

INVESTIGATING THE IMPACT OF ENVIRONMENTAL CONTAMINANTS ON
PERICONCEPTUAL ENVIRONMENT AND EARLY REPRODUCTIVE EVENTS

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Abstract

The modern environment in which we live is replete with chemicals that have accumulated to levels that have been proposed to impact various aspects of physiology. The impact of a selection of these environmental contaminants on the physiology of early reproductive events has been investigated. The contaminants investigated include Bisphenols A and S, as well as triclosan and acetamiprid. Firstly, the effect of individual exposure to these contaminants on periconceptual environment was explored, using bovine oviduct epithelial cells (BOEC) cultured in vitro. Using microfluorometric assays and high-performance liquid chromatography (HPLC), it was discovered that pyruvate concentration and amino acids composition in BOEC secretions were sensitive to treatment with these chemicals. Furthermore, the expression of the amino acids transporters was also altered after exposure. Subsequently, the bovine oocyte function and metabolic profile was assessed after exposure to environmental contaminants during in vitro maturation (IVM). The nuclear status, steroid hormone production, energy substrates consumption after IVM, mitochondrial activity, and the pronuclei formation were assessed. Exposure to Bisphenols decreased 17 β -oestradiol production and Triclosan increased progesterone production in a non-monotonic pattern. Meanwhile, both steroid hormones remained unchanged in the presence of Acetamiprid. However, Acetamiprid as well as Triclosan affected the metabolic status of pyruvate irrespective of the administrated dose. Furthermore, the mitochondrial activity of oocytes after Triclosan and Acetamiprid exposure was impaired. Finally, pronuclei formation was influenced in the presence of all environmental contaminants. Next, the effects of environmental contaminants on embryo developmental competence and metabolic activity after indirect and direct exposure were assessed. Reduced blastocyst rates and altered energy substrates consumption was observed in embryos derived from chemically exposed oocytes. Altered metabolic activity of embryos was also revealed after direct exposure during cleavage and blastocyst stage of development. The data generated in this work demonstrate the capacity of common environmental contaminants to alter early development at multiple stages in subtle but significant ways. It has been suggested that changes on the environment during the early stages of development could have an impact on the health in later on stages of life. This observation highlights the importance of our findings.

List of content	003
Abstract	002
List of content	003
List of tables	010
List of figures	012
Acknowledgment	022
Declaration	023
 Chapter 1: Introduction	 024
1.1 Overview	025
1.2 Early Reproductive events	028
1.2.1 Gametogenesis	028
1.2.2 Oestrus cycle	033
1.2.3 Fertilisation	036
1.2.4 Early-stage embryo development	038
1.3 Mammalian oviduct	041
1.3.1 Oviduct: the neglected organ	041
1.3.2 Morphology and function	042
1.3.3 Oviductal fluid	045
1.3.3.1 Oviduct fluid composition	046
○ <u>Electrolytes</u>	046
○ <u>Energy substrates</u>	047
○ <u>Amino Acids</u>	047
○ <u>Proteins and enzymes</u>	048
○ <u>Fatty Acids</u>	049
○ <u>Extracellular vesicles</u>	049
1.3.3.2 Mechanism of oviduct fluid secretion	050
1.4 The role of the oviduct in Reproduction	051
1.4.1 Oviduct: The dynamic microenvironment	051
1.4.2 Sperm-oviduct interactions	052

1.4.3	Oocyte-oviduct interactions	055
1.4.4	Oviduct as co-factor in embryo development	056
1.4.5	Embryo transit through the oviduct	058
1.5	Emerging Environmental contaminants	060
1.5.1	Endocrine Disrupting Chemicals	060
1.5.1.1	Bisphenols	061
1.5.1.2	Bisphenol A: Occurrence in the environment and Human exposure	062
1.5.1.3	Bisphenol S: Occurrence in the environment and Human exposure	064
1.5.1.4	Triclosan: Occurrence in the environment and Human exposure	066
1.5.2	Neonicotinoids	068
1.5.2.1	Acetamiprid: Occurrence in the environment and Human exposure	070
1.5.3	Environmental contaminants of interest and their effects on reproductive system	072
1.5.3.1	Bisphenol A and the effects on oogenesis, oocyte quality and fertilization	072
1.5.3.2	Bisphenol A and the effects on spermiogenesis and sperm quality	074
1.5.3.3	Bisphenol A and the effects on Embryo development	076
1.5.3.4	Bisphenol S and the effects on reproductive system	077
1.5.3.5	Triclosan and the effects on reproductive system	079
1.5.3.6	Acetamiprid and the effects on reproductive system	080
1.6	Metabolism in cellular level	083
1.6.1	Metabolism overview	083
1.6.2	Metabolic pathways	084
1.6.2.1	Glycolysis	084
1.6.2.2	Fatty Acid metabolism	085
1.6.2.3	Amino Acid metabolism	086
1.6.2.4	TCA cycle	087

1.6.2.5 Oxidative phosphorylation	088
1.7 Developmental Origin of Health and Disease	091
1.7.1 Linking DOHaD with epigenetic modelling of embryo	092
1.8 General Hypothesis and Aims	094
Chapter 2: Material and Methods	095
2.1 Bovine Oviduct Epithelial Cell culture	096
2.1.1 Bovine Oviduct Epithelial Cell harvest and maintenance	096
2.1.2 In vitro culture of BOEC and confirmation of a confluent epithelial monolayer in Transwells	097
2.1.3 Confirmation of epithelial monolayer integrity using Fluorescein rejection assay	098
2.1.4 In Vitro Derived Oviduct Fluid formation	102
2.2 Proliferation assay	102
2.2.1 Cell Proliferation on BOEC	102
2.3 Bovine oocyte maturation, sperm preparation and In vitro embryo production	103
2.3.1 Stocks, supplements, and additives preparation for in vitro production	103
2.3.2 Media Preparation for the in vitro production	106
2.3.3 Ovary harvest and preparation	108
2.3.4 Aspiration and in vitro maturation of bovine oocytes	109
2.3.5 In vitro fertilisation (IVF)	109
2.3.6 In vitro embryo culture (IVC)	110
2.3.7 Individual embryo culture	111
2.3.8 Optimisation of embryo production	112
2.4 Metabolic assays	113
2.4.1 Principles of Microfluorometric assays	114
2.4.2 Quantification of Glucose, Lactate and Pyruvate production or depletion using microfluorometric assays	116

2.4.3	High Performance Liquid Chromatography (HPLC) principle	118
2.4.4	Quantification of Amino Acids turnover using HPLC	119
2.4.5	Oxygen consumption rate assay	121
2.5	Relative gene expression	123
2.5.1	RNA extraction	123
2.5.2	Optimisation of RNA extraction	124
2.5.3	Complementary DNA (cDNA) synthesis	124
2.5.4	quantitative Polymerase Chain Reaction (q-PCR)	125
2.6	Enzyme-linked Immunosorbent Assay (ELISA)	127
2.6.1	General principles of ELISA	127
2.6.2	17b-estradiol and progesterone quantification using ELISA assay	128
Chapter 3: The impact of environmental contaminants of interest on BOECs		129
3.1	Introduction	130
3.1.1	Bovine epithelial cells	130
3.1.2	Transwell system	131
3.2	Hypothesis and Aims	132
3.3	Methods	133
3.3.1	Identification of the suitable solvent for the environmental contaminants stock preparation	133
3.3.1.1	Identify the suitable solvent for the metabolic experiments	133
3.3.1.2	Environmental contaminants stock preparation and storage	134
3.3.2	Experimental design overview	135
3.4	Statistical Analysis	138
3.5	Results	139
3.5.1	Pilot study: Impact of environmental contaminants on BOECs function	139

3.5.1.1	Impact on BOECs energy substrates uptake and release	139
3.5.1.2	Effects on BOECs proliferation	143
3.5.2	Impact of environmental contaminants on BOECs secretions	144
3.5.2.1	Effects on BOECs TEER	145
3.5.2.2	Impact on energy substrate transport after chronic exposure	148
3.5.2.3	Impact of environmental contaminant exposure on expression of amino acid transporters	158
3.6	Discussion	165
Chapter 4: The impact of environmental contaminants on bovine oocytes		172
4.1	Introduction	173
4.1.1	Oocyte metabolic function during maturation	173
4.2	Hypothesis and Aims	176
4.3	Methods	177
4.3.1	Environmental contaminants stock preparation and storage	177
4.3.2	Experimental design	177
4.3.3	Statistical Analysis	179
4.4	Results	180
4.4.1	Oocyte nuclear maturation and exposure to environmental contaminants	180
4.4.2	Effects of environmental contaminants on steroid production during IVM	181
4.4.3	The impact of environmental contaminants on oocytes' energy substrates	186
4.4.4	The impact on mitochondrial activity using Oxygen Consumption Rate (OCR) as a marker	190
4.4.5	The zygotes pronuclear status after exposure to environmental contaminants of interest	198
4.5	Discussion	201

Chapter 5: The impact of environmental contaminants on early stages of embryo development	207
5.1 Introduction	208
5.1.1 Embryo metabolism	208
5.2 Hypothesis and Aims	211
5.3 Experimental design	212
5.3.1 Environmental contaminants stock preparation and storage	212
5.3.2 Experiment 1: How does embryo development and metabolism respond when oocytes are exposed to environmental contaminants?	212
5.3.3 Experiment 2: How does exposure to environmental contaminants during embryo development embryo metabolism and subsequent development?	213
5.3.4 Experiment 3: Do environmental contaminants affect oxygen consumption rate during blastocyst stage?	214
5.3.5 Statistical Analysis	215
5.4 Results	216
5.4.1 The impact of environmental contaminants during oocyte maturation on subsequent embryo developmental competence and metabolic activity.	216
5.4.2 The impact on embryo metabolism after direct exposure to environmental contaminants on different stages of embryo development.	226
5.4.3 The impact of environmental contaminants on the oxygen consumption rate of bovine blastocysts	243
5.5 Discussion	251
5.5.1 Exposure to environmental contaminants during IVM impaired the developmental competence of the generated embryos.	251
5.5.2 Chemical exposure during different time points of development	

influences the glucose, pyruvate consumption and lactate production of embryos	254
5.5.3 Amino acids turnover was influenced by the presence of environmental contaminants during different time points of development	256
5.5.4 Environmental contaminants of interest influence the OCR consumption and its components	259
Chapter 6: General Discussion	262
6.1 Summary of findings	263
6.2 The effects of environmental contaminants on early reproduction	269
6.3 Strength and limitations	271
6.4 Importance of work	274
6.5 Future work	275
List of Reference	278
Appendices	328

List of tables

Table 2.1: Krebs - Ringer buffer stocks

Table 2.2: Krebs - Ringer buffer composition

Table 2.3: The final concentrations of the environmental contaminants used for the proliferation assay

Table 2.4: Stocks necessary for the Media preparation.

Table 2.5: Supplements and additives for the Media preparation.

Table 2.6: 50x Amino Acids supplements used in SOFaaBSA and SOFanalysis media for grouped and individual embryo culture respectively.

Table 2.7: Holding Media and Bovine Maturation Media.

Table 2.8: HEPES TALP and FERT TALP Media Composition.

Table 2.9: HEPES SOF and SOFaaBSA Media composition.

Table 2.10: Percoll gradients preparation.

Table 2.11: Buffers and stock solutions necessary for glucose, pyruvate and lactate cocktails preparation.

Table 2.12: Composition of glucose, pyruvate and lactate cocktail for the microfluorometric assays.

Table 2.13: Buffer A and Buffer B composition, used for Amino Acid quantification by HPLC.

Table 2.14: Buffer A and Buffer B ratio during the course of one run in the HPLC.

Table 2.15: The final concentrations of the environmental contaminants used for the OCR assay.

Table 2.16: The final concentrations of the mitochondrial inhibitors used for the OCR measure on bovine oocytes and embryos.

Table 2.17: Master Mix for cDNA libraries preparation.

Table 2.18: Conditions for cDNA synthesis.

Table 2.19: Reaction conditions.

Table 2.20: qPCR thermocycler conditions.

Table 2.21: Primers' sequences.

Table 3.1: Final concentration of the environmental contaminants, BOECs were exposed \

Table 4.1: The final concentration of environmental contaminants that COCs exposed

Table 4.2: Number of COCs tested for nuclear status

Table 4.3: Number of zygotes tested for pro-nucleus formation.

Table 5.1: The final concentration of environmental contaminants that COCs exposed

Table 5.2: Amino Acids with statistically significant differences per environmental contaminants per time of exposure

List of figures

Figure 1.1: a) The process of spermatogenesis (figure from and b) the final form of spermatozoa (figure adapted from (Yan et al., 2022).

Figure 1.2: Folliculogenesis and oocyte maturation (figure from (Mihalas et al., 2017).

Figure 1.3: Graphical illustration of a) oestrus cycle in bovine (Forde et al., 2011) and b) menstrual cycle in human (Draper et al., 2018) hormonal cyclicity

Figure 1.4: Schematic presentation of early stages of embryo development (Smith & Sturmey, 2013).

Figure 1.5: The oviduct anatomical regions connected with the ovary (Maillo et al., 2016).

Figure 1.6: Electron scanning micrograph of bovine oviduct epithelia a) surface and b) epon-embedded oviduct (Mondéjar et al., 2012).

Figure 1.7: The mechanism of oviduct fluid secretion (Leese et al., 2001).

Figure 1.8: Schematic presentation of oviduct interactions with sperm, oocyte, and embryo (Saint-Dizier et al., 2019).

Figure 1.9: Chemical structure of a) Bisphenol A, b) Bisphenol S, and c) 17β - Estradiol (Grignard et al., 2012)

Figure 1.10: Chemical structure of Triclosan and Thyroxine (T4) (Ruszkiewicz et al., 2017)

Figure 1.11: Chemical structure of a) Nicotine and b) Acetamiprid (ACE) (Kimura-Kuroda et al., 2012)

Figure 1.12: Metabolic pathways overview (Williams & O'Neill, 2018).

Figure 1.13: The electron transport chain (Muller et al., 2019).

Figure 2.1: Layout of Improved Neubauer haemocytometer.

Figure 2.2: BOEC culture in Transwell.

Figure 2.3. Fluorescein transport from the basal to apical compartment of BOECs over the course of 200 minutes when a) $TEER > 700 \Omega \cdot cm^2$ and b) $TEER > 1500 \Omega \cdot cm^2$.

Figure 2.4: Layout of wash dish and IVC dish.

Figure 2.5: Layout of individual embryo culture dish.

Figure 2.6: Glucose conversion to 6-Phosphogluconate.

Figure 2.7: Lactate conversion to pyruvate catalysed by Lactate Dehydrogenase (LDH) enzyme.

Figure 2.8: Pyruvate conversion to lactate catalysed by LDH enzyme.

Figure 2.9: Representative standard curves of a) glucose, b) lactate and c) pyruvate used for microfluorometric assays.

Figure 2.10: Example of amino acids standards as detected using HPLC

Figure 2.11: The measurement of Oxygen Consumption Rate using Seahorse XFp (Agilent Technologies).

Figure 2.12: Primer's efficiency

Figure 3.1: a) Glucose uptake, b) pyruvate and c) lactate release after BOECs exposed to ethanol (EtOH) or DMSO.

Figure 3.2: Graphical presentation of the experiment investigating the impact of environmental contaminants exposure on epithelial secretions (ivDOF).

Figure 3.3: Glucose uptake after BOECs exposed to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid.

Figure 3.4: Concentration of Pyruvate in spent medium after BOEC exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid.

Figure 3.5: Concentration of lactate in spent culture medium after BOECs treatment with a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid.

Figure 3.6: Cell proliferation of BOECs exposed to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid over the course of 7 days of culture.

Figure 3.7: TransEpithelial Electrical Resistance (TEER) of BOECs cultured in Transwell system and exposed to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid over the course of 7 days of culture.

Figure 3.8: Glucose concentration in the in vitro derived oviduct fluid (ivDOF) after BOECs chronic exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid.

Figure 3.9: Lactate concentration in the in vitro derived oviduct fluid (ivDOF) after BOECs chronic exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid.

Figure 3.10: Pyruvate concentration in the in vitro derived oviduct fluid (ivDOF) after BOECs chronic exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid.

Figure 3.11: Bisphenol A impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after 24-hours of BOECs cultured in air-liquid phase.

Figure 3.12: The impact of Bisphenol S on amino acid concentration in BOEC ivDOF after 24-hours culture in air-liquid phase.

Figure 3.13: The effect of Triclosan exposure on the amino acid composition of ivDOF after 24-hours of BOECs culture in air-liquid phase.

Figure 3.14: Acetamiprid impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after 24-hours of BOECs cultured in air-liquid phase.

Figure 3.15: Relative gene expression of SLC1A4 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase.

Figure 3.16: Relative gene expression of SLC1A5 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase.

Figure 3.17: Relative gene expression of SLC7A1 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase

Figure 3.18: Relative gene expression of SLC7A2 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase.

