses in this study (Supplemental Table S2). Despite the fact that the proportion of DEGs located on the X-chromosome was lower than those previously reported in the bovine blastocyst [13], similar numbers of DEGs were located on the X chromosome. Sexual dimorphic biological pathways in Day 19 conceptuses included cell cycle progression and chromosomal replication as well as stem cell pluripotency. Also overrepresented are genes involved in the pathways of glucocorticoid receptor signaling, androgen signaling and MIF signaling. These data are interesting in the context of a recent study by Dobbs *et al*. [30] that found a sex-dependent response of the embryo to colony-stimulating factor 2. Thus, embryo trophic factors produced by male and female conceptuses on Day 19 may affect pathways in the conceptus itself in a sex-specific manner.

Comparisons of transcripts affected by sex of the embryo at Day 7 to those affected in the Day 19 conceptus revealed that only a small proportion are conserved between these distinct morphological time-points (Figure 4). The timing and mechanism of XCI differs between germ line and somatic cells [12] [28] and this may account for the seemingly small overlap in sex-regulated genes. In particular, tissue analyzed on Day 7 consists of a mixture of approximately 2:1 trophoblast to inner cell mass whereas on Day 19 only trophoblast cells were analyzed. Within these 864 genes, less than half displayed a similar expression pattern on both days of pregnancy. Interestingly, a number of genes were decreased on both days 7 and 19 in the female compared to male embryo/conceptus *i.e*. when X-chromosome inactivation has occurred, given increased expression of *XIST* in the female embryo [14] and conceptus (this study). Evidence from other species shows that coordinate with XCI, portions of autosomal chromosomes can become inactivated during this process (reviewed by [31]). The decreased transcript abundance in female conceptuses on Day 19 may be autosomal genes inactivated during the process of XCI. In addition, it is possible that the transcripts only decreased on Day 19 in female conceptuses may be a late or delayed consequence of XCI. Some caution is needed with the interpretation of these comparisons however, given the two data sets are derived from somewhat different sources. The Day 7 blastocyst data were derived from in vitro fertilized embryos produced with sex-sorted semen. The current data were derived from AI with conventional (non-sex-sorted semen).

In contrast to the sex-induced differences in the conceptus, no differences in the endometrial transcriptomic response to either a male or female conceptus were detectable on Day 19. As has been shown on Day 18 [11] and Day 20 [10] in cattle and in humans [32], the endometrium does respond differently to conceptuses of differing quality and trajectories with regard to pregnancy outcome [10, 11]. Therefore, the lack of differences observed in the endometrial transcriptomic response to male and female conceptuses may simply reflect a lack of difference in developmental competency of a male versus a female conceptus after Day 19. It is interesting, however, that a number of the DEGs between male and female conceptuses are also

expressed in the endometrium (e.g. *MIF*, *OXT*, SI1). In addition, we know from previously reported studies that the endometrium expresses receptors for some conceptus-derived ligands but the fact remains that no differences in the pregnancy recognition signal occur, at least in the intercaruncular endometrium. However, it is possible that there may be protein differences or differences in the post-translational modifications of proteins in the endometrium in response to male and female conceptuses and could be an avenue of future study.

Both male and female Day 8 blastocysts [33] as well as Day 14 conceptuses [14] display differences in the type of IFNT transcripts expressed; however, there was no difference in the abundance of IFNT in the ULF containing male or female conceptuses on Day 19. Given that IFNT is predominantly responsible for the pregnancy recognition response in the endometrium of cattle, as there are no differences between male and female abundance of IFNT, this may explain in part, why there is no difference in the transcriptomic response of the endometrium to the conceptus. Differences in amino acid composition in the ULF on Day 19 were interesting. In a previous study by Sturmey *et al*., [16], male *in vivo*-derived blastocysts had a lower depletion of amino acids and lower amino acid turnover compared to female blastocysts i.e. increased amounts of amino acids in the media similar to increased amino acids in the ULF in this study. If increased amino acids in the ULF are indicative of reduced uptake by the Day 19 conceptus (likely given there are no differences in amino acid transporters in the endometrium) then it is reasonable that male conceptuses do not utilize amino acids to the same extent as female conceptuses. Differences in the amino acid composition of the ULF hosting cloned and *in vitro* produced conceptuses on Day 18 have been observed [34] but this is coordinate with reduced expression of amino acid transporters in the endometrium (likely due to the different endometrial response these conceptuses elicit [11]). In the present study differences in amino acid composition were not due to differences in transcript abundance for the amino acid transporters in the endometrium. Thus, despite a similar uterine environment male and female conceptuses utilize this environment in a sex-specific manner *in vivo* similar to the phenomenon observed *in vitro* with regard to amino acid uptake [16] as well as other energy substrates. Indeed, concentrations of the neutral amino acids asparagine, glutamine, and methionine were higher in the ULF hosting male compared to female conceptuses. Male conceptuses exhibited increased expression of the neutral amino acid transporter *SLC6A19*. This increased expression of the transporter in the male conceptuses may not necessarily translate into increased neutral amino acid uptake. Alternatively, this could be increased transcript abundance that may translate into increased amino acid uptake by the male conceptus at a later time point i.e. after Day 19 when these samples were taken.

In conclusion, the results of this study support the hypothesis that XCI is incomplete during the initiation of implantation in cattle. There is also significant sexual dimorphism in terms of amino acid consumption as well as gene expression in the conceptus at Day 19. Yet despite the significant difference in gene expression changes, the sex of the conceptus itself does not elicit a significantly different response in the transcriptome of the endometrium, at least on Day 19. Moreover, given the fact the conceptus is exposed to the same maternal environment (as there is no difference in endometrial response) we propose that conceptuses of different sexes utilize the same uterine environment but in a sex-dependent fashion.

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FIGURE LEGENDS

Figure 1. Correspondence analysis indicating the source of greatest variation in the overall transcriptional profile. Each dot represents an individual microarray for an individual sample. **A**) A clear segregation in the expression profiles of the different tissue types, i.e. endometrium and conceptus, was observed, but sex of the conceptus affected gene expression in the conceptus (right) but not the endometrium (left). **B**) Differences in overall gene expression profiles in male and female conceptus when plotted alone.

Figure 2. Concentrations (μM±SEM) of amino acids in the uterine luminal fluid (ULF) recovered on Day 19 of pregnancy from heifers with a male (black bars) or female (open bars) conceptus present (n=11 per sex). Significant differences in amino acid concentrations from the uterus containing a male versus a female conceptus are noted with an asterisk (*) when $P<0.05$.

Figure 3. Analysis of transcript expression for amino acid transporters and selected transcripts from the microarray study qRT-PCR in male and female conceptuses on Day 19 of pregnancy. All expression values are given as mean calibrated, normalized, relative expression values in arbitrary units (AU±SEM) for n=5 male (closed bars) and n=5 female (open bars) conceptuses on Day 19. Significant differences in expression between male and female conceptuses are noted by an asterisk (*) when $P<0.05$.

Figure 4. Graph depicting the number of genes on each chromosome as well as the frequency of chromosomal location of genes identified as differentially expressed between male and female conceptuses on Day 19 of pregnancy (this study) and embryos on Day 7 of pregnancy [13].

Figure 5. Venn diagram showing the overlap in differentially expressed genes (**A**) as well as the direction of fold change between male and female embryos (**B**) using the same analysis criteria (Day 7 [13]) and male and female conceptuses (Day 19: This study). All data was subjected to the same stringent data pre-processing prior to generation and comparison of lists of differentially expressed genes.

