Modelling oviduct fluid formation in vitro

Short Title
In vitro Derived Oviduct Fluid

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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, for the novel application of investigating 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. The culture system was optimized further to incorporate 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™ 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™ 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 fluid formation in vitro. With this system, we have confirmed the secretion of OVG1 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 ivDOF, 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·ml$^{-1}$ PenStrep, 20 μg·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 Ω·cm⁻² to ~ 800 Ω·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 Ω·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% CO₂. After 24 hours, a thin film of fluid formed in the apical chamber — termed in vitro Derived Oviduct Fluid (ivDOF) (Figure 1A).

**Dual Culture**

Bovine oviduct fibroblast cells were harvested by trypsinisation from tissue culture flasks after 5 days in culture. 1x10⁶ 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 ivDOF 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

**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 reagent and chloroform (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 ∆∆Ct 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™ inserts. The standard experimental design was to assign 3 Transwell™ membranes for treatment with the dependent experimental variable and the remaining 3 as negative controls. The ivDOF 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 ivDOF isolations.
In this study, *in vitro* derived oviduct fluid (*ivDOF*; Figure 1A) from untreated (native) bovine oviduct epithelial cells was analysed (Figure 3: A-D) and compared with previously reported *in vivo* observations. To interrogate the dynamicity of *ivDOF*, its composition 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 *ivDOF*. 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). Flasks were seeded in parallel for gene expression studies to complement *ivDOF* 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 ivDOF 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 ivDOF 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 ivDOF (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 ivDOF was present at 60 kDa.

Figure 3F shows that the amino acid composition of ivDOF 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 ivDOF (Figure 3F). Similarly the addition of P4 (Table 1) increased the apical flux of glutamine, 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 ivDOF 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 ivDOF produced (Figure 5E). Figure 5F shows that hypo and hyper treatments had no significant impact on the carbohydrate composition of ivDOF. 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 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 (ivDOF). 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 ivDOF amino acid composition (Figure 3G). In addition, analogous 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 ivDOF 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 µl·min⁻¹ previously reported in vivo by Hugentobler et al (2008). The osmolarity of native ivDOF 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 ivDOF vs in vivo.

OVGP1 in ivDOF 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-acetylglucosamine, required for glycosylation.

The amino acid composition of ivDOF (Figure 4F) resembled data on cannulated oviducts of anaesthetised 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 ivDOF 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 ivDOF pH. Addition of E2 caused histidine in ivDOF 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 ivDOF 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 ivDOF 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 ivDOF 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, Srsuma 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 ivDOF 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 ivDOF 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 ivDOF 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 ivDOF. 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 ivDOF 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 ivDOF generated to optimise embryo culture media.
Declaration of Interests

The authors declare no conflict of interest.

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**Figure and Table Legends**

**Figure 1** — (aA) Schematic representation of the culture system for *in vitro* Derived Oviduct Fluid (*ivDOF*) production. The basal chamber represents the bloodstream whilst the apical represents the oviduct lumen. (B) The technical method and apparatus innovated for seeding fibroblasts to the basal surface of Transwell™ membranes for establishing dual culture. Large 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 surface of the semi-permeable membrane.

**Figure 2** — (A) Haematoxylin and eosin stained bovine oviduct epithelial cells cultured to confluence on Transwell™ membranes and imaged at x20 magnification. (B) Transmission electron microscopy 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). (C) FACS analysis of cultured BOEC purity showing mouse IgG1 negative control (background noise), anti-Vimentin 1° antibody (BOFC population), and anti-Cytokeratin 18 1° antibody (BOEC population); all in combination with the Alexafluor 488 nm 2° antibody showing in excess of 95% epithelial purity (representative of n=2) at a fluorescence intensity (FLH-1) between $10^3$ – $10^4$. (D) FACS analysis of cultured BOFCs showing mouse IgG1 negative control (background noise), anti-cytokeratin 18 1° antibody (BOEC population), and anti-vimentin 1° antibody (BOFC population) in excess of 99% stromal purity (n=1).
Figure 3 — (A) The volume (n=6 ± SD), (B) osmolarity (n=3 ± SD), and (C) carbohydrate content (n=3 ± SD) of ivDOF 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 ivDOF (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 ivDOF. (F) The amino acid composition of ivDOF accumulated apically from native (N) BOECs (n=12 ± SD) vs. culture medium (C) supplied basally (n=3 ± SD) vs. ivDOF derived from BOECs basally supplemented with 29.37 pM 17β-oestradiol (E2; n=6 ± SD) vs. ivDOF from BOECs treated with 6.36 nM progesterone (P4; n=4 ± SD) vs. ivDOF from BOECs basally supplemented with 62.77 pM testosterone (T; n=3 ± SD). (G) The amino acid profile of native ivDOF (n=12 ± SD) vs. ivDOF from BOECs cultured with BOFCs basally adjacent in the dual culture arrangement (n=4 ± SD). All ivDOF accumulated over 24 hours and a = p ≤ 0.0001, b = p ≤ 0.001, c = p ≤ 0.01, and d = p ≤ 0.05.

Figure 4 — 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 ≤ 0.0001, *** = p ≤ 0.001, ** = p ≤ 0.01, and * = p ≤ 0.05.

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 ivDOF 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 ivDOF obtained
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Figures and Tables
Figure 1

Figure 2
Figure 3
Figure 4
Figure 5
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Table 1
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Table 2