Figure 3.19: Relative gene expression of SLC7A4 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase.

Figure 3.20: Gene expression of SLC2A1 glucose transporter in BOEC exposed to Triclosan for 24 hours in air-liquid phase.

Figure 4.1: Nuclear status of bovine COCs exposed to two different concentrations (10 nM and 1nM) of a) BPA, b) BPS, c) TCS and d) ACE during IVM.

Figure 4.2: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to two different concentrations of Bisphenol A and Bisphenol S at 10 nM or 1 nM during in vitro maturation.

Figure 4.3: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to two different concentrations of Triclosan at 10 nM or 1 nM during in vitro maturation

Figure 4.4: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to two different concentrations of Acetamiprid at 10 nM or 1 nM during in vitro maturation

Figure 4.5: Release or uptake of a) pyruvate and b) lactate from bovine COCs exposed to two different concentrations of Bisphenols (BPA and BPS) at 10 nM or 1 nM during in vitro maturation

Figure 4.6: Release or uptake of a) pyruvate and b) lactate from bovine COCs exposed to two different concentrations of Triclosan at 10 nM or 1 nM during in vitro maturation

Figure 4.7: Release or uptake of a) pyruvate and b) lactate from bovine COCs exposed to two different concentrations of Acetamiprid at 10 nM or 1 nM during in vitro maturation

Figure 4.8: The impact on OCR immature CEOs acutely exposed to Bisphenol A (BPA) immediately after isolation from the ovary. a) OCR in COCs in response to BPA, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPA treatment, c) mitochondrial parameters as a percentage of basal OCR after BPA injection.

Figure 4.9: The impact on OCR immature CEOs acutely exposed to BPS immediately after isolation from the ovary. a) OCR in COCs in response to Bisphenol S, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPS treatment, c) mitochondrial parameters as a percentage of basal OCR after BPS injection.

Figure 4.10: The impact on OCR immature COCs acutely exposed to TCS immediately after isolation from the ovary. a) OCR in COCs in response to Triclosan and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the TCS treatment, c) mitochondrial parameters as a percentage of basal OCR after TCS injection.

Figure 4.11: The impact on OCR immature CEOs acutely exposed to ACE immediately after isolation from the ovary. a) OCR in COCs in response to Acetamiprid and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the ACE treatment, c) mitochondrial parameters as a percentage of basal OCR after ACE injection.

Figure 4.12: Pro-nucleus formation of zygotes exposed to a) BPA b) BPS, c) TCS and d) ACE during in-vitro fertilisation.

Figure 5.1: Cleavage rate of embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation.

Figure 5.2: Blastocyst rate of embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation

Figure 5.3: Glucose consumption of blastocyst stage embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation.

Figure 5.4: Pyruvate consumption of blastocyst stage embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10nM or 1nM of each chemical during in vitro maturation.

Figure 5.5: Lactate production of blastocyst stage embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation.

Figure 5.6: Comparison of glucose, pyruvate consumption and lactate production between alive and dead blastocysts, derived from chemically exposed COCs to a) BPA, b) & c) TCS and d) & e) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation.

Figure 5.7: The turnover of amino acids with statistically significant difference on blastocysts derived from chemically exposed COCs to α) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation.

Figure 5.8: Glucose consumption of cleavage embryos (day 2 and day 3) exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture.

Figure 5.9: Pyruvate consumption of cleavage embryos (day 2 and day 3) exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture.

Figure 5.10: Lactate production of cleavage stage embryos (day 2 and day 3) exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture.

Figure 5.11: Glucose consumption of Day 2 and Day 3 embryos exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture.

Figure 5.12: Pyruvate consumption of Day 2 and Day 3 embryos exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture.

Figure 5.13: Lactate production of Day 2 and Day 3 embryos exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture.

Figure 5.14: The effects of α) & b) BPA and c) BPS, d) & e) TCS and f) & g) ACE on amino acids turnover during cleavage stage of embryos development. Embryos were exposed at 10 nM or 1 nM of each chemical during in vitro culture.

Figure 5.15: The effects of a) BPA and b) BPS, c) TCS and d) ACE on glucose consumption of during blastocyst stage exposure. 10 nM or 1 nM of each chemical was used during individual in vitro culture for 24 hours exposure during blastocyst stage.

Figure 5.16: The effects of a) BPA and b) BPS, c) TCS and d) ACE on pyruvate consumption of during blastocyst stage exposure. 10 nM or 1 nM of each chemical was used during individual in vitro culture for 24 hours exposure during blastocyst stage.

Figure 5.17: The effects of a) BPA and b) BPS, c) TCS and d) ACE on Lactate production of during blastocyst stage exposure. 10 nM or 1 nM of each chemical was used during individual in vitro culture for 24 hours exposure during blastocyst stage.

Figure 5.18: The effects of a) & b) BPA and c) BPS, d) & e) TCS and f) & g) ACE on amino acids turnover during blastocyst stage of embryos development. Blastocysts were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture for 24 hours.

Figure 5.19: OCR of blastocysts in response to acute injection of Bisphenol A (BPA) immediately on D7 of embryo culture a) The timeline of OCR in response to BPA, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPA injection, c) mitochondrial parameters as a percentage of basal OCR after BPA injection.

Figure 5.20: OCR of blastocysts in response to acute injection of Bisphenol S (BPS) on D7 of embryo culture a) The timeline of OCR in response to BPS, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPS injection, c) mitochondrial parameters as a percentage of basal OCR after BPS injection.

Figure 5.21: OCR of blastocysts in response to acute injection of Triclosan (TCS) on D7 of embryo culture a) The timeline of OCR in response to TCS, and the mitochondrial inhibitors: oligomycin, FCCP and Antimycin/Rotenone mix as comparison among the different treatment groups b) OCR as response to the BPS injection, c) mitochondrial parameters as a percentage of basal OCR after TCS injection.

Appendices

Figure 3.21_Appendix: Bisphenol A impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase.

Figure 3.22_Appendix: Bisphenol S impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase.

Figure 3.23_Appendix: Triclosan impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase.

Figure 3.24_Appendix: Triclosan impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase.

Figure 4.13_Appendix: Release of a) 17b-estradiol and b) progesterone from bovine COCs exposed to DMSO during in vitro maturation

Figure 4.14_Appendix: Release of a) 17b-estradiol and b) progesterone from bovine COCs exposed to DMSO during in vitro maturation

Figure 4.15_Appendix: Nuclear status of bovine COCs exposed to DMSO during IVM.

Figure 4.16_Appendix: The impact on OCR immature CEOs acutely exposed to Control-Vehicle immediately after isolation from the ovary. a) OCR in COCs in response to BPA, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPA treatment, c) mitochondrial parameters as a percentage of basal OCR after BPA injection.

Figure 4.17_Appendix: Pro-nucleus formation of zygotes exposed to DMSO during in-vitro fertilisation.

Figure 5.22_Appendix: a) Cleavage and b) Blastocyst rate of embryos derived from DMSO exposed COCs during in vitro maturation.

Figure 5.23_Appendix: a) Glucose, b) Pyruvate consumption, and c) Lactate production of blastocyst stage embryos derived from DMSO-exposed COCs during in vitro maturation.

Figure 5.24_Appendix: a) Glucose, b) Pyruvate consumption, and c) Lactate production of embryos exposed to DMSO during cleavage stage.

Figure 5.25_Appendix: a) Glucose, b) Pyruvate consumption, and c) Lactate production of embryos exposed to DMSO during blastocyst stage.

Figure 5.26_Appendix: The impact on OCR blastocysts acutely exposed to Control-Vehicle on D7 of embryo culture a) The timeline of OCR in response to Control-Vehicle, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the Control-Vehicle injection, c) mitochondrial parameters as a percentage of basal OCR after Control-Vehicle injection.

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This PhD is dedicated to my upcoming daughter, Vicky, who unexpectedly came into my life and made me see everything from a completely different perspective.

Declaration

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

Chapter 1

Introduction

1.1 Overview

Reproduction is essential process for all organism as it ensures the continuation of species. However, reproduction is susceptible to various challenge, including genetic, physiological challenges such as gene mutations, diseases (Smith et al., 2009), and insufficient nutrition (Allen & Ullrey, 2004), as well as anthropogenic challenges such as environmental pollution (Aulsebrook et al., 2020) and habitat destruction due to urbanization, agriculture, and deforestation (Milligan et al., 2009; Simkin et al., 2022).

Apart from those challenges, environmental factors such as climate change and pollution could potentially affect the reproductive process of all organisms (Carey, 2014). Due to climate change, alterations in temperature and weather patterns pose a threat on terrestrial and aquatic habitats existence (Boekhorst et al., 2024). Elevating temperatures could negatively impact reproductive ability of both male and female' individuals by disrupting hormonal balance, reducing sperm quality (Levine et al., 2023) and ovulation rates (Segal & Giudice, 2022). Additionally, extreme weather events such as heatwaves and droughts can lead to loss of environmental habitats and reduced food availability resulting in insufficient reproductive capability. Furthermore, alterations on the timing of reproductive cycles of species could be caused by climate changes leading to breeding mismatches and/or offspring malnutrition due to restricted food availability (Post & Forchhammer, 2007; Paoli et al., 2020; Segal & Giudice, 2022)/

Due to industrialization and modern lifestyle changes, humans similar to other organisms, are exposed to diverse classes of chemicals either indirectly due to their accumulation in the environment (Zhang *et al.*, 2021a) and food (Darnerud et al., 2006; Rashid et al., 2020) or directly through use of consumer products (Dionisio et al., 2018). On a daily basis, all organisms are exposed to, and interact with a variety of environmental factors and chemicals that affect the physiological processes and functions. One example of such an environmental chemical exposure is from xenoestrogens, which are extensively dispersed in the environment, meaning that the majority of people and wildlife population are exposed to them. A xenoestrogen is structurally or functionally related to human hormone 17 β -estradiol (E2) and consequently can bind Estrogen Receptors (ERs) but is not formed within the body. Xenoestrogens may be divided into two main classes based on their origins; either natural or

synthetic (Singleton, and Khan, 2003). Natural xenoestrogens are reported to have beneficial effects on health in the majority of cases (Paterni, et. al, 2017) although some data indicate dose-dependent effects such as the case of breast cancer (Kwon, 2014; Blei et al., 2015). On the other hand, synthetic xenoestrogens which are chemically produced molecules have been correlated with several health problems and are classified as endocrine disrupting chemicals (EDCs) (Paterni et. al, 2017).

One of the best characterised EDC in terms of impacts on human health are the bisphenols which are used during plastics and resins production. Among bisphenols, Bisphenols A (BPA) is extensively used and for this reason it has been studied in the most detail (Gramec Skledar and Peterlin Mašič, 2016). Studies point to a correlation between exposure to BPA and a number of human health problems including obesity, diabetes, and cardiovascular diseases (Gramec et. al, 2016; Paterni et. al, 2017), as well as abnormal reproductive function in both males and females (Murata and Kang, 2018). Even though BPA is considered a traditional pollutant and extensive research has been conducted about the effects on reproductive system, data of the effects on early stages of embryo development and the environment this process take place are limited.

After the evidence that BPA exposure can have adverse effects on human health, this chemical was replaced with structural analogs (BPS, BPF, BPAF, BPB). One of the major BPA substitutes is Bisphenol S (BPS) (Wu et al., 2018). BPS is now widely used for products that general population use daily, including cans, baby bottle and toys, and personal care products (Liao and Kannan, 2014). It is the main BPA substitute and is contained in all products labelled 'BPA-free'. BPS was first considered as "a safe BPA's substitute" because of the higher stability and the greater biodegradability resistance compared to BPA (Ike et al., 2006; Danzl et al., 2009). Environmental and epidemiological studies now confirm that BPS is widely dispersed in different environmental (sediment, surface and drinking water, indoor dust) and biological matrices (urine, serum, breast milk, cord serum) (Wu et al., 2018). However, little is known about the BPS mechanism of action in the human organism and how it may interfere with reproductive function.

In addition, despite the traditional pollutants such as those mentioned previously, organisms are exposed also to other environmental compounds that were not recognized before. Such compounds are described as emerging environmental compounds. Examples include per-

fluorinated chemicals (e.g., PFOA and PFOS), pharmaceuticals, illicit drugs, antibacterials, disinfection by-products (DBPs), pesticide reaction/degradation products, brominated flame retardants (PBDEs) perchlorate and nanomaterials. While these contaminants are characterized as “emerging”, many of them have been accumulating in the environment for many years, however they have not been identified because of lack of proper techniques and instruments (Richardson and Kimura, 2017).

In the category of antibacterial environmental contaminants, is Triclosan (TCS) an antimicrobial agent used in soaps, healthcare antiseptic, medical products (Rodricks et al., 2010; Dann and Hontela, 2011; Rochester and Bolden, 2015; Ye et al., 2015). Studies have shown that TCS is rapidly absorbed, mainly through ingestion and dermal absorption, is metabolised and distributed in the tissues. In vitro and in vivo studies have also identified that Triclosan is able to disrupt the endocrine function of organism. Importantly, TCS has been identified to accumulate in aquatic and terrestrial environment. However, the results of its action as reproductive toxicant are limited and, in some cases, conflicting.

A further example of emerging environmental contaminants is a class of pesticides named Neonicotinoids. Neonicotinoids have been widely used in crop protection against pest insects. Their mechanism of action is based on their ability to bind on the nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects (Matsuda et al., 2001). This category of neuro-active insecticide consists of 7 different compounds, acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid and thiamethoxam. Once, these were regarded as ‘environmentally friendlier’ than more traditional organophosphate and carbamate pesticides because they could bind on nAChRs’ insects with greater affinity compared to mammals (Tomizawa and Casida, 2005). However, recent reports have shown that neonicotinoids could be harmful for non-target organisms (Bonmatin et al., 2015) and negatively impact their reproductive system (Kapoor et al., 2011; Gu et al., 2013; Babel’ová et al., 2017). In 2017, following a risk assessment of the European Food Safety Authority (EFSA) the commission services completely banned the outdoor use of three neonicotinoids (clothianidin, imidacloprid and thiamethoxam) (EU, 2016, 2018).

Of the permitted neonicotinoids, EFSA concluded that acetamiprid posed a low risk to bees, so neither ban or further restriction was established for this substance (EFSA, 2016). However, scientific reports have pointed out that this compound is widely spread and accumulated in the

soil and water surface (Yi et al., 2019). Based on the high-water solubility and long degradation period in soils there is still the risk that acetamiprid may pose a threat to non-target organisms.

A critical period for the proper development of the organism is preimplantation development. Preimplantation period begins after a successful fertilisation process and occurs as the embryo travels along the fallopian tube to the uterus (Fig 1). During this ‘‘journey’’ the embryo undergoes consecutive cellular divisions; numerous physiological and biochemical alterations take place which are important for a normal development.

Recent evidence suggest that developmental stages of the preimplantation period might be affected by environmental contaminants (Choi, et. al, 2016). Furthermore, high (occupational or ambient residential) exposure to chemical is linked to changes in male and female reproductive function. Combining all this information, there is a need for further research on how environmental contaminants affect the reproductive process. More specifically, there is a research gap regarding the effects on the fallopian tube. The proper function of its environment is of major importance for a successful fertilisation and the subsequent early stages of embryo development.

The aim of this thesis is to examine how the processes of early development are affected by the exposure of reproductive system to environmental contaminants. For a better understanding of the chemicals’ mechanism of action in the reproductive system, the exposure of gametes, embryos, and the cells of the oviduct in environmental compounds, will be studied. Initially, the environmental contaminants which will be examined are BPA, BPS and Triclosan (TCS) representing a traditional and emerging environmental contaminant respectively. Finally, regarding the neonicotinoid pesticide, acetamiprid, it would be an interesting case of research as the accumulation of this compound has been increased in the environment and there is emerging evidence indicates human exposure to this compound. However, before we address these aims is necessary to describe the physiology of early reproductive events.

1.2 Early Reproductive events

1.2.1 Gametogenesis

Primordial germ cells (PGCs) are formed during embryonic development, usually around 3-4 weeks after fertilization in humans and are the precursors of male and female germ cells. Their proper development is essential for the successful development of the reproductive system. PGCs derivation seems to differ among the mammals. For instance, PGCs originate from the posterior proximal epiblast of the peri-implantation embryo in pig and mouse at embryonic day 11.5 (E11.5) and 6.25 (E6.25) prior gastrulation, respectively (Kobayashi *et al.*, 2017). Whereas in macaques PGCs are first identified in the extra-embryonic tissue known as dorsal amnion at E11 (Sasaki *et al.*, 2016). In mammals their formation is defined by bone morphogenetic proteins (BMP) which act through SMAD proteins (Hancock *et al.*, 2021).