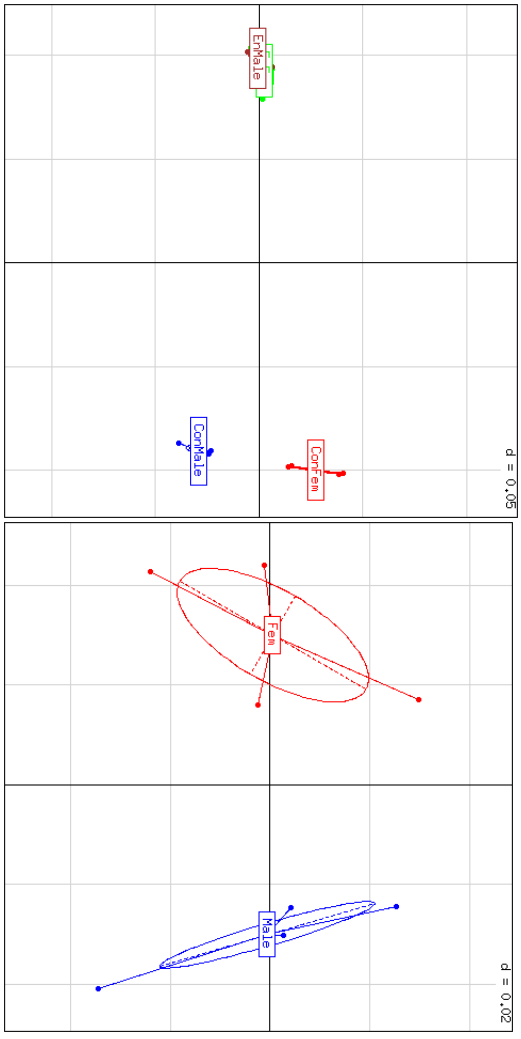


Figure 1(A). **(B).**

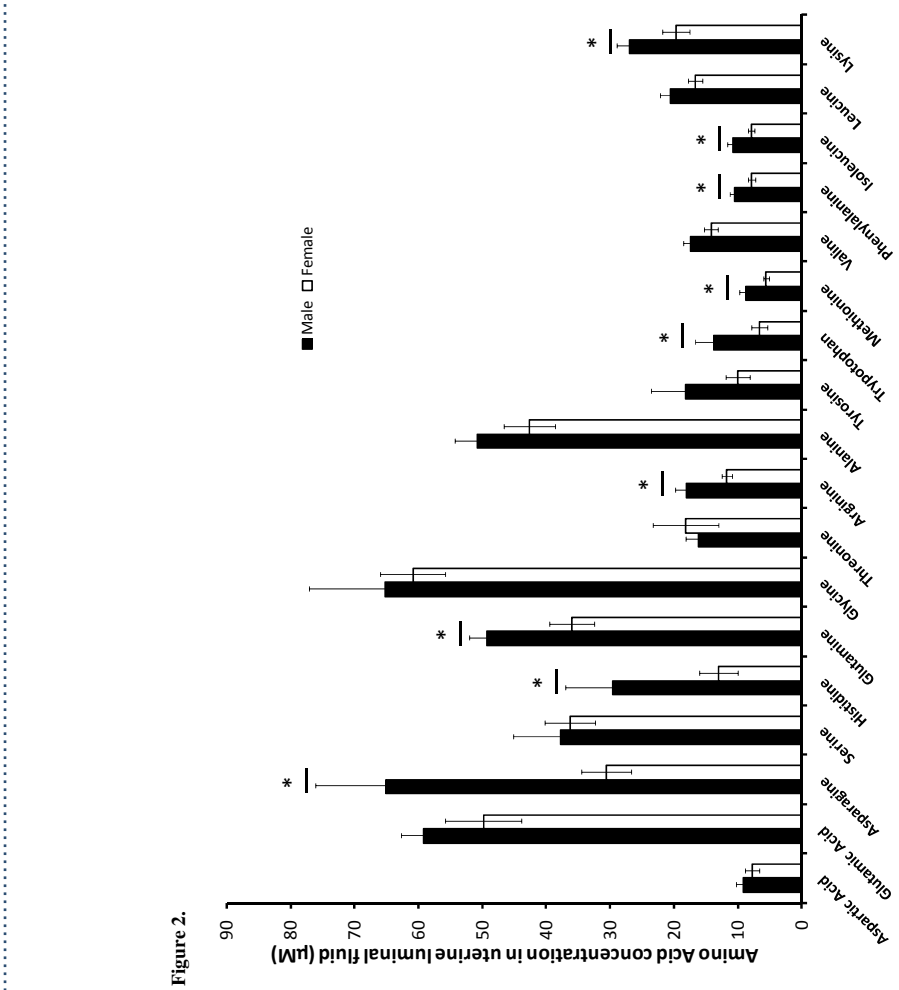
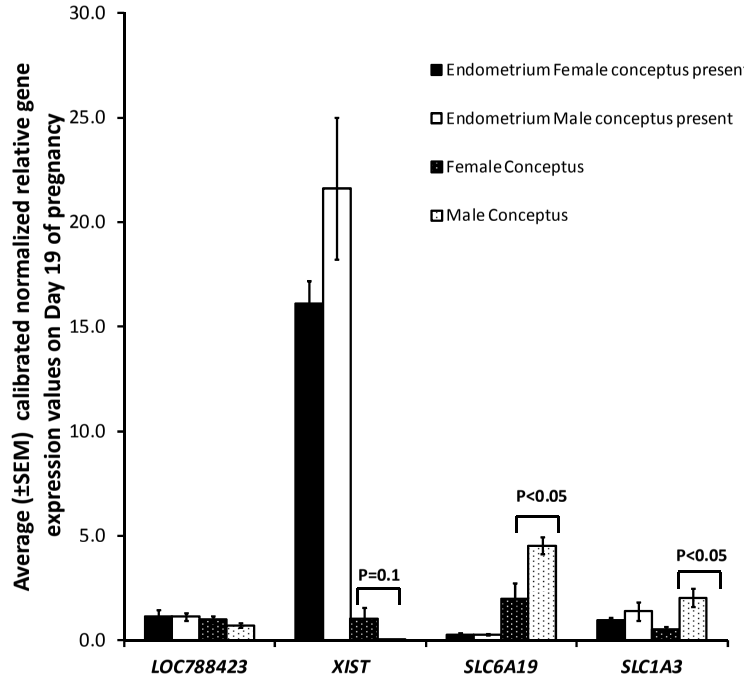

Amino Acid concentration in uterine luminal fluid (μM)
0
10
20
30
40
50
60
70
80
90
Male
Female
Aspartic Acid
Glutamic Acid
Asparagine
Serine
Histidine
Glutamine
Glycine
Threonine
Arginine
Alanine
Tyrosine
Tryptophan
Methionine
Valine
Phenylalanine
Isoleucine
Leucine
Lysine
*

Figure 2.

Figure 3.


30.0
25.0
20.0
15.0
10.0
5.0
0.0
Average (±SEM) calibrated normalized relative gene expression values on Day 19 of pregnancy
Endometrium Female conceptus presen
Endometrium Male conceptus present
Female Conceptus
Male Conceptus
P=0.1
P<0.05
P<0.05
LOC788423
XIST
SLC6A19
SLC1A3

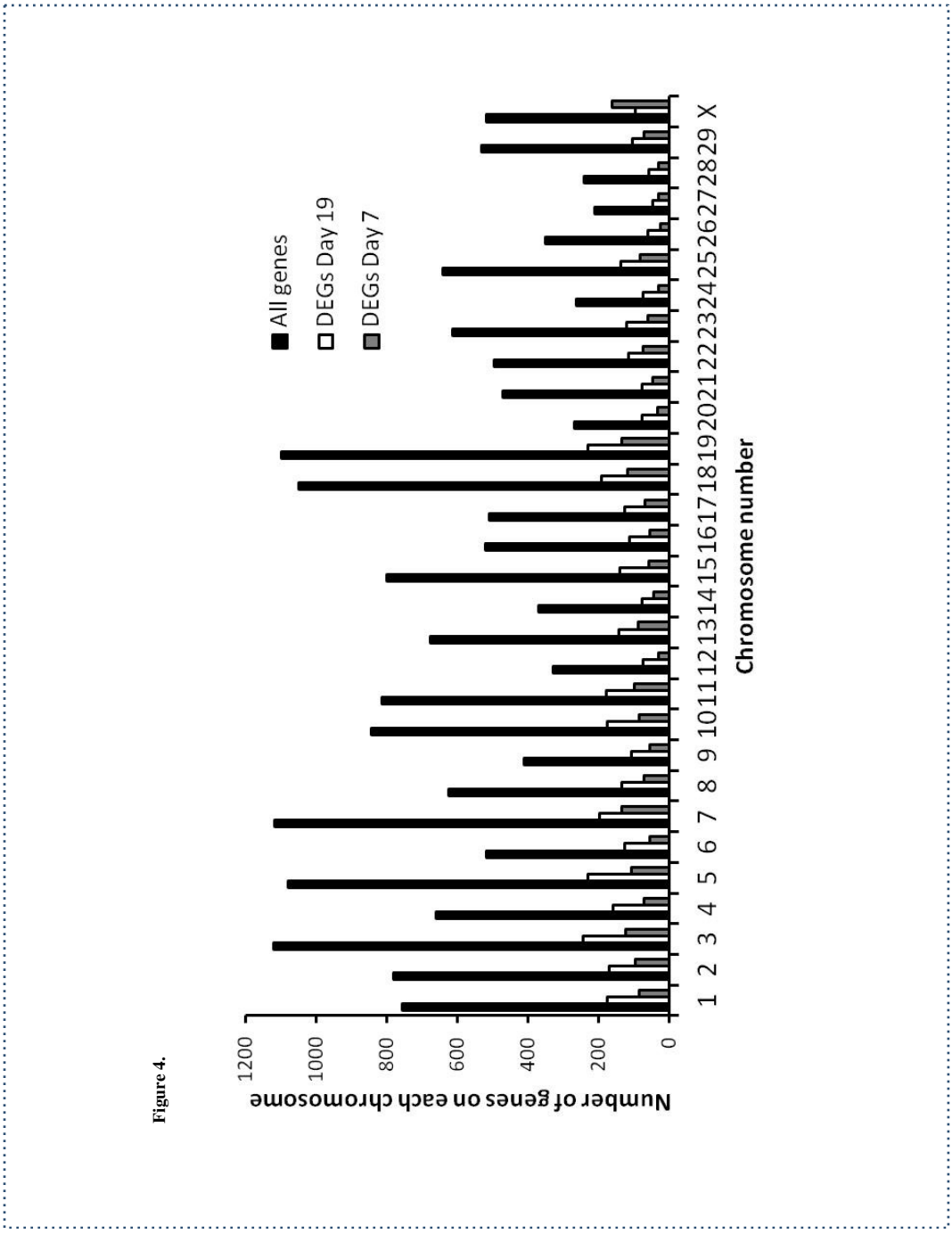

All genes
DEGs Day 19
DEGs Day 7
Number of genes on each chromosome
0
200
400
600
800
1000
1200
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 X
Chromosome number

Figure 4.

Figure 5A.

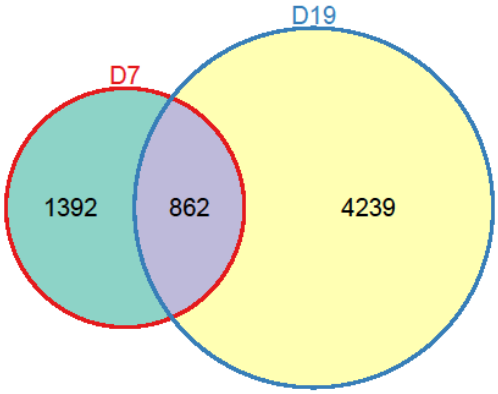
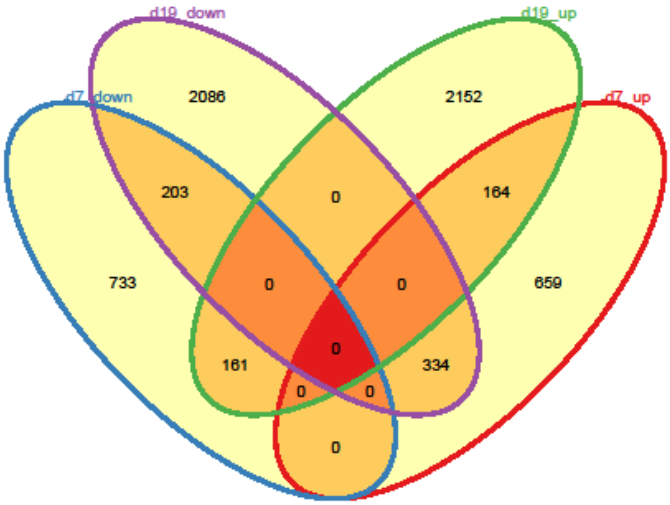


Figure 5B.



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PLOS ONE

Amino Acids in the Uterine Luminal Fluid Reflects the Temporal Changes in Transporter Expression in the Endometrium and Conceptus during Early Pregnancy in Cattle

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Abstract

In cattle, conceptus-maternal interactions are critical for the establishment and maintenance of pregnancy. A major component of this early interaction involves the transport of nutrients and secretion of key molecules by uterine epithelial cells to help support conceptus development during the peri-implantation period of pregnancy. Objectives were to: 1) analyze temporal changes in the amino acid (AA) content of uterine luminal fluid (ULF) during the bovine estrous cycle; 2) understand conceptus-induced alterations in AA content; 3) determine expression of AA transporters in the endometrium and conceptus; and 4) determine how these transporters are modulated by (Progesterone) P4. Concentrations of aspartic acid, arginine, glutamine, histidine, lysine, isoleucine, leucine, phenylalanine and tyrosine decreased on Day 16 of the estrous cycle but increased on Day 19 in pregnant heifers ($P<0.05$). Glutamic acid only increased in pregnant heifers on Day 19 ($P<0.001$). Asparagine concentrations were greater in ULF of cyclic compared to pregnant heifers on Day 7 ($P<0.05$) while valine concentrations were higher in pregnant heifers on Day 16 ($P<0.05$). Temporal changes in expression of the cationic AA transporters *SLC7A1 SLC7A4* and *SLC7A6* occurred in the endometrium during the estrous cycle/early pregnancy coordinate with changes in conceptus expression of *SLC7A4, SLC7A2* and *SLC7A1* ($P<0.05$). Only one acidic AA transporter (*SLC1A5*) increased in the endometrium while conceptus expression of *SLC1A4* increased ($P<0.05$). The neutral AA transporters *SLC38A2* and *SLC7A5* increased in the endometrium in a temporal manner while conceptus expression of *SLC38A7*, *SLC43A2*, *SLC38A11* and *SLC7A8* also increased ($P<0.05$). P4 modified the expression of *SLC1A1*, *-1A4*, *-1A5*, *-38A2*, *-38A4*, *-38A7*, *-43A2*, *-6A14*, *-7A1*, *-7A5* and *-7A7* in the endometrium. Results demonstrate that temporal changes in AA in the ULF reflect changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle, some of which are modified by P4.

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Introduction

A major cause of infertility in cattle is embryo mortality due to conceptus and/or uterine dysfunction during the pre-implantation period of pregnancy. In cattle, the majority of embryonic loss occurs prior to maternal recognition of pregnancy [1] which occurs on approximately Day 16 of pregnancy [2,3]. Thus, conceptus-maternal interactions are critical to the establishment and maintenance of pregnancy. Major components of this early conceptus-maternal interaction involve transporters for nutrients and secretions of key molecules by uterine epithelial cells that support conceptus development during the peri-implantation period of pregnancy [4,5,6,7]. These secretions and transported molecules make up histotroph that represents maternal contributions to uterine luminal fluid (ULF). The requirement for these uterine-derived secretions and transported nutrients for successful pregnancy is well established in the ovine uterine gland knockout model in which the conceptus fails to elongate [8]. In cattle, ULF is a prerequisite for development of the embryo beyond the hatched blastocyst stage in vivo, and attempts to artificially induce elongation of bovine conceptuses in vitro have been unsuccessful [9,10]. The ULF of ruminants is composed of both secreted and transported molecules that include numerous secreted proteins [11,12], glucose [13], ions [13], fatty acids [14] and amino acids [13,15]. In sheep, a significant increase in total amino acid content occurs between Days 10 and 16 of pregnancy [13]. Moreover, the expression of cationic [16], acidic and neutral amino acid transporters is altered in a spatial and temporal manner in both

the endometrium and conceptus of sheep during early pregnancy [13] and is modulated by ovarian progesterone (P4) and/or conceptus interferon tau (IFNT), prostaglandins and cortisol in vivo [17,18,19].

In spite of the importance of maternally-derived secretions, including amino acids, there are limited reports pertaining to the requirements for conceptus elongation and successful pregnancy recognition in cattle. What is known, however, is that amino acids are utilized by the early embryo both in vitro and in vivo [20,21,22,23]. In addition, total amounts of amino acids increase in ULF as the estrous cycle progresses [24] and are higher in ULF of pregnant compared to non-pregnant heifers on Day 18 [12]. Furthermore the abundance of individual amino acids (e.g., valine) is regulated by P4 [25].

The hypothesis tested was that the amino acid composition of bovine ULF changes in a temporal manner during the estrous cycle and early pregnancy due to alterations in the expression of their transporters in the endometrium and conceptus. The objectives were to: 1) analyze the temporal changes in the amino acid content of ULF during the bovine estrous cycle; 2) understand conceptus-induced alterations in amino acid content of ULF during critical windows of early pregnancy; 3) determine expression of the transporters in the endometrium and conceptus responsible for shuttling these amino acids into and out of the uterine lumen; and 4) determine how these transporters are modulated in the endometrium by P4.

Materials and Methods

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. Unless otherwise stated, all chemicals were sourced from Sigma (Dublin, Ireland).

Study 1: Analysis of the amino acid content of uterine luminal fluid and expression of amino acid transporters in the endometrium during the estrous cycle and peri-implantation period of early pregnancy

The estrous cycles of 100 cross-bred beef heifers were synchronized as previously described [26] by insertion of a controlled internal drug release (CIDR) device (1.38 g progesterone; InterAg, Hamilton, New Zealand) into the vagina for 8 days. A 2 ml intramuscular injection of a prostaglandin $F_{2\alpha}$ (PG) analogue (Estrumate, Shering-Plough Animal Health, Hertfordshire, UK: equivalent to 0.5 mg cloprostenol) was administered one day before CIDR removal. Heifers were checked for estrus and only those detected in standing heat (estrus = Day 0) were utilized further. In order to generate pregnant (P) and cyclic (C) tissues, heifers were assigned randomly to either an inseminated group (n = 59) or a non-inseminated cyclic control group (n = 24). Cyclic heifers were slaughtered on Day 7, 10, 13, or 16 of the estrous cycle and inseminated heifers were slaughtered on Day 7, 10, 13, 16 or Day 19 of pregnancy. These stages correspond in pregnant animals to the times of blastocyst formation, blastocyst hatching, initiation of elongation, maternal recognition of pregnancy and initiation of implantation, respectively. Cyclic heifers were not included for the Day 19 time-point as they had undergone luteolysis at this stage. At slaughter, the uterine horn ipsilateral to the corpus luteum (CL) was flushed with 20 ml of 10 mM Tris (pH 7.2) and in the inseminated group, only those flushings that contained an appropriately developed conceptus (i.e., correct stage for age) were processed further. Recovered ULF was centrifuged at 1,000 g for 15 min, supernatant decanted and immediately snap frozen in 1 ml aliquots in liquid nitrogen for subsequent amino acid analysis. Intercaruncular and caruncular endometrial tissues were dissected out separately from the uterine horn ipsilateral to the CL and snap-frozen in liquid nitrogen for RNA extraction and quantitative real-time PCR (qRT-PCR).

Study 2: Expression of amino acid transporters in the conceptus during key developmental stages

Analysis of the expression of amino acid transporters in the embryo/conceptus was carried out by screening RNA sequencing data generated as previously described [27]. Briefly, estrus synchronization of cross-bred beef heifers (n = 72) was performed as described for Study 1. Heifers observed in standing heat were inseminated. Animals were assigned randomly for slaughter on Day 7, 10, 13, 16 or 19 of pregnancy. At slaughter, each uterine horn was flushed with 20 ml phosphate buffered saline (PBS) containing 3% fetal calf serum (FCS), the embryo/conceptus recovered and snap frozen in liquid nitrogen. Only those uterine flushings with conceptuses at the correct morphological stage of development for their age were analysed. RNA was extracted from whole conceptuses, cDNA libraries were prepared and cluster generation and sequencing were carried out using standard procedures for the Illumina genome analyzer sequencer (www.illumina.com). The RNAseq samples were processed through the standard software pipeline for the Genome Analyzer (http://bioinfo.cgrb.oregonstate.edu/docs/solexa/SCS2_01_IPAR1_01_Release_Notes.pdf). The CASAVA module from Illumina software was used to process RNAseq data. All data were aligned against the BosTau4 genome and a pseudochromosome containing potential splice junction sequences was generated. This gene expression data set was then screened for expression of members of the solute-like carrier (SLC) gene family for transport of amino acids.

Study 3: Identification of changes in endometrial expression of amino acid transporters by manipulation of P4 concentrations in vivo

The estrous cycles of cross-bred beef heifers were synchronized as described in Study 1 and only those observed in standing estrus (n = 52) were used. Heifers were assigned randomly to one of three treatments, (i) high P4 (n = 12), (ii) normal P4 (n = 12) and (iii) low P4 (n = 28). Heifers in the high P4 group had a progesterone-releasing intravaginal device (PRID, CEVA, Libourne, France) inserted on Day 3 of the estrous cycle to elevate P4 concentrations [28], while heifers in the control group received no P4 manipulation. Heifers assigned to the low P4 group received three intramuscular injections of PG (Estrumate, Shering-Plough Animal Health, Hertfordshire, UK) on Day 3, 3.5 and 4 of the estrous cycle to reduce P4 output from the CL, as previously described [29]. Daily blood samples were obtained from all heifers up to day of slaughter on either Day 7 or Day 13 of the estrous cycle. Following slaughter, intercaruncular endometrial tissue from the tip of the uterine horn ipsilateral to the CL was recovered, snap frozen in liquid nitrogen for subsequent RNA extraction and qRT-PCR was performed for selected amino acid transporters.

Analysis of uterine luminal fluid amino acids. The amino acid content of ULF was measured by High Performance Liquid Chromatography (HPLC) as previously described [23]. In summary, the amino acids present in ULF were derivatised with *O*-Phthaldialdehyde reagent, supplemented with 1 mg/ml 2-mercaptoethanol. Reverse phase chromatography was subsequently performed on an Agilent 1100 Series HPLC system coupled

with a Phenomenex HyperClone 5 mm C-18 ODS 250×4.6 mm column (Phenomenex, Macclesfield, UK). A gradient elution with two buffers comprised of (A) 80% 83 mM sodium acetate, 19.5% methanol, 0.5% tetrahydrofuran, and (B) 80% methanol and 20% 83 mM sodium acetate was used to separate OPA-amino acid derivatives at 30°C for 60 min at a flow rate of 1.3 ml/min. Concentrations (μM) were determined by comparing the area under the curve for each peak to those given from certified standards.