As the epiblast undergoes gastrulation, a small group of cells separate from the rest and migrate through the gut cavity to genital ridge where gonads will eventually develop, a process that is directed by a series of chemical signals. The formation and migration of the PGCs to the final position consists of the first phase of this developmental process and the time of completion vary among the species. The second phase starts once the PGCs reach the genital ridge. During this phase, PGCs undergo proliferation and the differentiation is initiated through a series of signals that are received from their environment to form the male or female sex cords.

This second phase follows two distinct pathways depending on the presence or the absence of the SRY gene on the Y chromosome (Johnson and Everitt, 2007). The expression of the Sry, in males, leads to vigorous proliferation of the sex cord cells resulting to the deep penetration of mesenchyme where they surround the PGCs and eventually form the progenitors of the Sertoli cells (Nishimura and L'Hernault, 2017). However, the absence of SRY, in female foetus, allows the sex cords cells to remain in clusters around the PGCs; at this time, they are known as oogonia. While the initial fate decision whether an ovary or a testis will be created depends on the expression of Sry in order to develop Sertoli cells, the development of the gonad, particularly of the ovary and its follicles, depends on the existence of a population of normal germ cells (Tanaka, 2016).

The spermatogonial stem cells generated in male foetus enter mitotic arrest until puberty where they are reactivated, and the spermatogenesis is initiated. Spermatogenesis is the process through which spermatozoa are generated in the testis assisted by the Sertoli cells. It consists of 3 phases. 1) mitotic *proliferation* where large number of cells are generated, 2) two meiotic *divisions* where the haploid cells with genetic diversify are created and 3) the

cytodifferentiation during which the sperm acquire the final characteristic form (fig 1.1a). The result of the first phase is the generation of a large number of cells known as primary spermatocytes. After the first meiotic division, the secondary spermatocytes with one set of chromatin are created and finally the haploid early spermatids are generated following the second meiotic division. Finally, the spermatids undergo extreme remodelling including cytoplasmic changes, nucleus elongation, acrosome establishment and flagellum formation. This process is known as spermiogenesis and concludes with the production of the spermatozoa (Johnson and Everitt, 2007). The final form of spermatozoa (fig 1.1b) includes three main parts: the head, the mid-piece and the tail. The head contains the nucleus and the acrosome region with the proteolytic enzymes necessary for the acrosome reaction during the fertilisation process. The mid-piece includes the mitochondria which are necessary for the energy production of spermatozoa and finally the tail (or flagellum) consists of microtubules which give to the spermatozoa the ability to move/swim (Johnson and Everitt, 2007; Nishimura and L'Hernault, 2017)

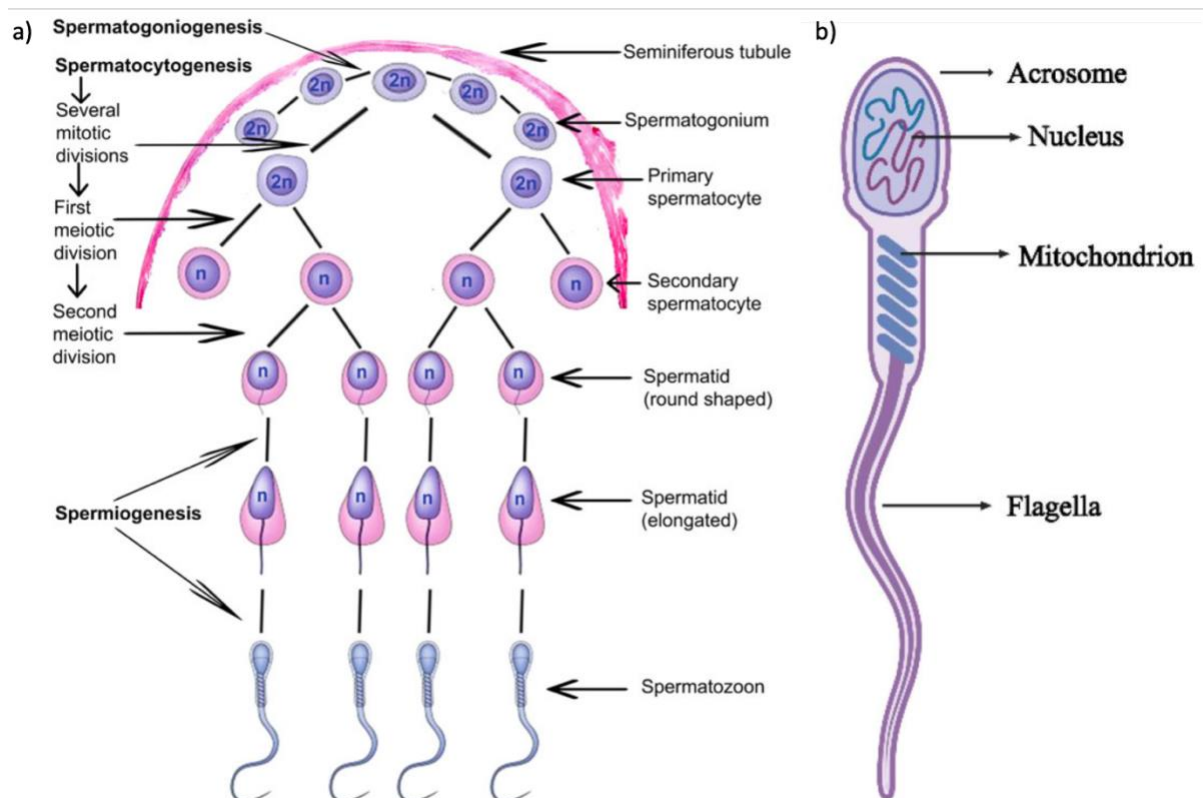


Figure 1.1: a) The process of spermatogenesis (figure from (Das et al., 2023) and b) the final form of spermatozoa (figure adapted from (Yan et al., 2022)). Through several mitotic and two meiotic divisions from spermatogonium four haploid round spermatids are created a process known as spermatogenesis. Subsequently, through spermiogenesis the round spermatids are transformed into elongated spermatids and then to spermatozoa. The final structure of spermatozoa includes the head, the mid-piece, and the flagella.

The process during which the mature oocyte is generated in the ovary, is called oogenesis, and shares several similarities to spermatogenesis, but with key differences. For instance, like spermatogenesis, oogenesis consists of three phases, proliferation through mitosis, genetic remodelling by generation of haploid gametes using meiosis divisions, before finally, a stage of cytodifferentiation during the final oocyte maturation (Johnson and Everitt, 2007). However, the proliferation phase of female PGCs is limited compared to males and occurs only while the female fetus is in utero. During this time, the PGCs complete mitotic expansion and begin to differentiate to primary oocytes, surrounded by pre-granulosa cells. This structure is called the primordial follicle. A characteristic difference compared to spermatogenesis is that the primordial follicles (equivalent to primary spermatocytes), which form as the foetus is in utero, are the only source of oocyte development during adulthood, meaning that the number of gametes in the female ovary is fixed at, or shortly after birth. The primary oocyte, situated within the primordial follicle arrests at the pro-phase stage of the first meiotic division (pro-phase I) (Gilbert and Gilbert, 2000; Johnson and Everitt, 2007; Jaffe and Egbert, 2017). At this stage, the oocyte is characterised by a large nucleus, and is referred to as the germinal vesicle (GV) stage. In humans, follicles can wait for up to 50 years arrested at the pro-phase I (Coticchio *et al.*, 2014), until they are recruited during the reproductive cycle.

The systematic resumption of the primordial follicle development occurs during puberty, similarly to spermatogenesis. Primordial follicles fall into 3 distinct stages of development towards to ovulation. Initially, they progress to primary or pre-antral follicles. This stage is characterised, depending on the species, by a 10 to 20-fold increase in follicle diameter due to granulosa cells proliferation. At this stage the theca cells begin to form, derived from ovarian stroma progenitor cells (Magoffin, 2005) surrounding the granulosa cells of the follicle. In parallel, the diameter of the oocyte begins to increase, reaching the 120µm in human, and extensive mRNA synthesis and organelle generation, necessary for the subsequent stages of development (Coticchio *et al.*, 2014), occur during this stage. In addition, an extracellular matrix is formed of glycoproteins; known as zona pellucida, this surrounds the oocyte and plays an important role during fertilisation. However, during the progress of primordial to pre-antral follicle the oocyte remains in meiotic arrest at prophase I (Jaffe and Egbert, 2017).

The next stage of the follicle development is characterised by a further size increase as the proliferating granulosa cells start to secrete follicular fluid, forming the follicular antrum. At this stage the follicle is known as secondary or antral follicle. During the progression from pre-

antral to antral follicle, theca and granulosa cells further differentiate. The former differentiates to theca interna, highly vascularised cells participate in the process of steroidogenesis, and the theca externa, loosely organised cells with structural role as they separate the theca interna from the interfollicular stromal region in the ovary. The latter differentiates to contribute cumulus cells which surround the oocyte, and the mural granulosa cells which reside on the wall of the follicle. The cumulus cells support the survival, nourishment and regulation of the oocyte maturation and fertilisation. Importantly, progression of follicle development at this stage is supported by steroids production from theca and granulosa cells which act synergistically with Follicle Stimulating Hormone (FSH) from the anterior pituitary gland, to promote granulosa cells proliferation. The mechanism of this process is discussed further in the following section.

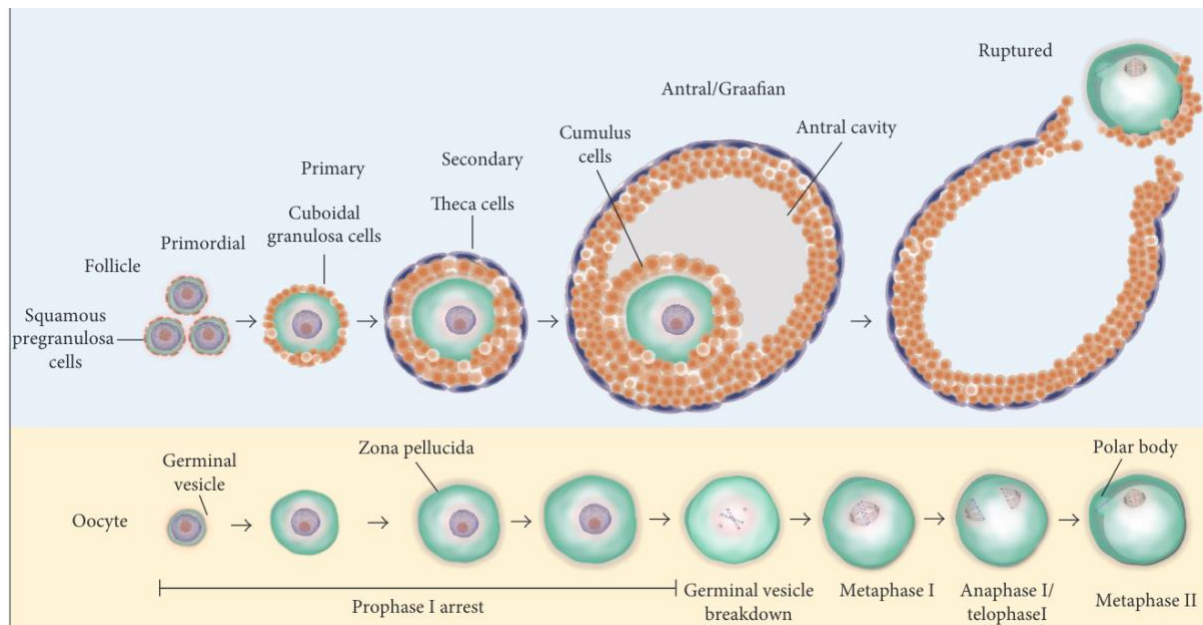


Figure 1.2: Folliculogenesis and oocyte maturation (figure from (Mihalas et al., 2017)). While in the uterus, PGCs of female fetus after mitotic expansion differentiate to primary oocytes, which are characterised by a large nucleus known as germinal vesicles (GV stage) and surrounded by pre-granulosa cells. This structure is called primordial follicle and is arrested at pro-phase of first meiotic division. At puberty, primordial follicles progress to primary follicles where theca cells start to form. Meanwhile zona pellucida which surrounds the oocyte is formed. Primary follicle progress to secondary follicle where the follicular fluid starts to form. Next one of the secondary follicles become dominant (graafian follicle) and the oocyte complete the first meiotic division and the first polar body is extruded. He oocyte enters the second meiotic division and arrests at metaphase.

In each oestrus cycle in mammals or menstrual cycle in human several follicles are recruited, although in mono-ovulatory species like the human or cattle one will become the dominant

follicle depending on the responsiveness of the granulosa cells to the FSH. Following the LH surge, as the recruited follicle progresses from antral phase towards to ovulation. At this time, the oocyte within the dominant follicle resumes and completes the first meiotic division, extruding a structure called the first polar body. This enables chromosome segregation whilst minimising loss of cytoplasmic contents (Maro and Verlhac, 2002). Subsequently the oocyte commences the second meiotic division, but it enters a second arrest at the metaphase II (MII). Finally, the MII oocyte surrounded by the cumulus cells will be released from the antral follicle and collected from the fimbriae of the oviduct during the ovulation.

The second meiotic division will be completed only if fertilisation occurs. When the second division is completed a second polar body is formed. The occurrence of these two uneven meiotic divisions resulting in a large oocyte and a small polar body each time allows the oocyte to retain the necessary mRNA, proteins and organelles such as mitochondria which will be necessary to support the subsequent preimplantation embryo development (Johnson and Everitt, 2007).

1.2.2 Oestrus cycle

In mammals such as the cow, the oestrous cycle represents the behavioural changes that females undergo due to the internal cyclicity of the ovarian activity whilst in higher primates such as humans this cyclicity of the ovarian activity is determined as menstrual cycle (Johnson and Everitt, 2007). Oestrous and menstrual cycle share some similarities but also have some differences.

The ovarian function of both cycles is regulated by the same hormones based on positive and negative feedback mechanism which act on the hypothalamus-pituitary-ovary (HPO) axis. Gonadotropin Releasing Hormone (GnRH) is produced and secreted in a pulsatile nature by hypothalamus in the brain and transferred via the hypophyseal portal blood system to pituitary gland where it stimulates the production of gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary gland. Subsequently, these two gonadotrophins induce the steroid production in the ovary by the two-cell two gonadotrophins mechanism (Johnson and Everitt, 2007; Forde *et al.*, 2011)

Initially, LH acts on the theca cells to stimulate androgen synthesis. These androgens are then converted in granulosa cells to oestrogens. FSH acts on the granulosa to stimulate the production of aromatase, the enzyme which catalyses the conversion of androgens to oestrogens through the cAMP-dependent signalling cascades which regulate the transcription of the CYP19A1 gene. Apart from oestradiol production, FSH stimulates the expression of LH receptors in the granulosa cells of the ovary acting as a positive feedback mechanism for the continuous production of oestrogen. The resulting oestrogen along with FSH stimulates the proliferation of the granulosa cells leading to the increase of oestradiol. Oestrogens have a regulatory action in the whole process, at low levels, oestradiol 17 β inhibits the gonadotrophins release by acting through a negative feedback mechanism on GnRH. By contrast, high levels of oestrogen triggers a rapid increase of and LH, this surge is leading to the final stage of the dominant follicle's maturation and finally ovulation (Forde *et al.*, 2011; Barbieri, 2014)

At ovulation, the oocyte is expelled from the ovary, and the remaining granulosa and theca cells of the follicle form the corpus luteum (CL) which produce progesterone. The progesterone acts on hypothalamus to reduce GnRH and subsequently gonadotrophins release. If fertilisation take place, the CL is preserved to support pregnancy by sustaining progesterone production and interrupting resumption of the ovarian cycle. However, luteolysis of the CL take place in response to prostaglandin secretion from the uterus if fertilisation does not occur (Forde *et al.*, 2011; Barbieri, 2014)

The whole process described can be divided into two distinct phases based on the appearance of the ovary. The *follicular phase* describes the period of final follicle maturation and ovulation, whereas the *luteal phase* describes the period when corpus luteum is formed after ovulation. In bovine the initial stage of luteal phase is also determined as met-oestrous and lasts around 3 to 4 days (Forde *et al.*, 2011). Finally, both species, human and bovine are mono-ovulatory with waves of follicles growth and the formation of a single dominant follicle taking place per cycle and the whole process is initiated at puberty (Johnson and Everitt, 2007).