Quantitative real-time PCR analysis (qRT-PCR). For both Studies 1 and 3 total RNA was extracted from 100 mg of endometrial tissue using Trizol reagent as per manufacturer's instructions. RNA clean-up and on-column DNase treatment were performed (Qiagen, Crawley, Sussex, UK). Both the quality and quantity of extracted RNA was determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Nanodrop 1000 (Thermo Fischer Scientific, DE, USA), respectively. One microgram of total RNA was converted to complementary DNA (cDNA) using Superscript III (Applied Biosystems, Foster City, CA, USA) and random hexamers as per manufacturer's instructions. All primers were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries where possible. Each qRT-PCR reaction was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems) with 50 ng cDNA, optimized primer concentrations (Table S1), and 7.5 μl FAST Sybrgreen mastermix (Applied Biosystems) in a final reaction volume of 15 μl. Cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 sec and 60°C for 1 min and were carried out with the inclusion of a dissociation curve to ensure specificity of amplification. A standard curve was included for each gene of interest as well as for the normalizer gene to obtain primer efficiencies. All raw cycle threshold values were then imported into qbase^{plus} software (Biogazelle, Zwijnaarde, Belgium) where data were calibrated, normalized and expression values for each gene were determined in arbitrary units (CNRQ).

Data analysis. All data were analysed using the SAS statistical package (SAS Institute Inc., Cary, NC, version 9.1.3). For gene expression analysis, the log of the calibrated, normalized, relative expression values (CNRQ) in arbitrary units from the endometrium or transcripts per million from the embryo/conceptus were inputted into the SAS program while total concentrations of amino acids in the ULF were used for amino acid analysis. Data were checked for normality and homogeneity of variance by histograms, qqplots, and formal statistical tests as part of the UNIVARIATE procedure of SAS. Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. The transformed data were used to calculate P values. Gene expression values were analyzed using the general linear model procedures (PROC GLM) with day, pregnancy status, P4 concentration and/or tissue type (i.e., caruncular vs intercaruncular) where appropriate as the main effects. Treatment effects on gene expression were separated by Tukey's test and a p value of ≤ 0.05 was considered significant. Concentrations of amino acids were analyzed with the MIXED procedure of SAS. Fixed effects included experimental treatment (cyclic and pregnant), day, and their interaction. The interaction term, if not statistically significant ($P>0.10$), was subsequently excluded from the final model. Heifer within treatment was included as a random effect. The type of variance-covariance structure used was chosen depending on the magnitude of the Akaike information criterion for models run under compound symmetry, unstructured, autoregressive, or Toeplitz variance-covariance structures. Differences between treatments were determined by F-tests using Type III sums of squares. The PDIFF command incorporating the Tukey test was applied to evaluate pairwise comparisons between treatment means.

Results

Amino acid content of ULF during the peri-implantation period of pregnancy in cattle

Analysis of the ULF detected the presence of 18 amino acids of which threonine and glycine were most abundant on all days examined. The acidic amino acids, aspartic and glutamic acids, were detected in the ULF throughout the estrous cycle and early pregnancy. Aspartic acid concentrations decreased on Day 16 in both pregnant and cyclic heifers compared to all other days; however, on Day 19 of pregnancy, concentrations were higher ($P<0.05$) than for Day 16 (Table 1). Glutamic acid concentrations did not change between Days 7 and 16 ($P>0.05$), but increased from Day 16 to Day 19 in pregnant heifers ($P<0.001$).

The basic amino acids arginine, histidine and lysine, displayed similar changes in abundance as the concentration of all these amino acids decreased on Day 16 of the estrous cycle compared to other days of the estrous cycle. This decrease did not occur in pregnant heifers; indeed, the concentrations of all three basic amino acids in ULF was greater on Day 19 compared to Day 16 ($P<0.05$).

Of the small neutral amino acids, concentrations of alanine, glycine and serine in ULF from both pregnant and cyclic heifers were similar for all days examined ($P>0.05$). Concentrations of asparagine were highest in cyclic heifers on Day 7, declined significantly by Day 10 and remained low thereafter, while in pregnant heifers, despite an initial decline in concentrations on Day 10, concentrations were relatively stable throughout early pregnancy. Concentrations of asparagine were greater in ULF of cyclic compared to pregnant heifers on Day 7 ($P<0.05$). Concentrations of threonine were elevated on Day 10 compared to all other days in cyclic heifers while concentrations in pregnant heifers were lower ($P<0.05$) on Day 16 and 19 compared with earlier time-points.

Of the large neutral amino acids in the ULF, glutamine, isoleucine, leucine, phenylalanine, tyrosine and valine exhibited similar trends in cyclic heifers with a decline in concentration on Day 16 of the estrous cycle. In pregnant heifers, concentrations of these amino acids as well as tryptophan were stable from Day 7 to Day 13, but increased ($P<0.05$) on Day 16 (glutamine) or Day 19 (isoleucine, leucine, phenylalanine, tryptophan, tyrosine, valine) of pregnancy. In contrast, concentrations of methionine decreased ($P<0.05$) from Day 13 onwards in cyclic heifers, while in pregnant heifers concentrations were lowest on Day 16.

Expression of cationic amino acid transporters in the endometrium and conceptus during the peri-implantation period of pregnancy

Endometrial expression of *SLC7A1* was affected by day with an increase ($P<0.01$) in expression at the latter stages of the estrous cycle and early pregnancy while all other members of this transport family (*SLC7A4* and *SLC7A6*) decreased ($P<0.01$) from Day 10 to 16 in the intercaruncular region of the endometrium (Table 2). In addition, expression of *SLC7A1* and *SLC7A4* mRNAs was less and *SLC7A6* expression was greater ($P<0.05$) in caruncular compared to intercaruncular regions of the endometrium (Figures 1A–C). Pregnancy significantly affected *SLC7A6* expression which was less abundant in intercaruncular regions of

Table 1. The effect of day and pregnancy status on the abundance of amino acids (μM) in bovine uterine luminal fluid during the peri-implantation period of pregnancy on Days 7, 10, 13, 16 for cyclic heifers and Days 7, 10, 13, 16 and 19 for pregnant heifers (n = 5 per treatment per time-point).

Amino acid	Treatment	Day 7	Day 10	Day 13	Day 16	Day 19	Day effect	Pregnancy effect	Day x Pregnancy
Basic amino acids									
Arginine	Cyclic	12.34±2.77ax	8.34±2.48ax	13.32±2.48ax	7.31±2.77bx	-	***	*	ns
	Pregnant	7.89±2.26ay	5.97±2.26ay	7.32±2.26ay	7.14±2.77ax	15.94±7.15b			
Histidine	Cyclic	5.67±1.21ab	4.23±1.21ab	5.79±1.21a	2.37±1.21b	-	ns	ns	ns
	Pregnant	3.69±0.99a	4.26±0.99a	5.01±1.40ab	4.33±1.21a	8.54±2.88b			
Lysine	Cyclic	16.38±2.92a	9.78±2.90ab	13.57±2.61a	5.58±1.75b	-	ns	ns	ns
	Pregnant	10.38±2.95a	7.23±2.76a	8.43±1.77a	9.55±10.70a	21.47±8.51b			
Acidic amino acids									
Aspartic acid	Cyclic	12.52±2.92a	12.62±2.61a	12.25±2.61a	4.50±2.92b	-	*	ns	ns
	Pregnant	9.08±2.38a	9.70±2.38a	8.93±2.38a	4.13±2.92b	9.76±4.37a			
Glutamic acid	Cyclic	47.11±10.66a	43.84±9.54a	43.54±10.74a	22.06±16.60a	-	ns	ns	ns
	Pregnant	38.58±7.60a	39.53±4.72a	40.40±7.74a	21.76±2.42a	69.43±27.18b			
Small neutral amino acids									
Alanine	Cyclic	35.27±8.09a	39.35±7.24a	42.54±7.24a	23.90±8.09a	-	ns	ns	ns
	Pregnant	26.63±6.61a	27.54±6.61a	30.28±6.61a	30.59±8.09a	51.13±17.99a			
Asparagine	Cyclic	3.12±0.35ax	1.63±0.35b	1.44±0.31b	0.80±0.40b	-	***	ns	0.1
	Pregnant	2.14±0.29ay	1.33±0.29b	1.28±0.40ab	1.68±0.35ab	3.41±1.47ab			
Glycine	Cyclic	114.12±42.78a	150.84±92.73a	65.91±28.07a	52.35±30.50a	-	ns	ns	ns
	Pregnant	129.90±57.83a	133.98±77.48a	57.41±36.66a	30.41±6.00a	54.78±22.77a			
Serine	Cyclic	21.63±3.94a	20.42±3.52a	17.27±4.55a	14.89±3.94a	-	ns	ns	ns
	Pregnant	15.78±3.22a	13.13±3.22a	22.23±3.52a	22.90±3.94a	27.64±9.26a			
Threonine	Cyclic	177.03±14.41ax	268.62±12.89bx	213.52±12.89ax	181.33±14.41ax	-	***	0.06	***
	Pregnant	208.61±11.77ax	195.70±12.89ay	212.91±11.77ax	151.07±14.41bx	124.53±5.96b			
Large neutral amino acids									
Glutamine	Cyclic	24.37±4.25ax	17.08±4.45abx	24.18±4.17ax	12.74±3.74bx	-	***	***	***
	Pregnant	14.60±7.07ax	16.47±7.45ax	21.97±8.20ax	18.17±1.62by	36.79±11.89c			
Isoleucine	Cyclic	8.81±1.58a	5.87±1.41ab	7.36±1.41ab	3.27±1.58b	-	ns	ns	ns
	Pregnant	5.79±1.29a	4.45±1.29a	4.39±1.29a	5.15±1.58a	9.78±3.82b			
Leucine	Cyclic	17.60±3.26a	12.27±2.91ab	15.73±2.91a	6.57±3.26b	-	ns	ns	ns
	Pregnant	11.78±2.66a	9.18±2.66a	9.73±2.66a	10.45±3.26a	20.28±9.32b			
Methionine	Cyclic	10.98±1.68ax	11.83±1.94ax	5.92±1.50bx	2.85±1.68bx	-	***	*	ns
	Pregnant	7.83±1.37ax	6.97±1.37aby	4.48±1.37abx	3.43±1.68bx	7.66±3.39a			
Phenylalanine	Cyclic	7.46±1.22a	5.84±1.22ab	6.14±1.09a	2.77±1.22b	-	ns	0.07	ns
	Pregnant	5.14±1.00a	3.69±1.00a	3.59±1.00a	4.59±1.22a	9.48±3.82b			

Table 1. Cont.

Amino acid	Treatment	Day 7	Day 10	Day 13	Day 16	Day 19	Day effect	Pregnancy effect	Day x Pregnancy
Tryptophan	Cyclic	3.46±0.83a	3.42±0.83a	2.89±0.96a	1.23±1.18a	-	ns	ns	ns
	Pregnant	2.03±0.68a	2.65±0.68a	2.03±0.96a	2.43±0.96a	5.49±2.64b			
Tyrosine	Cyclic	7.55±1.45a	6.06±1.45ab	6.15±1.30ab	2.66±1.45b	-	ns	ns	ns
	Pregnant	4.82±1.19a	5.41±1.19a	3.66±1.19a	4.43±1.45a	8.45±3.14b			
Valine	Cyclic	15.57±3.90ax	10.07±3.81ax	13.15±2.97ax	6.33±0.00bx	-	***	ns	***
	Pregnant	10.88±1.08ax	8.81±0.66bx	7.89±0.53bx	9.31±0.00cy	17.10±6.31d			

Significant overall effects of day, pregnancy status and their interactions are noted by an asterisk (*). Temporal differences are indicated by different superscript letters a, b, c, i.e. significant differences in amino acid abundance between sequential days of the estrous cycle or early pregnancy when P<0.05. Differences between pregnant and cyclic heifers on a given day are denoted by x,y when P<0.05.
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pregnant compared to cyclic endometria by Day 16. In the conceptus, *SLC7A4* was approximately 10-fold more abundant than the other cationic transporters. *SLC7A2* expression decreased with increasing conceptus age while *SLC7A6* and *SLC7A7* remained unchanged. The transporter *SLC7A1*, increased (P< 0.01) as the conceptus developed from Day 7 to Day 13, but declined on Day 16 while *SLC7A4* expression was lower (P<0.01) on Day 10 compared to Day 7 (Figures 1 D&E).

Expression of acidic amino acid transporters in the endometrium and conceptus during the pre-implantation period of pregnancy

In the endometrium, the expression of *SLC1A1*, *SLC1A2* *SLC1A3* and *SLC1A4* mRNAs was not affected by day. Expression of *SLC1A3* and *SLC1A4* mRNAs was lower (P<0.05) in caruncular compared to intercaruncular regions during the latter stages of the estrous cycle and early pregnancy (Table 2). The expression of *SLC1A5* in the endometrium increased (P<0.0001) as the estrous cycle and early pregnancy progressed and was affected by pregnancy status (P = 0.06: Figure 2A). In contrast, *SLC1A1*, *SLC1A3* and *SLC1A5* in the conceptus decreased with increasing conceptus age. The expression of *SLC1A4* increased (P<0.05) as the conceptus elongated while *SLC1A2* was not affected by stage of conceptus development (Figure 2B).

Expression of neutral amino acid transporters in the endometrium and conceptus during the peri-implantation period of pregnancy

In the endometrium, there was an overall increase in expression of *SLC38A2* and *SLC43A2*, while *SLC38A7* and *SLC6A14* expression decreased as the estrous cycle and early pregnancy progressed (P<0.05). Expression of *SLC38A2*, *SLC38A7*, *SLC43A2*, and *SLC38A11* mRNAs was lower while expression of *SLC38A4* and *SLC6A14* mRNAs was greater (P<0.05) in caruncular compared to intercaruncular regions of the endometrium on both Days 16 and 19 of pregnancy. In pregnant heifers, *SLC38A2* expression increased from Day 10 to Day 19 while S*LC38A4* and *SLC6A14* expression was lower (P<0.05) in pregnant heifers on Day 16 compared to cyclic controls (Figure 3A–C). In contrast to the endometrium, expression of *SLC38A2* decreased, while *SLC38A7*, *SLC43A2*, *SLC38A11* increased (P<0.05) as conceptus development progressed (Figure 3D). Expression of *SLC6A14* was less than one transcript per million at all stages of conceptus development (data not shown).

Expression of the neutral amino acid transporter *SLC7A5* increased (P<0.01) in the endometrium as the estrous cycle and early pregnancy progressed with a co-ordinate decrease (P<0.05) in expression as the conceptus developed. Endometrial expression of *SLC7A8* decreased to Day 16 in both pregnant and cyclic heifers, but expression increased (P<0.05) on Day 19 in pregnant caruncular and intercaruncular tissue. *SLC7A8* in the conceptus was affected by day (P<0.05) with greatest expression on Day 13 of pregnancy.

Modulation of amino acid transporters by progesterone in vivo

Expression of three acidic amino acid transporters was modulated by concentration of P4 in vivo. On Day 7 of the estrous cycle, expression of *SLC1A1* and *SLC1A4* was higher (P< 0.05) in high P4 heifers compared to control heifers and *SLC1A1* expression was lower (P<0.05) in the low P4 group (Figure 4A). In contrast, *SLC1A5* expression was higher (P<0.05) in the low P4 group on Day 7 compared to control heifers (Figure 4A). The

Table 2. The effect of day, pregnancy status, progesterone concentration and/or caruncular v intercaruncular endometrial tissue on expression of solute like carrier family member genes for amino acid transporters in the embryo/conceptus and endometrium as determined by RNAseq and quantitative real-time PCR (qRT-PCR) analysis respectively.

Gene	Embryo/Conceptus	Endometrium			
	Day	Day	Caruncular v Intercaruncular	Pregnancy Status	P4 Concentration
SLC1A1	**	-	-	-	*
SLC1A2	NS	-	-	-	NS
SLC1A3	*	NS	***	NS	NS
SLC1A4	***	NS	***	NS	*
SLC1A5	***	***	NS	P = 0.06	*
SLC38A11	***	**	NS	NS	NS
SLC38A2	**	***	**	**	*
SLC38A4	-	***	NS	*	*
SLC38A7	*	***	***	NS	*
SLC43A2	***	***	***	NS	*
SLC6A14	NS	**	***	P = 0.06	*
SLC7A1	***	***	*	NS	*
SLC7A2	***	-	-	-	NS
SLC7A4	***	***	**	NS	NS
SLC7A5	**	**	NS	NS	*
SLC7A6	NS	**	***	**	NS
SLC7A7	NS	***	NS	NS	*
SLC7A8	P = 0.06	**	NS	NS	NS

Significant differences are noted by an astrisk (*). Significance set at P<0.05 (*), P<0.01 (**) or P<0.001 (***). A dashed line indicates that gene was not detectable in the specific tissue type.

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cationic amino acid transporters *SLC7A1* and *SLC7A7* were more abundant on Day 7 in the high P4 group, while on Day 13, *SLC7A5* and *SLC7A7* were higher in the high and low P4 groups, respectively (P<0.05: Figure 4B). Manipulation of P4 concentrations had no effect on the expression of *SLC38A2*, *SLC38A4* or *SLC43A2* on Day 7 (P>0.05). However, P4 supplementation increased (P<0.05) *SLC38A7* and *SLC6A14* expression on Day 7. On Day 13 of the estrous cycle, heifers with low P4 had greater expression of *SLC38A2* and *SLC6A14*, while *SLC43A2* expression decreased as compared to values for control heifers (P<0.05: Figure 4C). The expression of *SLC38A4* was greater (P<0.05) in high P4 heifers while *SLC38A7* expression was similar amongst the three treatment groups.

Discussion

This study is the first report on temporal changes in the amino acid content of ULF during the bovine estrous cycle and at key stages of the pre- and peri-implantation periods of pregnancy. Results of this study indicate that the expression of solute-like transporters responsible for the active transport of these amino acids from the endometrium into the ULF is distinct from the expression of transporters that move these nutrients into the conceptus. In addition, expression of certain amino acid transporters in the endometrium was modified by P4 and may therefore play a role in the the capacity of the uterus to support conceptus elongation.

In sheep, glycine was the most abundant amino acid detected in ULF followed by serine [13]. In the current study, threonine and glycine were the most abundant amino acids (10–20 fold greater than most other amino acids). Consistent with our study, data from sheep indicate that most amino acids increase after pregnancy recognition (e.g., arginine, histidine, glycine, glutamic acid, isoleucine, leucine, phenylalanine, tryptophan, tyrosine). In contrast, others do not follow the same pattern in both species; for example, threonine concentrations decreased on Day 16 and 19 of pregnancy in cattle but was reported to increase from Day 12–15 in sheep [13].Whether such discrepancies between cattle and sheep reflect true species differences or are artefacts of different technologies remains to be clarified.