Despite the similarities on the endocrine regulation of the menstrual and oestrus cycle described above there are also significant differences among them. For instance, the menstrual cycle is characterised by menstruation, a process through which the endometrial lining of the uterus is excreted whilst in the oestrous cycle the endometrium is absorbed. The duration of the two cycles also differs with an average of 21 and 28 days for menstrual and oestrous cycle,

respectively. (Johnson and Everitt, 2007; Forde *et al.*, 2011) In cattle, day 1 of the oestrous cycle is considered the initiation of oestrus with the LH surge (Forde *et al.*, 2011) whereas in human is the first day of menstruation and the LH surge take place in the middle of the cycle between day 13 and 15 (Johnson and Everitt, 2007). Furthermore, in cattle, the females are receptive to males only during oestrus (Forde *et al.*, 2011) whilst in human, females are receptive through the whole menstrual cycle. Additionally, a key difference between menstrual and oestrous cycle is that the latter is disrupted by anoestrus phases. Anoestrus normally appears before puberty, during pregnancy and up to 60 days after labour and characterised by lack of oestrus cycle or manifestation of heat (Mwaanga and Janowski, 2000). Finally, even though menstrual and oestrus cycle are regulated by the same hormones through the HPO axis the duration and timing of each hormone differs, the differences are illustrated in figure 1.3 .

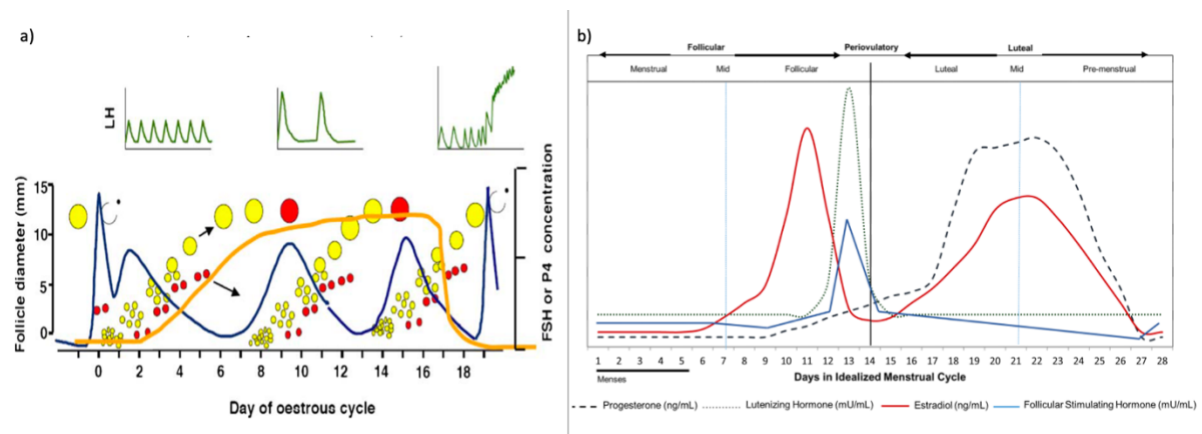


Figure 1.3: Graphical illustration of a) oestrous cycle in bovine (Forde *et al.*, 2011) and b) menstrual cycle in human (Draper *et al.*, 2018) hormonal cyclicality. In bovine Day 0 is the starting point of the early luteal phase while Day 1 is when menstruation begins in humans. In bovine a transient increase of FSH (blue line) is observed in every follicular growth wave. Meanwhile LH (green line) is secreted rhythmically. Progesterone (orange line) starts increasing on Day 2 of the cycle, from Day 8 to day 17 remains steady and then drops rapidly, right before ovulation. Ovulation occurs on Day 21. In human, after menstruation the follicular phase starts where the highest levels of oestradiol (red line) are observed, FSH (blue line) slightly increases during this phase and reaches a peak right before ovulation. LH surge occurs before ovulation, and both (LH and FSH) drop rapidly immediately after ovulation. Meanwhile, the luteal phase of the cycle starts and progesterone along with oestradiol increases, with progesterone concentration being higher than oestradiol until both decrease in the pre-menstrual phase.

1.2.3 Fertilisation

During coitus, sperm are deposited in the vagina, from where they travel through the vagina to the uterine cavity, ultimately reaching the oviducts. Throughout this journey, there are heavy losses of sperm, such that only 10s to 100s reach the oviduct (Sakkas *et al.*, 2015; Holt and Fazeli, 2016). Fertilization takes place in ampulla of the fallopian tube. Once the sperm enter the female reproductive system, it needs to undergo the final step of maturation to be able to successfully fertilise the oocyte; a process known as capacitation. During capacitation, a coating of glycoprotein is removed from the cell membrane that covers the acrosomal region of the spermatozoa. Sperm capacitation typically occurs in uterine cavity, or the isthmus of the oviduct (or fallopian tube in human) and the structural changes involved in capacitation result in a form of hyperactivated motility where the sperm motility pattern changes to vigorous and asymmetrical beating. Finally, capacitation appears to ultimately allow the influx of Ca^{2+} which is required to initiate the acrosomal reaction (Coy *et al.*, 2012).

Fertilization is a series of processes rather than a single event. It begins when the sperm initiates the penetration of the cumulus cell layer that surrounds the oocyte and ends with the fusion of paternal and maternal chromosomes. This process can be divided into three stages and has been summarised in a recently published review by (Siu *et al.*, 2021).

Initially, the first barrier that sperm encounters to meet oocyte is the dense layer of cumulus cells that surrounds the oocyte. The hyperactivation mode of the spermatozoa in combination with the enzyme hyaluronidase secreted by them assist penetration of the cumulus cells layer. Hyperactivation describes the new motility pattern that sperm acquire after capacitation and was described initially by Yanagimachi (1970) (Yanagimachi, 1970). Hyperactivated sperm have a vigorous motility pattern; the flagellum moves asymmetrically because of high-amplitude flagellar movements usually on the one side of the flagellum (Suarez and Ho, 2003). It has been suggested that the main trigger of the hyperactivation is the rise of intracellular Ca^{2+} , $[\text{Ca}^{2+}]_i$ and it seems that progesterone increases the concentration of $[\text{Ca}^{2+}]_i$ in human sperm (Publicover, *et al.*, 2008). The calcium ions move into the cytoplasm of the sperm through channels known as CatSper channels which are found in the principal part of the flagellum. It appears that the P4 combining with the slight alteration of intracellular pH activate the CatSper channels and assist to Ca^{2+} intake. (Lishko, Botchkina and Kirichok, 2011; Strünker *et al.*, 2011). The intracellular pH and subsequently CatSper channel activation are

possibly affected by the extracellular pH elevation in the oviduct fluid during the estrous (Suarez, 2008).

The zona pellucida is the second barrier encountered by spermatozoa and mainly comprises four glycoproteins in human (Lefievre, 2004) and three in bovine (Noguchi *et al.*, 1994). After penetrating the cumulus cell layer, spermatozoa bind the zona pellucida whereupon the spermatozoa undergo the acrosomal reaction. During penetration of zona pellucida, the acrosome reaction (AR) starts, and membrane fusion occurs (Gaddum-Rosse, 1985). During acrosomal reaction, proteolytic enzymes are secreted through exocytosis from the acrosome region located on sperm head. This allows the sperm to breach the glycoproteins zona pellucida shell. Subsequently sperm fuse to the oolemma, a process facilitated by membrane proteins of the sperm known as Izumo (Inoue *et al.*, 2005) and proteins from the oocyte known as Juno and Cas9 (Bianchi *et al.*, 2014). Following this, there is an increase of intracellular Ca^{2+} into the oocyte. Moreover, sperm-specific phospholipase C (PLC ζ) stimulates the calcium increase resulting on waves of calcium oscillations and zinc ‘spikes’ which support the subsequent events of fertilisation such as oocyte activation (Sanders *et al.*, 2018). Apart from the factors mentioned above, six more factors SPACA6, TMEM95, SOF1, FIMP, and DCST1/DCST2 expressed in sperm, have now been identified as important during the fertilisation process. The function of these factors are explained in the review published by (Siu *et al.*, 2021). Oocyte activation includes the block of further sperm binding, the resumption and completion of meiosis and concludes with pronucleus formation. The calcium oscillations are also involved in the hardening of the zona pellucida and the prevention of polyspermy by initiating the cortical reaction. Cortical reaction is the exocytosis of oocyte’s cortical granules. This induces modifications of the structural proteins of the zona pellucida, preventing further sperm binding, resulting on zona pellucida hardening and subsequently polyspermy prevention (Wakai *et al.*, 2011).

However, there are evidence which are suggesting that ZP is not the only place where AR take place. For instance, P4, which is secreted by the cumulus cells, promotes AR in several species. This could mean that AR might takes place before spermatozoa reach the ZP while they transit through the cumulus (Sun, Chung and Chan, 2011). Yanagimachi suggested that the point where this process starts in mammalian sperm is a debatable issue because nobody has ever traced a spermatozoon from the starting point of the acrosome reaction until the last part of

fertilization process. Due to that, the exact place and the triggers of spermatozoa acrosome reaction remains under investigation (Yanagimachi, 2011).

Irrespective of where the AR starts, the sperm-ZP binding is a crucial point for the success of fertilisation which is influenced by molecules presented in the oviductal secretions. Carrasco *et al.* 2008b has claimed that five estrous cycle-dependent glycosidases secreted in the oviduct fluid participate in the sperm-ZP binding. However, additional experiments are required to clarify the function of every glycosidase in the oviduct (Carrasco *et al.*, 2008).

At the final stage of fertilisation, a process known as syngamy take place. During syngamy, the female and male pronuclei, with the haploid genomes, line up and fuse restoring a diploid genome is created. Finally, the first mitotic division takes place resulting on 2-cell embryo.

As soon as fertilisation has been completed, the fertilised egg carries on its route into the oviduct until it reaches the uterus where it will implant. During this migration from the oviduct to the uterus the first stages of embryo development occur which is a period critical for the offspring's health.

1.2.4 Early-stage embryo development

Pre-implantation embryo development involves the early stages of development before the embryo is implanted in the uterus (fig 1.4). It begins after a successful fertilisation process and occurs as the embryo travels along the oviduct to the uterus (Johnson, 2019). Consecutive cellular divisions of zygote take place after fertilisation which could be synchronous or asynchronous (Prados *et al.*, 2012). The cellular divisions are time-sensitive and species-specific (Johnson, 2018). It has also suggested that the time between each cell division is of critical importance. It takes 18-20 hours for each cell division in normally developing embryos (Prados *et al.*, 2012). Until the stage of 8-cell, 3 cycles of cell division take place and at this point embryos are called cleavage stage embryos. During the cleavage stages, embryo is surrounded by the zona pellucida, which prevents the embryo from attaching itself to the wall of the lumen along the pathway until it reaches the uterus, thereby preventing an ectopic pregnancy (Wassarman and Litscher, 2018)

Up to the early stage of 8-cells the shape of the blastomeres remains distinct. The distinct appearance of the embryo could be explained by the negligible communication between the blastomeres up to this stage (Coticchio *et al.*, 2019). It also has been suggested that due to the negligible communication among the blastomeres, before the morula stage, the cells act autonomously in terms of their metabolic activity (Brison *et al.*, 2014). However, at the 8-16 cell stage, embryo compaction process take place, where blastomeres become compressed, maximising intercellular contacts through adherens and gap junctions' establishment (Fleming *et al.*, 2001; Bloor *et al.*, 2002). Furthermore, compaction causes a transformation from a spherical to an epithelial-like shape; by this time, the embryo is called morula (Coticchio *et al.*, 2019). It is around this stage that the embryo moves from the oviduct to the uterus (Lonergan *et al.*, 2016). Notably, up to this developmental stage the size of the whole embryo remains unchanged despite the fact that the number of the cell increases (Johnson, 2007)

During compaction, the embryonic cell population is positioned into inner and outer space of the embryonic structure. Depending on the cell polarisation, which occurs during compaction, the cells differentiate into either an inner cell mass (ICM) or the trophectoderm (TE) (Johnson and McConnell, 2004). It has been suggested that the determination of which cells will differentiate to TE or ICM is based not only on the cell allocation during the cleavage stages but that blastomeres at the 2-cell have a tendency to contribute more either to embryonic or extraembryonic tissue (Piotrowska *et al.*, 2001). This discovery that was further supported by the finding that 4-cell blastomeres have different developmental potential depending on whether originate from the first or the second dividing cell on the 2-cell stage blastomeres (Piotrowska-Nitsche and Zernicka-Goetz, 2005; Piotrowska-Nitsche *et al.*, 2005). However, these discoveries have been challenged by research studies suggesting that the cell fate for embryonic and extraembryonic axis resulting from the physical barrier of the ZP and is not prepatterned fate of blastomeres (Motosugi *et al.*, 2005; Kurotaki *et al.*, 2007). The cell fate specification during the preimplantation embryo development has been discussed in detail in a recently published review (Lamba and Zernicka-Goetz, 2023).

As the morula develops to the next stage, blastomeres, allocated at the outer surface, begin to pump Na⁺ ions into the intercellular space within the embryo. As this occurs fluid begins to accumulate, moving along a concentration gradient, and forming a cavity called blastocoel. At this developmental stage the embryo is named blastocyst. At this stage cell differentiation takes place with the formation of the trophoblast or trophectoderm (TE) and the inner cellular mass

(ICM) from the cells allocated at the external and the internal space of the cavity respectively. The blastocyst continues to expand as the cells continue to divide and more fluid accumulates in the cavity.

The TE, which are polarised, surround the blastocoelic cavity contain the blastocoelic fluid as well as the cells of the ICM. The ICM further differentiate to the epiblast cells (Epi), from which the embryonic tissue will be developed and the primitive endoderm (PrE) which with the TE will form the extraembryonic tissues (Xenopoulos, Kang and Hadjantonakis, 2012; White et al., 2018)

The early stages of pre-implantation embryonic development are controlled by the maternal genome, because the fertilized embryo is in a transcriptional arrest at the beginning of its development. During ovulation and later in the mature egg, proteins and maternal mRNAs accumulate, which facilitate the first cell cycles and the basic biosynthetic process in the cleavage embryo. Of critical importance for the normal embryo development is a process called embryonic genome activation (EGA). EGA is the process during which the transition from the use of maternal- derived mRNA to embryo-produced mRNA (Jansz and Torres-Padilla, 2019). This process is time-depending and species specific however in all cases take place at the early stages of embryo development where the embryo allocated in the oviduct. This time point is at the 8-to-16 cell stage for bovine (Sirard, 2012).

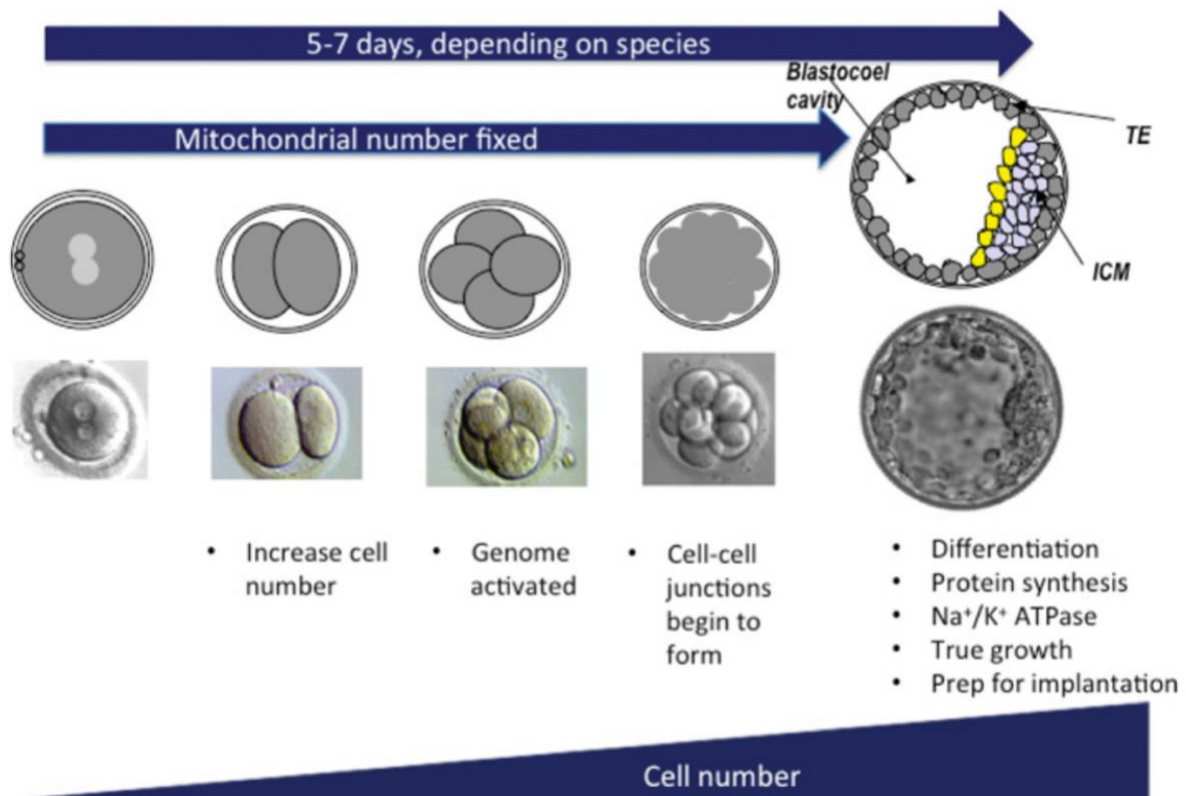


Figure 1.4: Schematic presentation of early stages of embryo development ((Smith & Sturmey, 2013). After fertilisation the zygote undergoes three consecutive division and increases the number of blastomeres without size increase. On the next stage around 8 to 16-cells the blastomeres undergoes compaction and morula is formed. After compaction the cells starts to secrete a fluid and blastocoel cavity formation initiating. During this stage, the blastomeres differentiate to TE and ICM. During early stage of development embryonic genome activation occurs; a process during which the embryonic genome is activated and the development transit from maternal control to embryonic. This process is species depended.

1.3 Mammalian oviduct

1.3.1 Oviduct: the neglected organ

The oviduct is the organ of the mammalian female reproductive system where the two gametes encounter to form the zygote, and the subsequent the first stages of embryonic development take place there. For many years due to the artificial reproductive techniques (ARTs) development, the research of the oviduct was not the primary aim of the scientific community since the fertilisation can be successfully achieved without an oviduct (Ménézo *et al.*, 2015; Pérez-Cerezales *et al.*, 2018). However, the scientific interest in understanding the function of

the oviduct and how it contributes to the reproduction process has been revitalized because of indications that a better understanding of the oviduct activity, and in particular of its micro-environment, will inform improvements of laboratory conditions, used in the IVF, especially by mimicking the *in vivo* nature environment the *in vitro* culture conditions will be optimized. Consequently, it is widely believed that this improvement will lead to greater embryos' quality (Ménézo et al., 2015; Binelli et al., 2018)⁴.

Aside from interest in its function, we should also strive to comprehend how it reacts to the consequences of environmental factors (e.g., environmental contaminants) that the human body is exposed to on a daily basis. Hence, one of primary aim of this thesis is to review the evidence on the question “Do environmental factors influence the function of the oviduct and to what extent does this affect the reproductive function?”. Before considering this question, it is necessary to describe the oviduct and why is considered as an important parameter for fertilization, and for the reproduction process in general.

1.3.2 Morphology and function

In 1561, the Italian anatomist Gabriele Fallopius described how the ovaries and the uterus are connected through a tubular organ, which he described as the “trumpets of the uterus” because of their resemblance to a musical instrument (Cappello et al., 2010). Nowadays, this tubal organ is known as the oviduct, although the term Fallopian tube is often still used in humans. Anatomically directed from the ovary to the uterus, the oviduct consists of 4 functional regions (fig 1.5): the infundibulum, the ampulla and the isthmus and the utero-tubal junction (UTJ) (Leese, 1988). These four sections are different in terms of morphology, function and cellular characteristics (Leese, 1988; Hunter, 1998). The infundibulum is the widest region of the oviduct and contains fimbriae which collect the oocyte after the ovulation. The ampulla is the region in which the final nuclear and cytoplasmic maturation of the oocyte, the fertilization and the first embryo cleavage stages take place. In the other two sections, the isthmus and the UTJ, pre-fertilisation processes such as the retaining and capacitation of spermatozoa occur, and the fertilised oocyte moves into the uterus through these sections (Li & Winuthayanon, 2017).

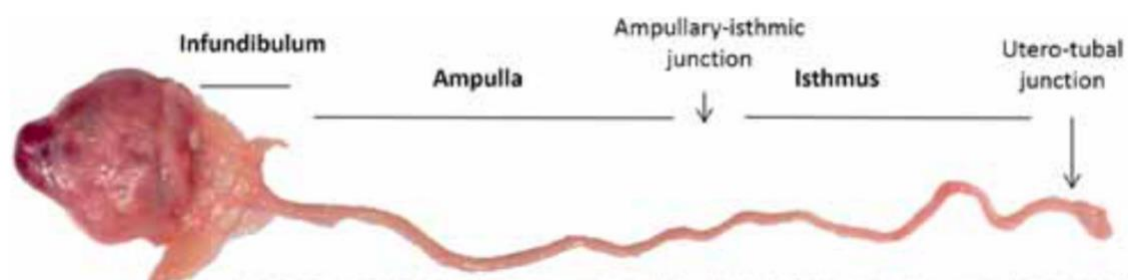


Figure 1.5: The oviduct anatomical regions connected with the ovary (Maillo et al., 2016). The regions of infundibulum, ampulla isthmus and utero-tubal junction are presented. Ampullary-isthmic junction is the fertilisation site.

Besides the functional regions of the oviduct mentioned above, histologically, in transverse section, the oviduct can be divided into 4 layers directed from inner to outermost: tunica mucosa (Tmuc), tunica submucosa (Tsub), tunica muscularis (Tmusc) and tunica serosa (Tser). Starting with the outermost layer, the Tser executes a structural role in oviduct with minor histological variation among species (Johnson et al., 1974). It is comprised of connective tissue which is connected to the uterine tunica serosa. The second layer, the Tmusc, contains the smooth muscle- responsible for the contractions in the oviduct. The thickness of this layer increases from infundibulum to isthmus and in response to ovarian hormones (Hafez & Hafez, 2000) that fluctuate during the estrous cycle (Ayen et al., 2012). Directly beneath the Tmusc is tunica submucosa. The Tsub is also known as the myosalpinx, and is a complex, thin high elastic and compressible lattice which provides tensile strength (Frantz et al., 2010). This layer is responsible for the fast transfer of compounds via diffusion between vasculature and epithelium. Through this layer, endocrine, metabolic, and lymphatic signals are transported to the oviduct. Furthermore, the expression of oviduct components is estrous cycle – dependent (Gabler et al., 2001). Finally, the fourth layer beneath Tsub is tunica mucosa is very highly folded longitudinally. This layer consists of the epithelial monolayer which sits on a stromal membrane consisting of collagen and laminin.

The tubal lumen is lined with a layer of epithelial cells (fig 1.6) in all four section which form a functional barrier. The epithelium mainly is combined of two types of cells: the ciliated and the secretory cells (Leese, 1988). Each type is involved in different activities of the oviduct with the ciliated cells participating in processes associated with reproduction (e.g. sperm capacitation, sperm and embryo transfer) and the secretory cells are involved with the production and secretion of the oviductal substances, (Gonella-Diaza et al., 2017) which create

the appropriate microenvironment for reproductive procedures such as the sperm selection and preimplantation embryo development (Pérez-Cerezales et al., 2018). The number and the percentage of these two types of epithelium cells varies across the length of the tube. The number of the ciliated cells decrease, whereas secretory cells increase in the direction from infundibulum to isthmus (Leese, 1983). However, the ampulla, where the fertilisation takes place, has greater secretory activity compared to those of infundibulum and isthmus (Buhi et al., 2000).

The lumen of the oviduct contains variable numbers of folds and invaginations. Interestingly, it has been found mucosal folding begins as the oviduct is forming during in utero development, yet and only in the adult life does the epithelium cells' reach the final level of organisation complexity by developing primary, secondary, and tertiary folds (Agduhr, 1927; Konishi et al., 1987; Kenngott & Sinowatz, 2007). Apart from the epithelial cells' differences among the anatomical sections of the oviduct, differences are also apparent during the days of the oestrus cycle; the quantity of folds as well as the folding grade reaches a peak during oestrus (Restall, 1966). It has been proposed that the percentage of the ciliated and secretory cells varies in oestrus cycle and only the secretory cells proliferate (Ito et al., 2016). It seems that the number and the size of secretory cells is increased during the oestrus cycle with the profile/phenotype of these cells to be related with the endocrine fluctuation of the reproductive cycle (Eriksen et al., 1994; Ayen et al., 2012).

Using an air-liquid interface (ALI) culture model of porcine oviductal epithelial cells (POECs), (Chen et al., 2013b) concluded that the cellular function and the proportion of POECs were affected by the sex-steroid (E2 & P4) treatment. Importantly, E2 and P4 controlled the transformation between ciliated and secretory cells and the induction of ciliogenesis (Chen et al., 2013a). The same team in 2018 by using an identical culture model suggested that changes of P4 levels (rather than E2) provoked the morphological and gene expression variations in the oviduct. However, E2 is required for proper immune response of the epithelium during the estrous cycle (Chen et al., 2018). Furthermore, a study of periovulatory endocrine profile of E2 and P4 based on the size of pre-ovulatory follicle in cattle suggested that greater E2 and P4 concentrations influenced the size of the follicle in a positive way, and this led to a greater oviductal remodelling (Gonella-Díaz et al., 2017), which is associated with improved fertility (Pugliesi et al., 2016). This idea is reinforced by studies carried out on sex-steroid receptors ESR1 and PGR in mice, which showed that abnormalities in these receptors provoke embryo

development failure and lack of estrous cycle/transcriptional changes in the oviduct, respectively (Kawakami et al., 2001; Töpfer-Petersen et al., 2002). Finally, Buhi et al shows that E2 stimulates the biosynthetic process of the secretory cells in a region-specific way. More specifically, macromolecules` synthesis and secretion in infundibulum and ampulla was increased whilst was remained unchanged in isthmus after injecting exogenous E2 in ovariectomized sows (Buhi et al., 2000).

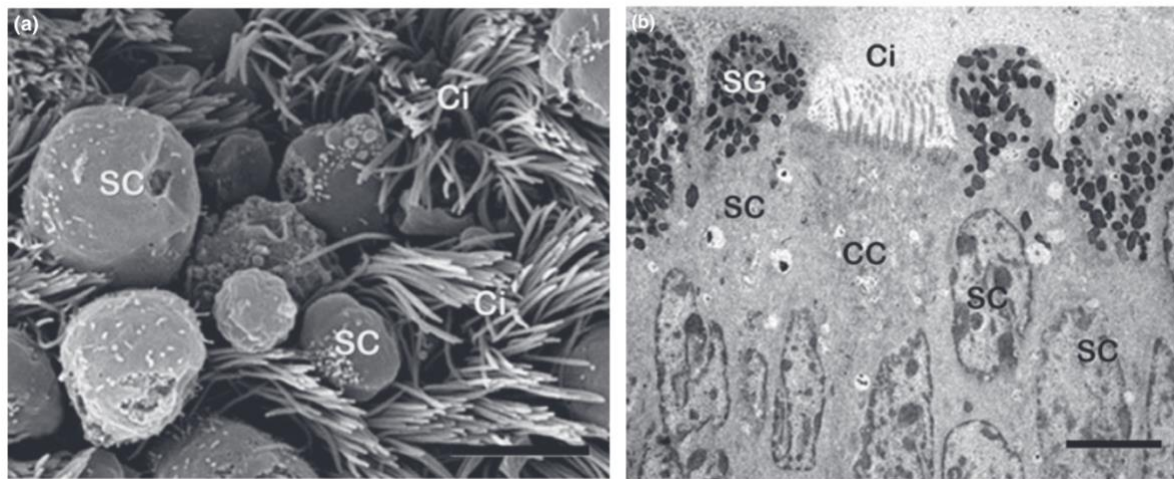


Figure 1.6: Electron scanning micrograph of bovine oviduct epithelia a) surface and b) epon-embedded oviduct. SC=secretory cells, CC and Ci = ciliated cells (Cilia) (Mondéjar et al., 2012).

1.3.3 Oviductal fluid

In 1956 Bishop reported that the *in vivo* oviductal secretions is an active process. Bishop cannulated the infundibulum of rabbit oviduct and measured the pressure of the oviduct fluid in oestrous, ovariectomized and pregnant rabbits. The quantity of oviductal fluid in oestrous was 5.5 times higher per 24h compared to those of ovariectomized rabbits; also, oviductal fluid secretion was decreased during pregnancy. Interestingly, the volume and the pressure of the oviduct fluid in ovariectomised rabbits returned to physiological levels after oestradiol injections (Leese, 1988). Nowadays, these active oviductal secretions are named oviductal fluid which is presumed to be a product of epithelial secretory cell activity. These cells secrete proteins and other factors which are combined with plasma-derived factors to create oviductal fluid. Electrolytes, proteins, lipids, catecholamines, steroids, vitamins, growth factors, macromolecules such as albumin and metabolic substrates such as glucose, pyruvate, amino

acids constitute the oviductal fluid (Leese, 1988; Leese et al., 2008; Ménézo et al., 2015). The concentrations of these factors are different of those in serum and uterine fluid (Leese et al., 2008) and despite the fact that the composition is influenced by the blood plasma, it seems that is regulated locally by the ciliated and secretory cells which form a cell monolayer lining the lumen (Pérez-Cerezales et al., 2018). Furthermore, this clue of the local regulation it seems to be supported by the great number of the oviduct-specific proteins recognized in the oviductal fluid (Avilés et al., 2010).

1.3.3.1 Oviduct fluid composition

The oviduct fluid is composed of proteins released from the epithelial cells and molecules derived from plasma (Leese, 1988). The composition has been described in different species (Aguilar & Reyley, 2005) and it mainly consist of 6 main groups: electrolytes, energy substrates, amino acids, proteins and enzymes, extracellular vesicles, and fatty acids.

- Electrolytes

Potassium, bicarbonate, calcium, magnesium and chloride ions are the main oviduct fluid's electrolytes. Potassium and bicarbonate are enriched in the oviductal fluid compared to the serum (Aguilar & Reyley, 2005) suggesting that the level of those electrolytes is not dependent on blood concentration. However, the same does not happen in the case of calcium and magnesium. Studies have shown that there is a positive correlation between the concentration of those two electrolytes in the oviduct fluid and in the blood (Aguilar & Reyley, 2005; Hugentobler et al., 2008). Regarding calcium, it also seems that its concentration within the oviduct differs among the oviduct sections and also is species depended (Aguilar & Reyley, 2005) however calcium levels in the oviduct are lower than those in plasma (Leese, 1988). Electrolytes in the oviduct fluid maintain a stable environment in terms of osmolarity and pH levels (Leese, 1988) and have been associated with the embryo development. For example, calcium is associated with embryo compaction (Miao & Williams, 2012) and potassium with higher development rates on 2-cell stage mouse embryos (Roblero & Riffo, 1986). Finally, the presence of bicarbonate ions in in vitro embryo production is associated with greater developmental progression by maintaining the extracellular pH. In summary, electrolytes promote embryo development in a direct and indirect way.

- Energy substrates

The main metabolic substrates in oviductal fluid are pyruvate, glucose, lactate and they mainly derive from blood (Leese & Gray, 1985). These substrates are used as energy sources, at levels which vary at different preimplantation stages (Edwards et al., 1997). Data describing concentrations of the metabolic substrates in the oviduct are limited because of the difficulty in accessing oviduct. However, Hugentobler et al (2008) in a study conducted in bovine, which is also the model that have been used during this thesis, has reported that the concentrations of these metabolites did not differ between ipsilateral (the side where the ovulation occur) and contralateral (the opposite) side of the oviducts. Moreover, secretion rates measured in $\mu\text{l}/\text{min}$ did not vary among the oestrus cycle days, only a small tendency to decline from Day 0 to Day 6 (Hugentobler et al., 2008) in bovine. However, the levels of these energy substrates in the lumen was significantly different compared to these in the plasma (Hugentobler et al., 2008). The concentration of these metabolites differ among the regions of the oviduct, for instance higher concentration in the ampulla compared to isthmus have been detected (Leese, 1983). Furthermore, lactate was found at a higher concentration compared to the other two metabolites suggesting that is partly synthesised, probably via the metabolism of glucose in the oviductal epithelium (Leese, 1988; Gardner & Leese, 1990). Finally, no association was observed between the concentrations of glucose, pyruvate or lactate and the concentrations of steroid hormones, oestrogen and progesterone (Hugentobler et al., 2008).

- Amino Acids

Apart from glucose pyruvate and lactate, amino acids are essential substrates during embryo development. For example, it has been found that blastocyst rates and the morphology of the embryos are significantly improved under cultured in media with physiological relevant amino acid concentration (Walker et al., 1996). Their concentration is species -studied depending and in some cases is also depended on the day on cycle (Aguilar & Reyley, 2005), fact which is not true is the case of bovine species (Hugentobler et al., 2007). Twenty-five amino acids were identified in the oviductal fluid of different animal models (bovine, porcine, ewes, rabbit and mouse) with glycine being the amino acid with the highest concentration among the species (Aguilar & Reyley, 2005). In bovine, following glycine, alanine and glutamate were the next most abundant amino acids in the oviduct fluid (Hugentobler et al., 2007). The amino acids

concentration in the oviduct fluid is significantly different to this of uterine fluid and plasma and their appearance in the oviduct fluid is a combination of epithelial cell secretion and transport from the blood stream (Leese et al., 2001a).

- Proteins and enzymes

Albumin and immunoglobulin G are the dominant proteins in the oviduct fluid, both of which are derived from the plasma (Oliphant et al., 1978; Leese, 1988). Albumin is able to bind peptides, catecholamines and lipids, as well as acting as a lipid transporter and exchanger and also plays an important role in sperm capacitation by facilitating changes in sperm membrane fluidity (Khatchadourian et al., 1987; Ehrenwald et al., 1990; Neill, 2006).