The basic amino acids arginine and lysine are transported by the cationic transporters *SLC7A1*, *SLC7A2*, *SLC7A3*, *SLC7A4*, *SLC7A6*, *SLC7A7*, and *SLC3A1* while *SLC38A6* transports histidine [16]. Of these transporters expression of *SLC7A1* and *SLC7A2* increased in response to P4 supplementation and IFNT in vivo in sheep [16], coordinate with increased recoverable amounts of arginine in the ULF [13]. In this study, the overall abundance of arginine in ULF was similar during the estrous cycle and early pregnancy, but increased significantly on Day 19 of pregnancy. This was coordinate with increased expression of its transporter *SLC7A1* which also increased in the endometria of pregnant heifers on Day 19. In addition, *SLC7A1* expression was greater in heifers with high P4 on Day 7 which is a model associated with advanced conceptus elongation in heifers [30]. The presence of the conceptus was associated with increased expression of transporters for arginine in the endometrium resulting in the increased abundance of arginine in ULF available to the developing conceptus. Arginine was found to stimulate proliferation, protein synthesis and/or migration of sheep and pig trophoblast cells in vitro (Pigs [31]: Sheep [32]). Given that

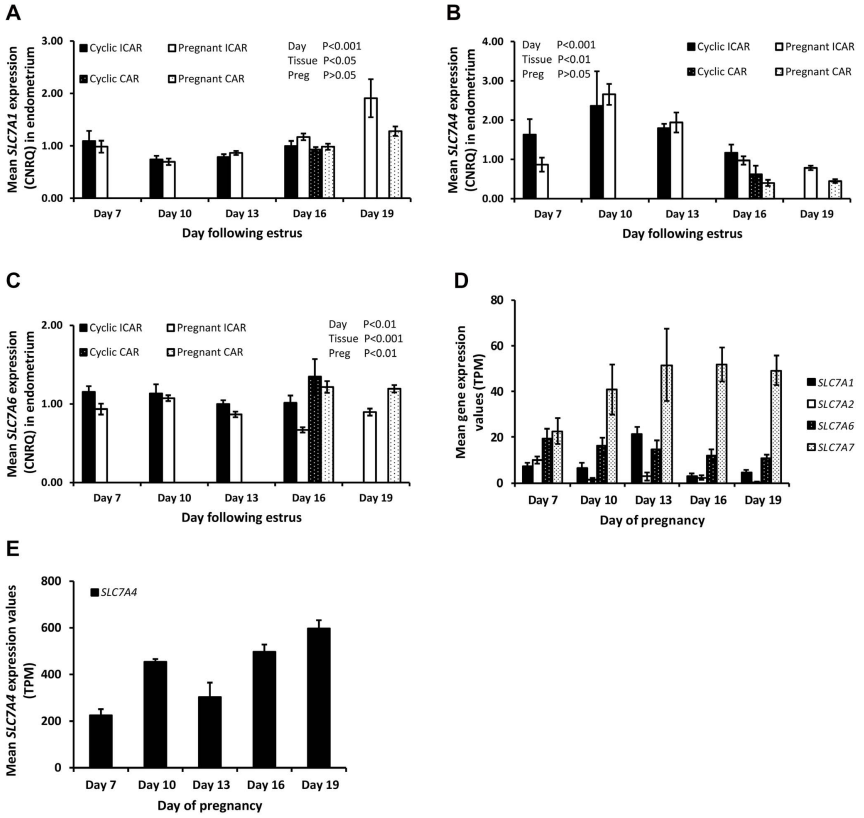

Figure 1. Gene expression values in the endometrium for cationic amino acid transporters as determined by qRT-PCR analysis. Data are displayed as mean calibrated, normalized, relative expression values in arbitrary units (CNRQ ± SEM) in the intercaruncular region of the endometrium from cyclic (solid bars) and pregnant (open bars) heifers and caruncular regions of cyclic (black bars white stipple) and pregnant heifers (while bars black stipple) (n = 5 per treatment per time-point). (A) *SLC7A1* CNRQ expression with a significant effect of day and tissue type (B) *SLC7A4* expression was significantly affected by day and tissue type and (C) *SLC7A6* whose expression was significantly affected by day, tissue type and pregnancy status. Significance was set at $P<0.05$. (D&E) Gene expression values for cationic amino acid transporters in the bovine conceptus during distinct developmental stages. Values were determined by RNA sequencing and are given as mean transcripts per million (TPM ± SEM) with n = 5 per time-point. A significant effect of stage of conceptus development was observed for all genes ($P<0.05$).
doi:10.1371/journal.pone.0100010.g001

conceptuses of those species also elongate, it is likely that arginine plays a similar role in cattle. What is less clear in cattle, however, is the route of uptake of arginine given the decreased expression of members of the y+ family of transporters (*SLC7A1* and -*7A2*) as the conceptus elongates. It is possible that arginine is transported into the conceptus by other members of the cationic amino acid transporters e.g. *SLC7A6* and *SLC7A7* whose expression was maintained or increased as the conceptus was undergoing elongation.

The acidic amino acids glutamic and aspartic acid are transported by *SLC1A1*, -*1A2*, -*1A3* and other acidic amino acids are transported by *SLC1A4* and -*1A5* from the ASC transport system [33]. Both aspartic and glutamic acid increased between Days 16 and 19 of pregnancy. There was no difference on Day 16, the day of pregnancy recognition [2,3]; however, endometrial expression of *SLC1A5* increased in pregnant heifers on Day 16 and was maintained to Day 19. It is likely that the increase in these amino acids on Day 19 and not before is due to a lag between the

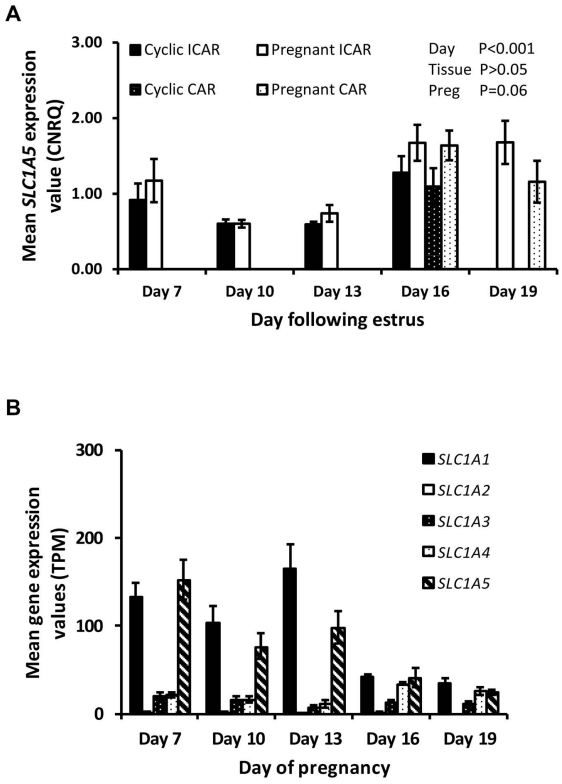

Figure 2. Gene expression values in the endometrium for acidic amino acid transporters as determined by qRT-PCR analysis. Data are displayed as mean calibrated, normalized, relative expression values in arbitrary units (CNRQ ± SEM) in the intercaruncular region of the endometrium from cyclic (solid bars) and pregnant (open bars) heifers and caruncular regions of cyclic (black bars white stipple) and pregnant heifers (while bars black stipple) (n = 5 per treatment per time-point). (A) *SLC1A5*CNRQ expression with a significant effect of day ($P<0.001$) and pregnancy status ($P = 0.06$). (B) Gene expression values for acidic amino acid transporters in the bovine embryo during distinct developmental stages. Values were determined by RNA sequencing and are given as mean transcripts per million (TPM ± SEM) with n = 5 per time-point. A significant effect of stage of embryo development was observed for all genes ($P<0.05$).
doi:10.1371/journal.pone.0100010.g002

increased expression of their transporters on Day 16 and the subsequent detection of an increased abundance of the amino acids. Although concentrations of these acidic amino acids increase in bovine ULF by the initiation of implantation (Day 19), the mechanism of transport of these amino acids seems to differ between the endometrium and conceptus. Given that *SLC1A5* increased in the endometrium with a corresponding increase in *SLC1A4*, we propose that the transport of glutamic and aspartic acid into the uterine lumen is mediated predominantly via *SLC1A5*; however, uptake of these amino acids by the conceptus is via *SLC1A4* in cattle. In addition, the early increase in *SLC1A5* expression in P4-supplemented heifers suggests that advanced conceptus elongation in this model is, in part, driven by increased transport of both aspartic and glutamic acids into the uterine lumen via increased expression of *SLC1A5* and not other members of the acidic amino acid transporter family.

The most abundant amino acids in bovine ULF in this study were the neutral amino acids. Of the neutral amino acid transporters analysed in the endometrium, increased expression of *SLC38A2* in the pregnant endometrium on Days 16 and 19 suggests that this gene is most likely involved in the transport of neutral amino acids into ULF during the pre-implantation period of pregnancy in cattle. Interestingly, asparagine and threonine were less abundant in ULF of pregnant compared to cyclic heifers

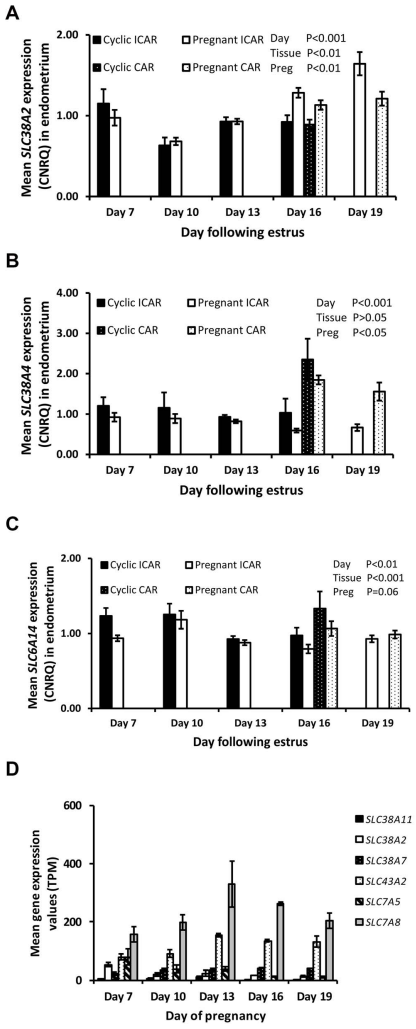

Figure 3. Gene expression values in the endometrium for neutral amino acid transporters as determined by qRT-PCR analysis. Data are displayed as mean calibrated, normalized, relative expression values in arbitrary units (CNRQ ± SEM) in the intercaruncular region of the endometrium from cyclic (solid bars) and pregnant (open bars) heifers and caruncular regions of cyclic (black bars white stipple) and pregnant heifers (while bars black stipple) (n = 5 per treatment per time-point). (A) *SLC38A2* expression with a significant effect of day, tissue type and pregnancy status (B) *SLC38A4* expression was significantly affected by tissue type and pregnancy status and (C) *SLC6A14* whose expression was significantly affected by day, tissue type and pregnancy status. Significance was set at $P<0.05$. (D) Gene expression values for neutral amino acid transporters in the bovine conceptus during distinct developmental stages. Values were determined by RNA sequencing and are given as mean transcripts per million (TPM ± SEM) with n = 5 per time-point. A significant effect of stage of embryo development was observed for all genes ($P<0.05$).
doi:10.1371/journal.pone.0100010.g003

on Days 7 and 10, respectively. This seems at odds with the fact that pregnancy recognition, has not occurred, and previous studies have shown that Days 15 and 16 of pregnancy are the earliest that differences in gene expression in the endometrium are detectable between cyclic and pregnant heifers [34,35].