However, a great number of the oviduct- specific proteins have been identified in oviductal fluid (Avilés et al., 2010). Among these proteins, the best characterised is oviduct-specific glycoprotein 1 (OVGP1). OVGP1 has been detected in different species, summarised in the review published by (González-Brusi et al., 2020) but strangely not in rat oviduct (Arias et al., 1994). Furthermore, it seems that OVGP1 levels differ depending on the oviduct region and the stage of oestrus cycle with highest levels on periovulatory period. Studies have reported that OVGP1 plays a role in sperm capacitation by interacting with the sperm acrosomal membrane, thus preventing polyspermy by covering the sperm-binding regions after fertilisation. Furthermore, OVGP1 interacts with zona pellucida (ZP) and this connection assists the “ZP hardening” process (Wegner & Killian, 1991; Coy et al., 2008; Algarra et al., 2016). Finally, it has been suggested that this protein could have a role in immunological protection of the embryo in vivo (Leese et al., 2001b). However, many species-specific differences have been described. For instance, differences among species have been found regarding gene expression during oestrus cycle and the length of the protein (Avilés et al., 2010). For this reason, we could say that many aspects of the role of this protein remain unidentified.

Beyond OVGP1, a range of other molecules such as growth factors, proteases, cytokines, proteases inhibitors, enzymes which are secreted by the oviduct epithelium (Pillai et al., 2017). Growth factors seem to support early stages of embryo development (Pillai et al., 2017). and contribute to embryo implantation (Simmen & Simmen, 1991). Other oviduct-derived

molecules that play a role in implantation include cytokines by promoting the uterus-embryo interaction (Simón et al., 1996). Finally, proteases and protease inhibitors play a role on homeostasis maintenance of the oviductal environment (Pillai et al., 2017).

- Extracellular vesicles

Epithelial- derived extracellular vesicles have been identified in the oviduct (Lopera-Vasquez et al., 2016). Extracellular vesicles (EV) have been previously detected in follicular fluid (da Silveira et al., 2012) and the uterus (Ng et al., 2013). It seems that extracellular vesicles facilitate the interactions and communication between the embryo and the oviduct. It has been shown that EV, derived from oviductal epithelial cells, in culture media improve embryo quality in terms of survival after verification as well as the trophoectoderm and total cell number in bovine (Lopera-Vasquez et al., 2016) and increase the developmental competence of porcine embryos (Fang et al., 2023). In recently published reviews the role of EVs in maternal -embryo communication has been discussed, the fact that oviduct-derived EVs are up taken by embryos and embryos-derived EVs might moderate the oviductal function suggest that EVs facilitate a bidirectional communication (reviewed by Bridi *et al.*, 2020) highlighting their potential contribution to reproductive health by improving our understanding fertility environment and developing diagnostics and therapeutics methods (reviewed by Fazeli and Godakumara, 2024)

- Fatty Acids

Fatty Acids are also another component of the oviduct fluid, however data regarding their presence and their effects in the oviductal environment are very limited. It has been found that the linoleic, stearic and oleic acid are the most abundant in the oviductal environment. Furthermore, arachidonic acid, palmitic acid and eicosapentaenoic acid have also been identified in the oviductal fluid and their concentration was higher compared to blood plasma (Drews et al., 2018). Furthermore, in an ex-vivo study conducted in bovine, it has been found that the concentration of non-esterified fatty acids (NEFA) in the oviduct fluid was 0.29mmol/L and there was a positive correlation to the concentration in the bloodstream (Jordaens *et al.*,

2017). The same group studied the NEFA transport in and out of the oviductal environment. The results showed that NEFA are transferred through the oviduct epithelial cells with a polarised way. Elevated levels of NEFA were transferred out of the lumen environment whereas the transport of the opposite direction was limited. These results suggested that oviduct epithelium act as a “gatekeeper” of the oviductal environment (L. Jordaens et al., 2017). Further to this study, Jordaens et al, suggested that elevated NEFA in the oviduct environment impair embryo development (Jordaens et al., 2020).

1.3.3.2 Mechanism of oviduct fluid secretion

In 2001 Leese et al., introduced mechanism of how the oviduct fluids are potentially secreted which is illustrated in Figure 1.7. This proposed mechanism was underpinned by the work of Quinton in 1990 regarding the ion transport which triggers water transfer through the epithelial monolayer in various epithelial tissues (Quinton 1990). The $\text{Na}^+/\text{K}^+/\text{-ATPase}$ enzyme is located in the basolateral membrane of the cells and regulates the exchange of intracellular Na^+ for K^+ ions in order to keep the levels of Na^+ low. At the same time the chloride ion flow is regulated through the electroneutral carrier $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ which is also located on the basolateral membrane of the epithelial. The apical membranes become permeable to Cl^- allowing influx to the lumen. Because of this movement, an electrochemical gradient is created which triggers the paracellular movement of Na^+ via the tight junction into the lumen. High osmotic pressure is created by the intracellular accumulation of ions which is equilibrated by the water influx to the lumen.

The volume of oviduct fluid might be driven by oestrogen levels during ovulation in rabbit (Gott et al., 1988) or after oestrogen treatment in ovariectomised sheep (McDonald & Bellve, 1969). Furthermore, it has been suggested that the increased levels of oviduct fluid during oestrus could be associated with the higher volume of ions transported via ions channel (Leese, 2001) due to higher height that epithelial cells acquire during this period (Chen, Einspanier and Schoen, 2013). This was investigated further in bovine epithelial cells by Simintiras et al., (2016) using the Transwell system of culture. In this study different concentration of steroid hormones, oestrogen, androgens, and testosterone shown to affect the bovine oviduct epithelial secretions as well as the gene expression.

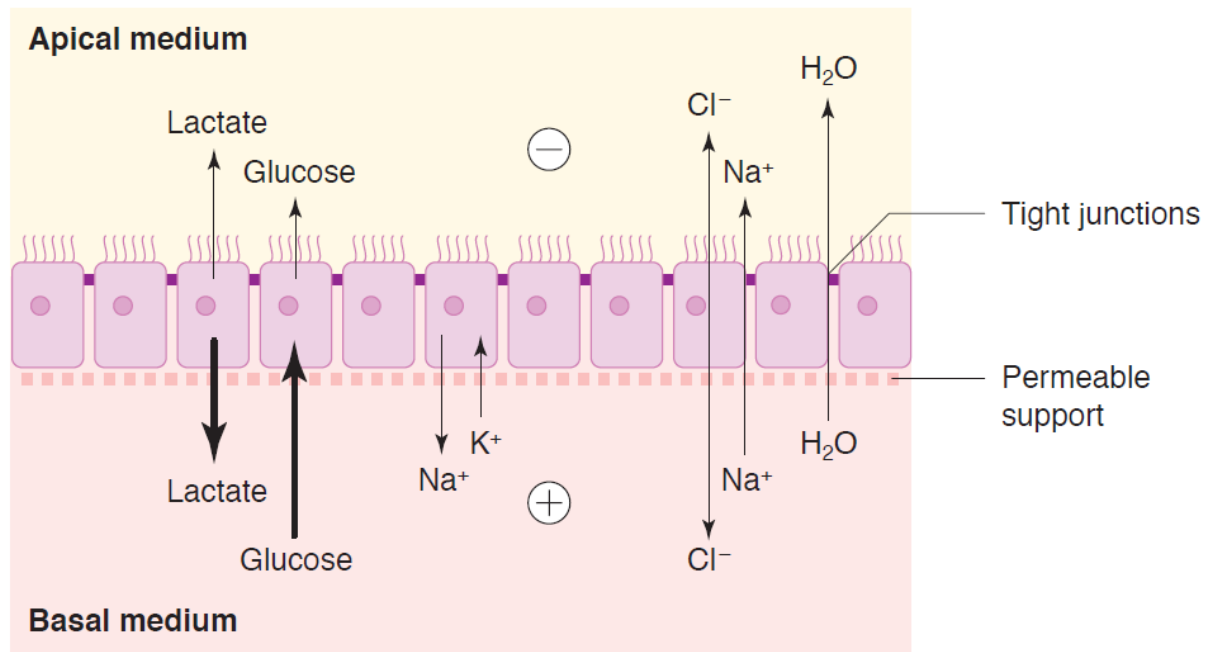


Figure 1.7: The mechanism of oviduct fluid secretion (Leese et al., 2001a). The mechanism of oviduct fluid secretion is described in detail in the section above (section 1.3.3.2)

1.4 The role of the oviduct in reproduction

1.4.1 Oviduct: The dynamic microenvironment

Although the reproductive function of the ovary and the uterus have studied extensively, the function of the oviduct in reproductive process is less well understood. The oviduct has been considered as a “pipeline” in which first the gametes and then embryo transit take place (Ménézo, et. al 2014). However, accumulated evidence over the last decades suggests that the oviduct interacts with gametes and embryos in a physical and molecular level and plays a role in several processes such as oocyte and sperm maturation, capacitation, sperm selection and preimplantation development by providing biochemical and biophysical support which is vital for a successful fertilisation outcome (Hunter, 1998; Wang *et al.*, 2004; López-Cardona *et al.*, 2017). In terms of the environment within the oviduct, a wide range of alterations in the proteome have been correlated with the presence of male and female gametes (Georgiou et al., 2005). It is possible that some of the proteins secreted in the oviduct contribute to sperm viability, while others, play a role in preparing the oviductal microenvironment for the forthcoming embryo (Holt and Fazeli, 2016). For instance, the presence of sperm in the human or mouse oviduct triggers the expression of adrenomedullin (Adm) and prostaglandin

endoperoxidase synthase 2 (Ptgs2), which have been implicated in ciliary motility and smooth muscle systole/relaxation (Li *et al.*, 2010). Furthermore, antioxidant proteins have been recognized in mammals' oviduct (cow and sheep) indicating its role in protecting gametes and early-stage embryos from oxidative stress produced by free radicals (Lamy *et al.*, 2016; Soleilhavoup *et al.*, 2016). However, this protection must be regulated in a way to facilitate and not prevent ROS-dependent processes, such as the acrosome reaction. The importance of the oviduct's role in reproduction is underlined by several studies which conclude that the quality of embryos which are produced *in vivo* in several mammals exceed those cultured *in vitro* in terms of physiological, genetical characteristics and the proportion of pregnancy after transfer (Fair *et al.*, 2001; Lazzari *et al.*, 2002; Rizos *et al.*, 2002, 2007; Tesfaye *et al.*, 2007).

As mentioned above, the procedures that occur in the oviduct are reliant on activities of steroid-regulated ciliated and secretory epithelial cell (Buhi, 2002). For this reason, the secretions of the epithelial cells can be considered essential moderator of the oviductal microenvironment which ensure gamete and zygote well-being, and preimplantation embryo development (Winuthayanon *et al.*, 2015).

1.4.2 Sperm-oviduct interactions

The sperm is deposited in different section of female reproductive tract depending on the species. However, the sperm must traverse the reproductive tract and enter the oviduct in order to encounter the oocyte (Coy *et al.*, 2012). In the majority of mammals, even if millions of spermatozoa are deposited in the vagina, only thousands of them manage to reach the isthmus and tens or hundreds reach the ampulla (Williams *et al.*, 1993). In addition there are subpopulation of sperm of different quality in every ejaculate (Ramon *et al.* 2014) so the superior quality fraction is selected through the oviductal transit (Holt and Van Look, 2004). During the transit in the female reproductive system, the spermatozoa must overcome a range of physical and anatomical barriers such as vaginal pH, cervical mucus resistance, the narrow utero-tubal junction and maternal immune response, all which constitute a selection mechanism for spermatozoa (Suarez and Pacey, 2006; Sakkas *et al.*, 2015).

In a study of mouse sperm, it was observed that the DNA fragmentation level of spermatozoa harvested from the uterus was higher compared to this in the vagina (Hourcade *et al.*, 2010)

This clue suggests that the lower reproductive tract is considered as the first obstacle for the sperm. Due to aggressive microenvironmental conditions (pH, viscosity and the immune response) extensive damage to the majority of the spermatozoa occurs during the transit from the vagina to the uterus (Hourcade *et al.*, 2010). Nevertheless, the same experiment detected that the spermatozoa which have high-quality genetic content are concentrated in the oviduct, so, it seems that the final selection of spermatozoa appears in the oviduct (Hourcade *et al.*, 2010).

A second anatomical barrier for sperm is the uterotubal junction because spermatozoa are impeded by the high mucosal folds (reviewed by Suarez and Pacey, 2006; Miller, 2024). In a study by Tokuhiro *et al.* on genetically modified mice it was shown that the first specific selection takes place in this region because the spermatozoa should possess certain specific proteins in their head plasma membrane to pass through this junction (Tokuhiro *et al.*, 2012). Sperm analysis studies of male knock-out mice for 8 different genes (such as those which encode the ADAMS proteins, angiotensin-converting enzyme (ACE), calreticulin3 (Carl3) and calmegin (Clgn) and protein-tyrosine sulfotransferase 2 (TPST2)) have shown problems in uterotubal junction transport (reviewed by Coy *et al.*, 2012)). It has been suggested that the function of these protein gives the sperm the ability to move forward to the oviduct. The common feature of these studies was the absence of ADAM protein, for this reason, this protein is considered as the central molecule of the uterotubal sperm transport and the additional proteins participate to its regulation (Coy *et al.*, 2012; Pérez-Cerezales *et al.*, 2018). This hypothesis is reinforced by the fact that ADAMS are able to bind to CD9 protein of the epithelial cells creating a protein-protein interaction, which could be a mechanism of sperm selection (Chen *et al.*, 1999).

Once the spermatozoa pass through the UTJ, they enter to the oviduct, where they bind the ciliated epithelial cells of isthmus. This location is also known as sperm reservoir. Epithelial cells and sperm are endowed with ligands and receptors respectively, which assist the development of carbohydrate-protein interactions in order for the binding to be established (Töpfer-Petersen *et al.*, 2002). The carbohydrates that are exposed on the membrane of oviduct cell interacts with the lectin-like proteins of the membrane of sperm head (Suarez, 2008). In this binding process, the molecules which participate differ among species in terms of the key sugar signals and the complementary sperm receptor molecules (Töpfer-Petersen *et al.*, 2002). Evidence in support of this is the observation that the key sugar signal are fucose residues and

recognized by spermadhesin BSP, in cattle (Ignotz *et al.*, 2001; Gwathmey, Ignotz and Suarez, 2003; Sostaric *et al.*, 2008). The formation of a functional sperm reservoir has been reported in several species of mammals including hamsters (Demott and Suarez, 1992), rabbits (Harper, 1973; Overstreet and Cooper, 1978), horses (Dobrinski *et al.*, 1996), sheep (Hunter and Nichol, 1983), pigs (Ekhlasi-Hundrieser *et al.*, 2005), lamas (camelid) (Apichela *et al.*, 2010). The biological explanation for the sperm reservoir remains elusive but it may contribute to the preservation of sperm fertility during storage and the timed release of a small quantity of spermatozoa during the periovulatory period in order to decrease the possibility of polyspermy (Hunter and Léglise, 1971; Suarez, 2008). Although the mechanism of sperm-release is not well understood, there is strong evidence to indicate that this procedure is correlated with changes in sperm during capacitation, (Smith and Yanagimachi, 1991; Suarez, 2008) the process through which the spermatozoa acquire the final maturation status and become able to fertilise (Austin CR, 1951; Chang, 1951). The sperm are released from epithelial cells either after they lose the binding proteins or because of the hyperactivation pattern- acquired at the capacitation- by escaping the attachment due to shear force (Demott and Suarez, 1992; Pacey *et al.*, 1995).

Spermatozoa are released after the capacitation from sperm reservoir because they acquire the hyperactivation motility pattern and finally move towards the ampulla in order to find the oocyte and fertilisation will occur. However it is still unclear how the spermatozoa are able to ‘‘know’’ the right direction to swim and how they recognise the oocyte.

It has been suggested that spermatozoa have a ‘‘navigation system’’. Based on in vitro studies three ‘‘navigation’’ mechanisms have been proposed; rheotaxis, thermotaxis, and chemotaxis. Rheotaxis is a passive physical process (Zhang *et al.*, 2016) in spermatozoa which are able to swim against a fluid flow due to the hydrodynamics of motile spermatozoa (Miki and Clapham, 2013; Zhang *et al.*, 2016). Thermotaxis has been defined as the procedure in which the sperm navigate according to a temperature gradient, from colder to warmer (Eisenbach and Giojalas, 2006). This has been observed in rabbits, mice (Pérez-Cerezales *et al.*, 2015) and human (Bahat *et al.*, 2003). Chemotaxis describes a mechanism by which the spermatozoa swim along a concentration gradient of a chemoattractant (Chang and Suarez, 2010). This has been reported in rabbits (Fabro *et al.*, 2002), mice (Oliveira *et al.*, 1999) and humans (Ralt *et al.*, 1994) Both thermotaxis and chemotaxis are active mechanisms that only capacitated

spermatozoa can undertake (Eisenbach, 1999; Bahat and Eisenbach, 2006). For this reason, these two processes could be considered as mechanisms for selecting capacitated spermatozoa.