Altering circulating concentrations of P4 in vivo can either advance (in the case of high P4 [36]) or delay (in the case of low P4 [29]) conceptus elongation following transfer of a blastocyst to heifers on Day 7 of the estrous cycle. This study clearly demonstrates that amino acids are an important component of ULF during the estrous cycle and early pregnancy and that the transport of these molecules into ULF from the endometrium occurs throughout this period. An early increase in P4 concentrations increased endometrial expression of *SLC1A5*, *SLC38A7*, *SLC6A14*, *SLC7A1* on Day 7 post-estrus, with an early (Day 7) and sustained increase in expression to Day 13 for *SLC38A4*, *SLC7A5* and *SLC7A7* compared to control heifers, suggesting that one way in which conceptus elongation is advanced, is through effects on the maternal amino acid transport system. Conversely, in heifers with a delay in the post-ovulatory increase in P4 (associated with smaller conceptuses), expression of *SLC43A2*, *SLC6A14*, *SLC7A6* and *SLC7A7* for amino acid transport into the ULF was suboptimal. These P4-regulated changes in expression of the transporters in the endometrium are consistent with lower concentrations of histidine and asparagine in ULF of low P4 heifers on Day 13 [37]. Therefore, in vivo manipulation of P4 alters the expression of genes for amino acid transporter from the endometrium into the ULF which has clear consequences for conceptus elongation in vivo which supports findings from studies with ewes [38].

Previous studies have demonstrated that in vitro derived blastocysts have a higher amino acid turnover than their in vivo derived counterparts and, overall, expanded blastocysts deplete more amino acids than those that do not undergo expansion [23]. This is interesting in the context of this study in which the amount of detectable amino acids in ULF increased as development of the blastocyst progressed to an elongated filamentous conceptus. However, once hatched from the zona pellucida the substantial increase in the composition of specific amino acids in the ULF suggests increased requirements for these amino acids to drive conceptus elongation. Moreover, substantial increases in the abundance of amino acids in the gravid as compared to the non-gravid uterine horn on Day 18 of pregnancy [15] indicate that the presence of the conceptus stimulates transport of amino acids into the uterine lumen. Interestingly, the amounts of amino acids in ULF were reduced in heifers with a developmentally compromised conceptus (i.e. cloned embryos) [39] and in subfertile animals [40]. The fact that we demonstrated a significant increase in the amount of amino acids in ULF on Day 19 of pregnancy, along with increased expression of their transporters in the endometrium, is consistent with the notion that increased abundance of amino acids in ULF is required for successful pregnancy establishment in cattle. In conclusion, results of this

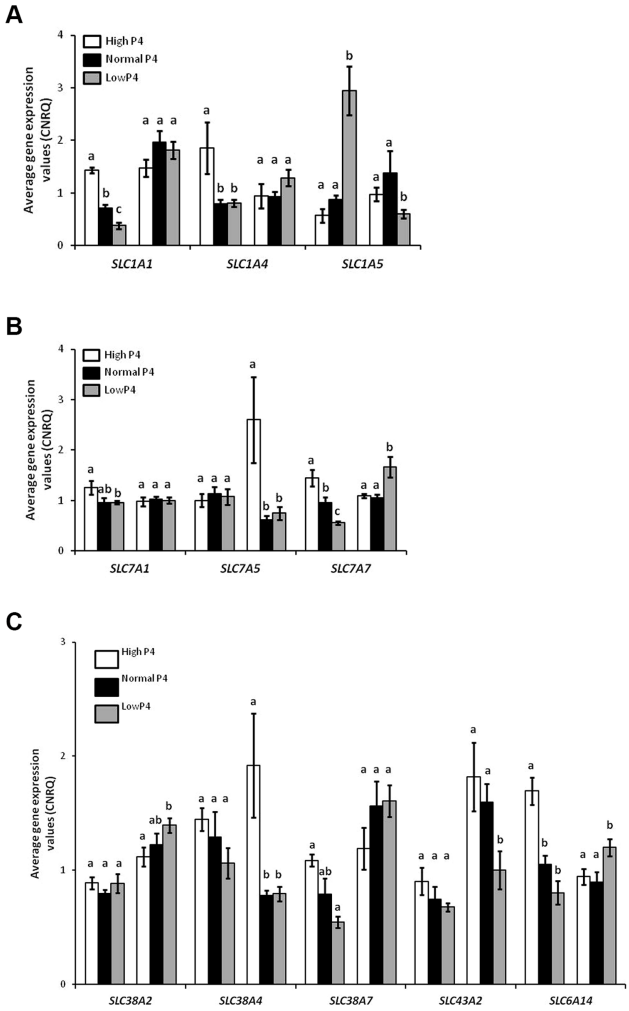

Figure 4. Gene expression values in the endometrium for heifers with high P4 (black bars: n = 5), normal P4 (white bars: n = 5) and low P4 (gray bars: n = 7) on Days 7 and 13 of the estrous cycle. All data are displayed as mean calibrated, normalized, relative expression values in arbitrary units (CNRQ ± SEM) for acidic amino acid transporters (A), basic amino acid transporters (B) and neutral amino acid transporter (C). Differences between treatments on a given day are denoted by a, b, c, when $P<0.05$.
doi:10.1371/journal.pone.0100010.g004

study demonstrated that most amino acids increase in ULF between Days 16 and 19 of pregnancy which is after pregnancy recognition has occurred. The amino acid transporters are temporally regulated in a tissue-specific manner in the endometrium and conceptus during the peri-implantation period of pregnancy and we propose that the transport mechanisms for amino acids into ULF from the endometrium are distinct from those of the conceptus. Moreover, expression of the amino acid transporters in the endometrium in vivo under conditions where conceptus elongation is advanced (elevated P4) or retarded (low P4), may alter the transport of acidic, neutral and cationic amino acids into ULF. We propose that transport of amino acids into the uterine lumen contributes to the capacity of the uterus to stimulate elongation of the conceptus during the peri-implantation period of pregnancy in cattle.

Supporting Information

Table S1 Primer information used for quantitative real time PCR analysis of candidate genes. All primers were used at a concentration of 300 nM in a final reaction volume of 15 µl. A dissociation curve was included to ensure specificity of each primer pair.
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: NF TES FWB PL. Performed the experiments: NF CAS RS SM PL. Analyzed the data: NF CAS RS AKK. Contributed reagents/materials/analysis tools: CAS RS. Contributed to the writing of the manuscript: NF PL. Critically revised the manuscript and approved the final draft: NF CAS RS SM AKK TES FWB PL.

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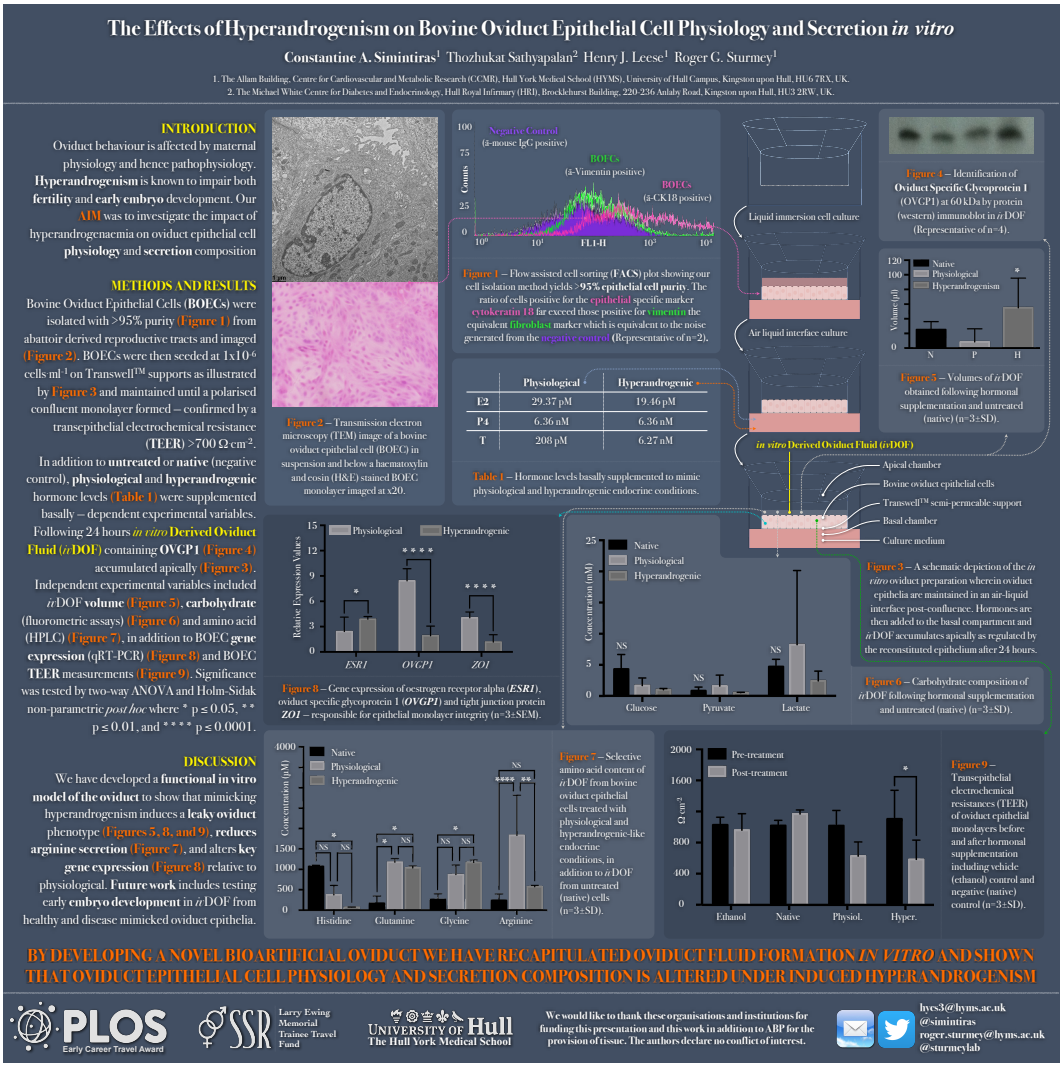
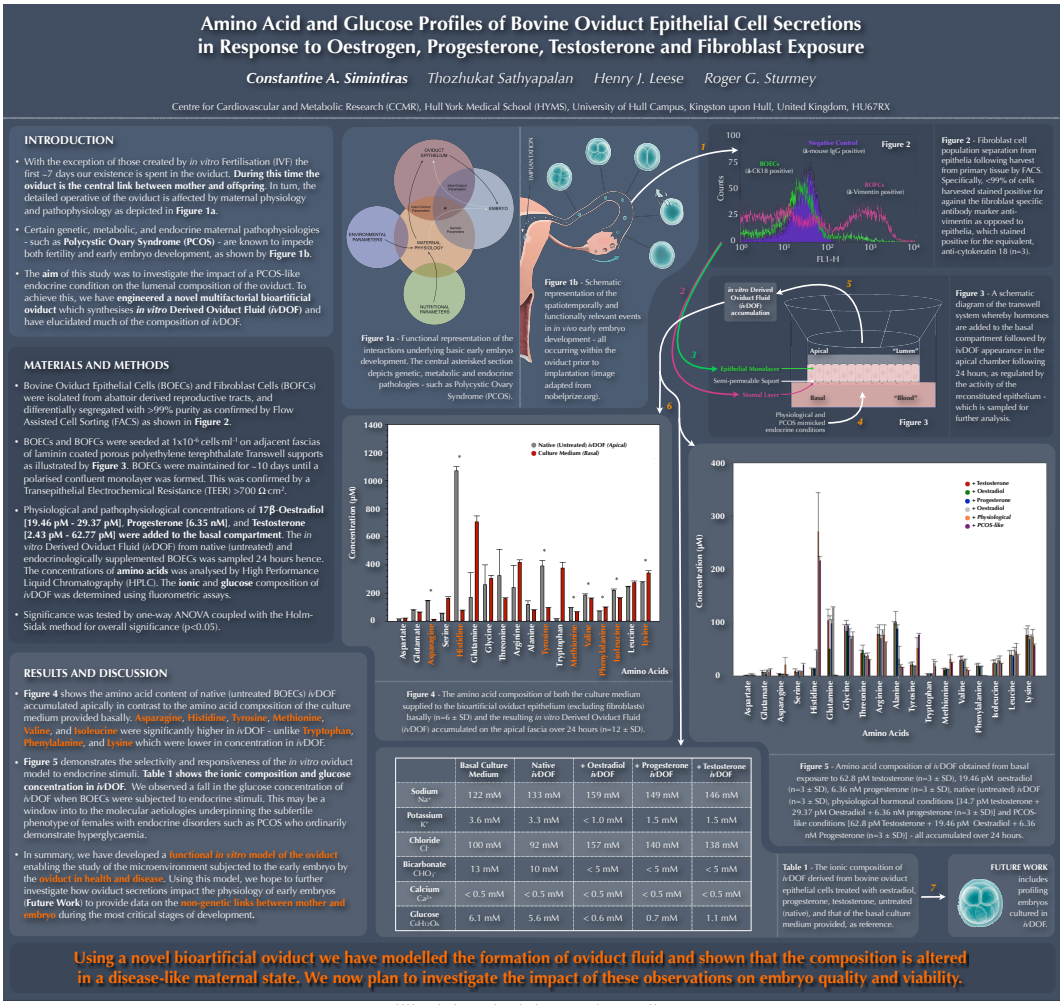
9.7. Poster for the *Society for the Study of Reproduction* (2016)



9.8. Poster for the *World Congress of Reproductive Biology* (2014)

	Basal Culture Medium	Native ivDOF	+ Oestradiol ivDOF	+ Progesterone ivDOF	+ Testosterone ivDOF
Sodium Na^+	122 mM	133 mM	159 mM	149 mM	146 mM
Potassium K^+	3.6 mM	3.3 mM	< 1.0 mM	1.5 mM	1.5 mM
Chloride Cl^-	100 mM	92 mM	157 mM	140 mM	138 mM
Bicarbonate CHO_3^-	13 mM	10 mM	< 5 mM	< 5 mM	< 5 mM
Calcium Ca^{2+}	< 0.5 mM	< 0.5 mM	< 0.5 mM	< 0.5 mM	< 0.5 mM
Glucose $C_6H_{12}O_6$	6.1 mM	5.6 mM	< 0.6 mM	0.7 mM	1.1 mM

9.9. Poster for the *Society for the Study of Reproduction* (2014)



	Basal Culture Medium	Native ivDOF	+ Oestradiol ivDOF	+ Progesterone ivDOF	+ Testosterone ivDOF
Sodium Na^+	122 mM	133 mM	159 mM	149 mM	146 mM
Potassium K^+	3.6 mM	3.3 mM	< 1.0 mM	1.5 mM	1.5 mM
Chloride Cl^-	100 mM	92 mM	157 mM	140 mM	138 mM
Bicarbonate CHO_3^-	13 mM	10 mM	< 5 mM	< 5 mM	< 5 mM
Calcium Ca^{2+}	< 0.5 mM	< 0.5 mM	< 0.5 mM	< 0.5 mM	< 0.5 mM
Glucose $C_6H_{12}O_6$	6.1 mM	5.6 mM	< 0.6 mM	0.7 mM	1.1 mM

9.10. Poster for the *International Embryo Transfer Society* (2012)

Genistein Transport Across the Bovine Oviduct Epithelium

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1. INTRODUCTION

- ★ This study has investigated the barrier properties of the bovine oviduct epithelial monolayer to the dietary compound, genistein.
- ★ Genistein is a naturally occurring soya isoflavone and 17β-estradiol analogue that has been reported to exert teratogenic effects on the early embryo [1].
- ★ The oviduct is of paramount importance in regulating the environment surrounding the gametes and early mammalian embryo.
- ★ However, the permeability of the oviduct to circulating dietary-derived compounds such as genistein remains relatively unknown.

2. MATERIALS & METHODS

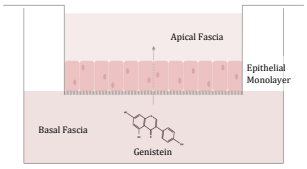

Figure 1: A schematic diagram of the transwell system whereby compounds of interest are added to the basal compartment, and their appearance in the apical compartment, as regulated by the activity of the reconstituted epithelium, can be measured.

- ★ Bovine oviduct epithelial cells (BOECs) were isolated from abattoir derived reproductive tracts at the mid luteal phase.
- ★ BOECs were seeded at a density of $1x10^{-6}$/ml on semi-permeable polyethylene terephthalate transwell porous supports (Corning) as depicted in Figure 1.
- ★ BOEC purity at time of cell harvest was confirmed using Flow Assisted Cell Sorting (FACS) – Figure 2.
- ★ The BOEC monolayer was maintained for ~10 days until a polarised confluent monolayer was formed, as confirmed by a transepithelial resistance (TER) greater than 700 Ωcm^2.
- ★ A physiological concentration of 100μM of genistein was added to the basal compartment, and apical samples were taken at 20 min. intervals prior to analysis by High Performance Liquid Chromatography (HPLC) as shown in Figures 3 and 4.

3. RESULTS

Figure 2 shows <95% of cells harvested stained positive for the epithelial antibody marker anti-cytokeratin-18 (ᾱ-CK18) as opposed to fibroblasts, which stained positive for anti-Vimentin (ᾱ-Vim) (n=3). Figure 3 shows the apical accumulation of 100 μM genistein at physiological (39°C) and room (21°C) temperature compared to genistein transport across a blank transwell support lacking BOECs. Genistein crossed the monolayer at a linear rate that was higher than spontaneous diffusion across a blank support. Furthermore, the rate of transport was unchanged at room temperature. Figure 4 shows the kinetic profile of a genistein equilibrium (50 μM apical and 50 μM basal) in addition to the total genistein present.

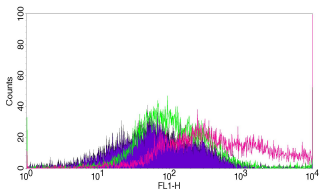

Figure 2: Analysis of Epithelial *vs.* Fibroblast populations by FACS: Mouse IgG1 Antibody Negative Control (background *noise*) = 786.43 Counts; ᾱ-Vim + AlexaFluor-488nm antibody (Fibroblast) = 629.07 Counts; ᾱ-CK18 + AlexaFluor-488nm antibody (BOECs) = 2096.2 Counts.

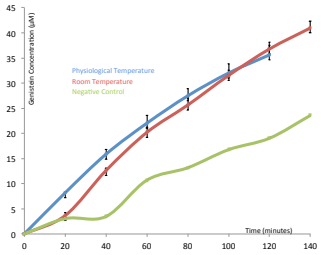

Figure 3: The basal to apical accumulation of genistein (100 μM) across the BOEC monolayer at room (~21°C) $12.7x10^{-3}$ $cm^{-2}\mu M^{-1}$, physiological (39°C) temperature $13.11x10^{-3}$ $cm^{-2}\mu M^{-1}$, and across a blank $7.32x10^{-3}$ $cm^{-2}\mu M^{-1}$ membrane support (n=3) ± SEM.

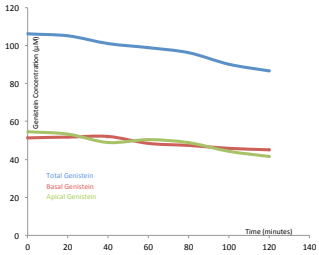

Figure 4: Genistein (50 μM basal and 50 μM apical) transport across the BOEC monolayer at room temperature (~21.5°C) over 120 minutes, and the indirect total genistein concentration present [calculated as ∑ (apical + basal)] (n=3) ± SEM.

4. DISCUSSION

These data show that bovine oviduct epithelium facilitates the transport of genistein *in vitro*. This transport is faster than diffusion in the absence of cells, but unchanged by temperature, which may indicate a passive, trans-cellular, physicochemical mechanism, as opposed to an active biological process. Furthermore, genistein has no specific directionality in relation to the apical and basal oviduct fasciae. These data show for the first time that the oviduct epithelium is permeable to the dietary compound genitsein, which has been reported to have teratogentic effects. The results furthermore demonstrate the potential use of this epithelial model in characterising the transport or barrier properties of the oviduct epithelium towards a range of circulating compounds which may be embryo-toxic.

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6. REFERENCE

[1] Newbold et. al. 2001 *Cancer Res* Vol. 61, pp. 4325-4328.