However, the exact process through which the sperm acquire the ability to react to these stimuli is poorly understood. It has been suggested that the reorganization of plasma membrane that occurs during capacitation is related to the subsequent thermo- and chemo- stimuli. According to this hypothesis, initially the capacitated spermatozoa released from sperm reservoir respond to temperature stimuli of thermotaxis and they are guided through the relatively long distance of the oviduct towards the ampulla (Eisenbach and Giojalas, 2006). Temperature changes provoke the increase of the sperm intracellular Ca^{2+} and subsequently affects flagellar bending (Bahat and Eisenbach, 2010). As soon as this subpopulation of sperm reach the area of fertilization, only those which can respond to chemoattractants will interact with the cumulus oocyte complex (COC) (Eisenbach and Giojalas, 2006). Potential chemoattractants include progesterone (P4) produced by the COC during the ovulation (Chang and Suarez, 2010) and natriuretic peptide precursor A (NPPA) (Bian *et al.*, 2012). Both of these substances are related to hyperactivation by modifying the sperm pattern motility and enhancing $[\text{Ca}^{2+}]_i$ levels (Yoshida and Yoshida, 2011; Bian *et al.*, 2012). After reaching the oocytes at the fertilisation site, the sperm cross the cumulus cells of oocyte. Sperm assisted by chemotaxis reach the ampulla as well as pass through cumulus cells' layers towards to egg (Teves *et al.*, 2006). It has been described that some hexosaminidases in oviductal fluid and SPAM1, an enzyme with hyaluronidase activity secreted by the oviduct, could play a role in cumulus cell disaggregation helping spermatozoa to exceed this obstacle (Carrasco *et al.*, 2008; Griffiths *et al.*, 2008).

The cells and the secretions of the oviduct combine to form the microenvironment for gamete protection, sperm maturation (capacitation and hyperactivation) and sperm guidance before fertilisation occurs and have been also discussed in recently published reviews (Soto-Heras *et al.*, 2023; Miller, 2024)

1.4.3 Oocyte- oviduct interactions

Through digital video microscopy of the bovine oviduct, it has been shown that the mature COCs released from the ovary travel to the site of fertilisation through a combination of cilia beating and the contractions of smooth (Kölle, Reese and Kummer, 2010). The oviduct

responds to the oocyte's arrival at the site of fertilisation by releasing the sperm from the sperm reservoir. Besides the fact that previous studies have shown that zona pellucida (ZP) is altered during folliculogenesis in numerous species (Oehninger *et al.*, 1991; Avilés *et al.*, 2000), more recent approaches have suggested that the ZP is reformed further after ovulation during its journey in the oviduct (Funahashi *et al.*, 2001; Gonçalves *et al.*, 2008). During the cumulus expansion and disaggregation, the oocyte becomes more receptive to oviductal fluid, facilitating alterations of the ZP. Furthermore, variances in extracellular oocyte coat among oviductal and follicular oocytes were referred too (Kolbe and Holtz, 2005; Lyng and Shur, 2007). It is speculated that the OVGPI and heparin like glucosaminoglycans (GAGs) secreted in the oviductal fluid contribute in ZP resistance to enzymatic digestion and polyspermy prevention (Coy *et al.*, 2008).

1.4.4 Oviduct as co-factor in embryo development

After fertilisation, the epithelial cells that line the oviductal lumen assist the early stage of embryo development producing the components needed for the embryo cleavage. Gandolfi *et al.* in 1987 first observed that the existence of the epithelial cells and no other kind of somatic cells improve embryo development in sheep models (Gandolfi and Moor, 1987). These findings were subsequently confirmed in pig (White *et al.*, 1989), cattle (Eyestone and First, 1989), mouse (Sakkas and Trounson, 1990), and human (Yeung *et al.*, 1992).

The transit of the embryo from the oviduct takes between 3-5 days depending on the species. Despite the fact that maternal recognition of the pregnancy take place in the uterus, several studies have shown that pre-implantation embryos might secrete "signals" recognised by the oviduct which responded differently according to the embryo presence or not. This claim is supported by studies in several species that identify that the unfertilised eggs either remain in the oviduct or transit to the uterus slower compared with the embryos (Van Niekerk and Gerneke, 1966; Ortiz, Lladós and Croxatto, 1989; Weber *et al.*, 1991). The oviductal response to embryos' presence is also confirmed by transcriptomic studies which have identified differences in genes expression between ampulla and isthmus during the period the embryo encounters these environments (Maillo *et al.*, 2016).

The oviductal fluid, created by the actions of epithelial cells of the oviduct, provides the nutrients to support the very early stages of embryo cleavage. At these developmental stages, oxidative metabolism is used by pre-implantation embryos for energy production, and pyruvate and lactate provided by the oviductal fluid serve as the main energy supply (Gardner and Leese, 1990; Dumollard, Duchen and Carroll, 2007; Absalón-Medina, Butler and Gilbert, 2014). As embryos develop, their metabolism switches from oxidative to glycolytic at the blastocyst stage, although the majority of ATP is synthesised through oxidative metabolism (Smith & Sturmey, 2013; Malkowska et al., 2022). Glycogen, which is an additional source of glucose is present in the ampulla and isthmic epithelial cells in primates models (Odor, Gaddum-Rosse and Rumery, 1983; Schultka and Cech, 1989). Furthermore, enzyme amylase AMY2B, which converts glycogen to glucose, is produced within oviductal epithelial cells offering a route by which glycogen-derived glucose may be used in oviductal and embryonic metabolism. During the last stage of preimplantation development, the mitochondria have been fully developed and generate their own energy by using oxygen and glucose (Sathananthan and Trounson, 2000).

In addition, embryotropic growth factors are released by oviduct epithelia and thus support cleavage and growth of embryo. Growth factors including epidermal growth factor (EGF) (Adachi *et al.*, 1995), fibroblast growth factor (FGF) insulin-like growth factor (IGF) (Pfeifer and Chegini, 1994), and transforming growth factor (TGF) (Chegini *et al.*, 1994) have been identified in the oviduct. The inhibition of growth factors such as TGF and EGF in co-culture of embryos with epithelial cells resulted in decrease of the blastocyst rate (Daliri *et al.*, 1999).

Oviductal fluid has also been proposed to contribute to prevention of oxidative stress and adverse immune response. Non-enzymatic and enzymatic antioxidant compounds participate in the protection against oxidative stress (Guérin, El Mouatassim and Ménéz, 2001). The major non-enzymatic antioxidants which have been found in the oviduct secretions of several model species including mouse, cow, sheep and rabbit, include glutathione (GSH), taurine, hypotaurine and cysteamine (CSH) (Guérin and Ménéz, 1995; Gardiner *et al.*, 1998). The main enzymes involved in embryo protection are catalase, superoxide dismutases (SOD1/2) and glutathione peroxidase (GPX4) (Guérin, El Mouatassim and Ménéz, 2001). Catalase, SOD1 and SOD2 are present in the oviduct fluid of mouse, cattle, and human and act by binding on ROS or ROS by-products (Harvey *et al.*, 1995; El Mouatassim, Guérin and Ménéz, 2000; Jang *et al.*, 2010). GPX4 is known for its function against hydrogen peroxide (Agbor *et al.*, 2014). Furthermore, the concentration of oxygen into the mammalian oviduct is considerably

lower than that of the atmosphere (Fischer and Bavister), thought to minimise ROS level and subsequently offer embryo protection (Catt and Henman, 2000).

As far as the maternal immune response to the presence of embryo is concerned, studies in pig, horse, and cattle oviducts have shown slight differences in activity of immune function (Almiñana *et al.*, 2012; Maillo *et al.*, 2015; Smits *et al.*, 2016). Given the semiallogenic nature of the embryo, this reduced reaction of the maternal immune system is expected otherwise the embryo could be rejected. However, it has been suggested that the embryos are able to avoid immunological rejection because of the expressed paternal antigens (Billington, 2003).

We have analysed so far how the environment of the oviduct contributes during the first stage of embryo development by providing it with the nutrients and protecting it from oxidative stress. However, embryo development and transport occur simultaneously, is essential to describe the embryo transport and how the oviduct participate in this process.

1.4.5 Embryo transit through the oviduct

Embryo transport along the oviduct is accomplished by a combination of ciliary beating, smooth muscle contraction, and flow of tubal secretions (Croxatto, 2002). Furthermore, it seems that the transport speed is different between fertilised embryos and unfertilised eggs. In hamsters, when the oocytes remained unfertilised, they were transported one day later compared to those that were fertilised (Ortiz *et al.*, 1986). Furthermore, it seems that advanced embryos can influence their transit to the uterus, according to a study in rats. More specifically, 1-cell or 4-cell in vitro produced embryos were transferred to the recipient rats's oviduct on Day 1 of pregnancy. After evaluation of the embryo's location on Day 4, the 1-cell group remained in the oviduct whilst the 4-cell embryos had already reached the blastocyst stage and transit to the uterus (Ortiz, *et al.*, 1989). However, in mares the unfertilised eggs were retained in the oviduct and only the embryo which secreted prostaglandin E2 was transferred to the uterus (Weber *et al.*, 1991).

By using video-microscopy in a cattle model, the mechanism of transport of the fertilised embryo was shown in the ampulla to be ciliary-driven transport taking place among the folds, whereas in the isthmus transport occurred along apical ridges of the folds (Kölle *et al.*, 2009).

As mentioned above, embryo transport is composed of ciliated epithelial cells and tubal muscle contraction. The beating of the ciliated cells produces the flow of oviductal fluid which promotes the transport of the embryo. Besides of ciliary beating, smooth muscle contractions ensure embryo transport in the oviduct. Oviductal smooth muscle is characterized by episodic and tonic contraction. The episodic contractions are frequent yet brief and promote the embryo transition in the ampulla, whereas the tonic are sustained contractions which happen in the ampullar-isthmus junction as well as the uterus-tubal junction UTJ (Croxatto, 2002). Estrogen (E2) and progesterone (P4) are key factors for the transport of embryo because they regulate the ciliary beating and the tubal muscles contraction. E2 generally increases the ciliary beat frequency, tubal fluid secretion, and tubal muscle contraction whereas P4 causes muscle relaxation and decreases ciliary beat frequency (Orihuela *et al.*, 2006; Bylander *et al.*, 2010;). Finally, it seems that cannabinoid receptors CNR1 and CNR2 play an important role in embryo transport because studies in double-knockout mice have shown that the majority of embryos retained in the oviduct compared with those of the control group (Wang *et al.*, 2004).

To sum up, during the development and transport of embryos, the oviduct responds to the secreted embryos' signals by altering its environment in order to provide the necessary nutrients, protection against oxidative stress and the maternal immunological response as well as facilitating embryo transfer to the uterus. In other words, we could suggest that the oviduct assists the initial stage of embryo cleavage during transit (Hess *et al.*, 2013)

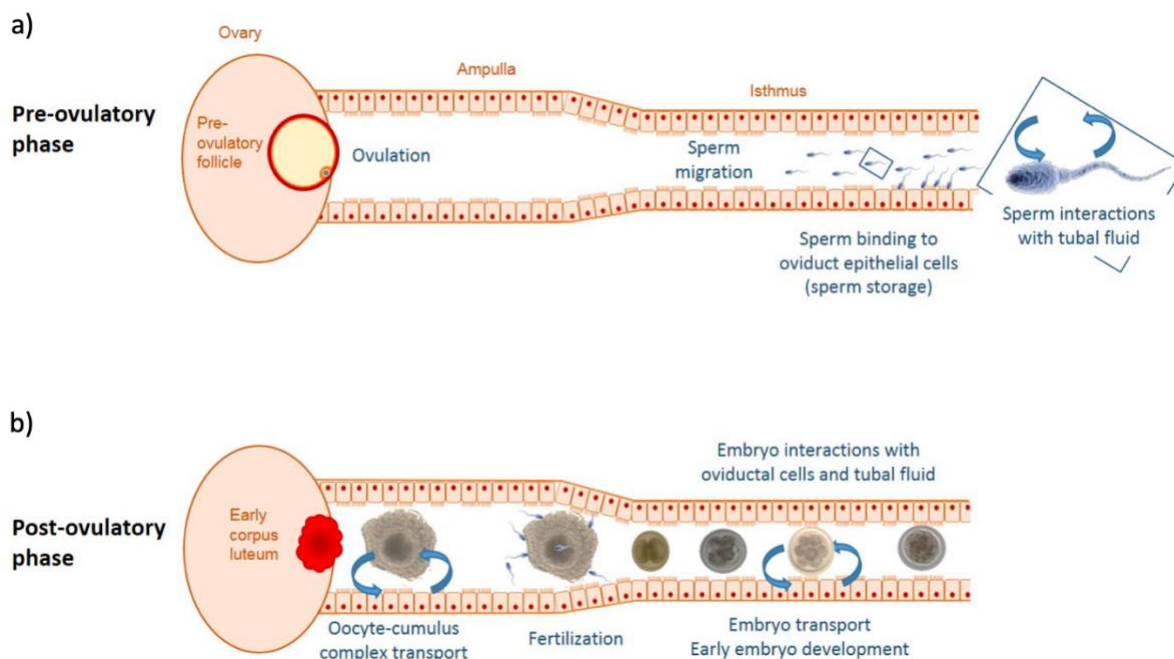


Figure 1.8: Schematic presentation of oviduct interactions with sperm, oocyte, and embryo (Saint-Dizier et al., 2019). a) at the pre-ovulatory phase, the sperm reach the oviduct through the uterus and binds on the epithelial cells until ovulation. b) after ovulation the oocyte meets the sperm in the ampullary-isthmic junction and the generated embryo develops as it transits through the oviduct. The oviduct provides the appropriate environment, (nutrients, structural support, and paracrine signalling) to assist the early reproductive events.

1.5 Emerging environmental contaminants

1.5.1 Endocrine Disrupting Chemicals

Endocrine Disrupting Chemicals (EDCs) are a class of chemicals defined by the Endocrine Society as “an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action”. EDCs includes a wide range of substances such as industrial solvents, pharmaceutical agents, pesticides, plasticizers, phytoestrogens and metals with over than 1000 manufactured chemicals showing endocrine-disrupting activity.

The majority of EDCs have lipophilic properties thus they tend to accumulate in the adipose tissues resulting on an extended half-life in the human organism. Interference with the synthesis and action of steroid hormones as well as with hypothalamo-pituitary-thyroid axis has been proposed as the mechanism by which EDCs elicit their effects. More specifically, EDCs’ estrogenic, anti-estrogenic, androgenic, and anti-androgenic activity has been confirmed in in

vivo and in vitro studies in different animal models. The main route for EDCs exposure in humans seems to be primarily through ingestion, and secondly through inhalation and dermal absorbance (Siracusa et al., 2018). Critically, EDCs have been correlated with developmental and reproductive toxicity such as infertility, reproductive organs abnormalities and cancer, puberty alterations and changes in male to female rates in births (reviewed by Ghosh *et al.*, 2022). Finally, there are also reports of causing metabolic disorders, alterations on insulin activity, obesity and diabetes type II (reviewed by (Le Magueresse-Battistoni et al., 2017).

All these reports and findings synthesise the picture of EDCs as a class of chemicals that have a great impact on human health. Among the different EDCs one of the most well studied in terms of impacts on human health is Bisphenol A. Its action has been correlated with a number of human health problems including obesity, diabetes, and cardiovascular diseases (Gramec Skledar & Peterlin Mašič, 2016; Paterni et al., 2017). After the evidence that BPA exposure can have adverse effects on human health, this chemical was replaced with structural analogs (BPS, BPF, BPAF, BPB). Bisphenol S (BPS) is one of the major substitutes of BPA and initially was introduced as the “safe substitute” (Wu et al., 2018). BPS is now widely used for products that general population use daily (Liao & Kannan, 2014a), yet despite this, it has been reported as a potential EDCs (Rochester et al., 2017). Additionally, Triclosan (TCS) is an antimicrobial agent used in personal care products (Rodricks et al., 2010; Dann and Hontela, 2011; Rochester and Bolden, 2015; Ye et al., 2015). TCS is rapidly absorbed, metabolised and distributed in the tissues. In vitro and in vivo studies have also identified that Triclosan is able to disrupt the endocrine function of organism (Wang & Tian, 2015). However, the results of its action as reproductive toxicant are limited and, in some cases, conflicting. Bisphenol A, Bisphenol S and Triclosan will be discussed further in the following sections of this chapter as are the EDC that have been chosen to be investigated in this thesis.

1.5.1.1 Bisphenols

Bisphenols represent a well-known class of chemicals which are used widely in the production of plastics and resins (Paterni et al., 2017). As a consequence of their use, large quantities of bisphenols have accumulated in the environment and they have been identified in soil and sediments, water (surface water, drinking water), and indoor dust (Vandenberg et al., 2007; Liao, Liu, Guo, et al., 2012; Wan et al., 2018). Furthermore, they have been detected in a range of human fluids and tissues, including serum, urine, breast milk and umbilical cord serum

(Ikezuki et al., 2002; Liao, Liu, Alomirah, et al., 2012; Liu et al., 2017; Mercogliano & Santonicola, 2018).

Their chemical structure is composed of two phenol rings combined with a bridging atom (Schmidt & Mašič, 2012) and they have functional homology to the steroid hormone 17 β -estradiol (E2) (fig 1.9). Indeed, bisphenols are characterised as *xenoestrogens* and potential endocrine disrupting chemicals (EDCs) because they could “interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the development, behaviour, fertility and maintenance of homeostasis”(Crisp et al., 1998).

1.5.1.2 Bisphenol A: Occurrence in the environment and Human exposure

Bisphenol A (BPA) is an organic compound which has been used as monomer for the manufacture of polyesters and polyacrylates and more specifically in epoxy resins and polycarbonate plastics production. Structurally, it is comprised of two methyl groups and two phenol rings which all are attached on a carbon bridge (Murata & Kang, 2018). (fig 1.9).

Concerns about the impact of environmental BPA on human health have arisen because it has been identified in the surface and drinking water, in soil, indoor dust and in food. Furthermore, in humans, a range of BPA concentrations have been identified in urine of adults and children as well as in maternal serum (Corbel et al., 2014). Beyond ambient exposure, there are growing concerns about dietary exposure to BPA (Vandenberg et al., 2007), since it has been identified in food samples at a mean concentration of 4.38 ng/g. In this research the tested food samples were divided into 9 groups: beverages, dairy products, fats and oils, fish and seafood, cereals, meat and meat products, fruits, vegetables, and “others” (Liao & Kannan, 2013). The highest concentrations of Bisphenols (730 ng/g) was observed in samples of canned foods (Noonan et al., 2011; Liao & Kannan, 2013; Fang et al., 2014). The estimated daily dietary intake of BPA varies from >1 μ g/kg body weight/day to 5 μ g/kg body weight/ day (Murata & Kang, 2018). The health effects of such exposures remain unclear. However, there is growing evidence that in pregnancy, the developing fetus is exposed to BPA through placental transport (Troisi et al., 2014; Strakovsky & Schantz, 2018). Studies on pregnant sheep have shown that 4.5% of a BPA dose administrated to the female is received by the fetus at the late stage of pregnancy (Corbel

et al., 2013). Startlingly, exposure continues postnatally through breast milk (Niu et al., 2017; Mercogliano & Santonicola, 2018).

BPA is metabolized rapidly in the liver to two principal metabolites in-vivo; BPA-glucuronide and BPA-sulfate. These represent the major and the minor metabolic pathway, respectively (Gramec Skledar & Peterlin Mašič, 2016). In a worrisome study of matched maternal and cord serum in humans, the total amount of these BPA-metabolites was significantly elevated in the cord serum. One interpretation of these data is that BPA clearance is slower in cord serum than maternal serum which could lead to higher dose exposure during perinatal development (Liu et al., 2017). This result has also been confirmed in study conducted in pregnant rhesus monkeys (vom Saal et al., 2014).

BPA is classified as a xenoestrogen and an EDC, and most studies have focussed on this activity. This organic compound is able to bind both of the Estrogenic Receptors alpha and beta however its affinity is remarkably lower than 17β -oestradiol. Furthermore, BPA can interfere with androgen, glucocorticoid and thyroid receptors. However, no estrogenic activity (Elsby et al., 2001) has been reported for BPA-metabolites (BPA- glucuronide and BPA-sulphate). However, these results do not ensure that the BPA metabolites could not have any other impact on biological function. For instance, it has been shown that BPA- glucuronide influences adipogenesis (Boucher et al., 2015).

Importantly, BPA exposure has been correlated to numerous health issues and diseases such as diabetes, cardiovascular diseases, obesity, and infertility. Several studies have identified that BPA levels were higher in serum to infertile compared to those of fertile women (Caserta et al., 2013; La Rocca et al., 2014). Additionally, the BPA concentration in serum of healthy women was significantly lower compared to women with polycystic ovary syndrome (PCOS) (Takeuchi & Tsutsumi, 2002) and in women with unexplained miscarriages (Zheng et al., 2012). In both studies, the BPA concentration in serum in “unhealthy” groups was double compared to the healthy groups. Additionally, exposure to BPA has been linked with abnormal reproductive function in both males and females, including effects on spermiogenesis and sperm quality, oocyte maturation as well as effects on fetal health through exposure of placenta (Murata & Kang, 2018).

1.5.1.3 Bisphenol S: Occurrence in the environment and Human exposure

After the BPA usage restriction on baby bottles at Canada and EU in 2010 and 2011 respectively, chemicals with similar chemical structure were used as substitutes. One of the major BPA substitutes is Bisphenol S (BPS) (Wu et al., 2018), which is used mainly as the monomer in synthetic polymers, epoxy resins and paper products manufacture, especially in thermal paper products (Liao, Liu, Guo, et al., 2012; Liao, Liu & Kannan, 2012; Pivnenko et al., 2015). BPS has also been identified in daily used products such as cans, baby bottles, toys, and personal care products (Liao & Kannan, 2014a).

BPS differs in structure to BPA (Fig 1) by virtue of the presence of a sulfone group instead of a carbon group bond between the two phenol rings which give higher stability. Furthermore, BPS is more resistant to heat, sunlight and biodegradability than this of BPA and for this reason was first considered as “a safe BPA’s substitute (Ike et al., 2006).

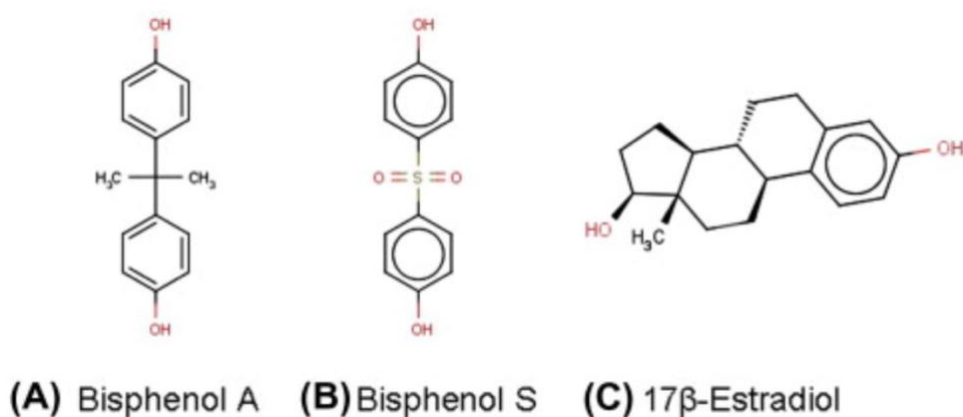


Figure 1.9: Chemical structure of a) Bisphenol A, b) Bisphenol S, and c) 17β- Estradiol (Grignard et al., 2012)

In recent years, studies conducted mainly in Asian countries (Korea, Japan and China) and U.S have reported that BPS is persisting in the environment including in surface water, liquid waste (Wan et al., 2018) and indoor dust (Liao, Liu, Guo, et al., 2012). Furthermore, BPS has been identified in a wide range of foodstuffs including vegetables, fruits, and meat, the detection frequency in the tested food samples were 21% and 23% in US (Liao & Kannan, 2013) and China (Liao & Kannan, 2014b) respectively. Yang et al (2014) concluded that canned food

contains higher concentration of bisphenols compared to the same categories of food which were packed in different kinds of packaging material (Yang et al., 2014) however the concentration of BPS was lower than the other bisphenols such as BPA and BPF.

These results indicate that BPS is now abundant in the environment in a way that will increase the likelihood of regular human exposure. Such exposures may include inhalation, dermal absorption through the usage of the personal care products and ingestion via diet and household dust. Similar to BPA dominant exposure route, dietary ingestion seems to be the dominant way of exposure to BPS (Gramec Skledar & Peterlin Mašič, 2016).

BPS has been confirmed in a range of biological fluids in humans. Initially, BPS was identified in urine samples from male and female adults (Liao et al 2012) with geometric mean 0.17 ng/ml (Liao, Liu, Alomirah, et al., 2012). In a later study in adults with no recorded occupational exposure, BPS was detected in 78% of urine samples studied with a mean concentration 0.13 ng/ml (Zhou et al., 2014). Furthermore, BPS exposure appears to be increasing over time: BPS was detected in urine samples collected after 2009 but was not detectable in urine samples between 2000 and 2007 (Ye et al., 2015). More recently, BPS has been identified in blood serum (Wan et al., 2018), breast milk (Dualde et al., 2019; Tuzimski et al., 2019) as well as umbilical cord serum (Liu et al., 2017). Importantly, the concentrations of BPS reported in these body fluids is much lower than those in urine sample. However, the fact that BPS has been detected in breast milk and cord serum provide the concerning evidence that human is exposed to BPS from the early stages of development.

Despite reports of the presence of BPS in physiological fluids, few studies have evaluated the metabolism of BPS. By 2016, only 4 studies (1 in-silico and 3 in-vitro) had been conducted. In these studies, 3 different metabolites of BPS were identified; BPS glucuronide (Gramec Skledar et al., 2015; Le Fol et al., 2015; Gramec Skledar & Peterlin Mašič, 2016; Skledar et al., 2016), BPS sulfate (Le Fol et al., 2015) and ortho-hydroxylated BPS (Skledar et al., 2016). All in vitro studies confirmed that the BPS glucuronide is the major metabolite, and its metabolism take place primarily in the liver. In 2018, Gys et al, used liquid chromatography coupled to high-resolution quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) to confirm the existence of these three metabolites. Furthermore, they detected three additional metabolites: dihydroxy-BPS, two isomers of hydroxy-BPS-glucuronide and two isomers of hydroxy-BPS-sulfate (Gys et al., 2018).

Given that the structure of BPS closely resembles BPA, it has been also characterized as potential endocrine disrupting chemical. Many studies have been conducted in order to identify the endocrine activities of BPS. A systematic review published in 2015, which include in vivo and in vitro studies, concluded that the mechanism of action of BPS is similar to BPA and it also has endocrine-disrupting effects (Rochester & Bolden, 2015). More specifically, BPS is able to bind to both ERs with greater chemical affinity to ER β (Molina-Molina et al., 2013). However, there are only two in vitro studies which examine the endocrine disrupting action of BPS's metabolites (Kang et al., 2014; Skledar et al., 2016). The results of these two studies are conflicting, Skledar et al., 2016 identify a weak estrogenic activity of ortho-hydroxylated BPS and no endocrine disrupting activities of BPS glucuronide whereas Kang et al., 2014 detect estrogenic activity of BPS glucuronide. Clearly additional research is required.

1.5.1.4 Triclosan: Occurrence in the environment and Human exposure

Triclosan (TCS) is a broad-spectrum agent with antimicrobial activity which is used as ingredient in routinely used products, including soaps, healthcare antiseptic (mouthwash, toothpaste), personal hygiene products (acne cream, skin cream, sunscreens and deodorant) and medical products (surgical sutures, catheters, ureteral stents) (Rodricks et al., 2010; Dann & Hontela, 2011; Ye et al., 2011). Initially, exposure to TCS was underestimated and was considered insignificant importance. However, due to the extensive use on commercial products combined with studies shown the accumulation of TCS in aquatic and terrestrial environment and bioaccumulation in aquatic biota (Houtman et al., 2004; Chalew & Halden, 2009; Fair et al., 2009) concerns have been raised about the impact on the human health and the environment (Olaniyan et al., 2016)

Potential routes of exposure to TCS for organism include ingestion and dermal absorption (Moss et al., 2000; Rodricks et al., 2010; Ye et al., 2011; Fang et al., 2014), mucosal absorption has also been reported (Bagley & Lin, 2000; Lin, 2000). It is estimated that the dermal absorption is between 3% and 7% of the total dose (Moss et al., 2000; Queckenberg et al., 2010). In a study in which Triclosan absorption on rat skin in vivo was compared with rat and human skin in vitro, the penetration of triclosan in vitro through rat skin was approximately 5 times faster than through human skin (Moss et al., 2000). In another study in which mouthwash

contained 0.03% TCS was used, the retained percentage of TCS was 7.33% of the total dose which participants were exposed (Lin, 2000). However, this percentage of TCS absorption in gastrointestinal tract increased when the individuals swallowed the mouthwash. (Bagley & Lin, 2000). Finally, in a study where the subjects applied cream that contained 2% Triclosan, the absorption rate did not exceed 10% (Queckenberg et al., 2010).

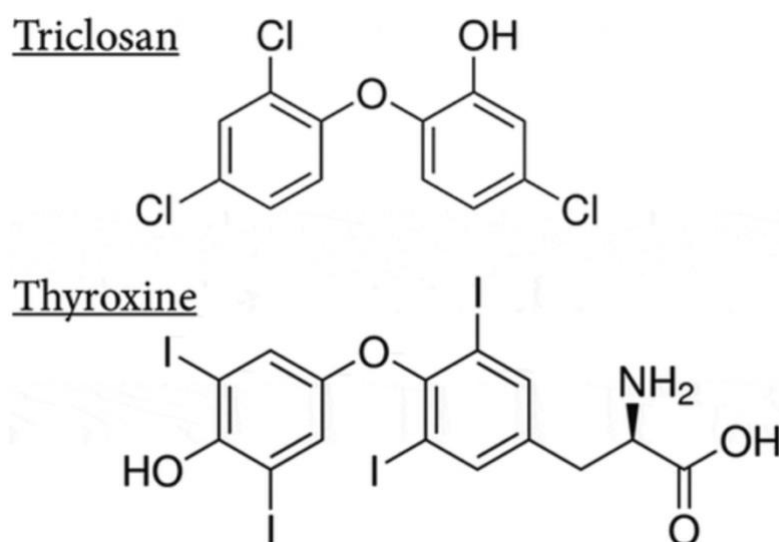


Figure 1.10: Chemical structure of Triclosan and Thyroxine (T4) (Ruszkiewicz et al., 2017)

TCS has been detected in the urine of cohorts in the USA (Calafat *et al.*, 2008) India (Xue *et al.*, 2015) and Spain (Calafat *et al.*, 2008; Xue *et al.*, 2015; Azzouz *et al.*, 2016). The concentrations of TCS in urine varies widely (8.3–13.09 nM) (Calafat *et al.*, 2008). Moreover, in a study where 1,890 first-trimester urine samples of pregnant women were tested, free TCS and TCS-metabolites were detected in 80% and 99% of the urine samples studied. The results of this study also identified a positive correlation between urinary TCS concentration, and age and socioeconomic status of the participants (Arbuckle *et al.*, 2015). Furthermore, TCS has been identified in serum (4.1–41.4 nM) (Azzouz *et al.*, 2016), plasma (0.035–1200 nM) (Allmyr *et al.*, 2008) and breast milk in a wide range of concentration (0.062–252 nM) (Adolfsson-Erici *et al.*, 2002; Dayan, 2007; Allmyr *et al.*, 2008; Azzouz *et al.*, 2016). In addition, TCS has been detected in a number of human tissues, including adipose tissue, brain, liver, (Geens *et al.*, 2012) as well as toe and finger nails (Yin *et al.*, 2016). The fact that TCS has been detected in the brain tissue indicates the fact that this compound is able to cross the

blood-brain barrier. These data suggest that TCS is distributed in human tissues and bio-accumulated in the organism.

It appears that after absorption, TCS is metabolised rapidly in the human. TCS-glucuronide and TCS- sulphate has been detected in urine and feces (Black et al., 1975; Moss et al., 2000; Fang et al., 2014). Some studies using animal models concluded that the main TCS metabolites is TCS- glucuronide (Black et al., 1975; Moss et al., 2000). However, in an in vitro study where human liver microsomes were exposed to environmentally relevant concentrations of TCS, metabolites were produced by the same rates (Wang et al., 2004). Pharmacokinetic studies in human suggest that TCS maximum concentration in plasma is demonstrated after 1-3 hours of a single oral dose. It was also estimated that the TCS half-life in the plasma is 21 hours, and this is independent of the mode of administration. TCS was primarily excreted through the urine and the TCS excretion half-life was eleven hours after intake (Sandborgh-Englund et al., 2006). Despite the fact that there is evidence that TCS is metabolised rapidly after absorption, there are no studies regarding the effects of the primary TCS-metabolites on the physiological function of the human.

This kind of evidence led to TCS restriction from certain wash products in 2016 (FDA, 2016) and from products used in hospital in 2017 (FDA, 2017). However, the usage of Triclosan is still permitted in personal care products, cosmetics, clothes, and toys. For this reason, it is estimated that the daily TCS exposure of general population is still high.

1.5.2 Neonicotinoids

Neonicotinoids is a category of chemicals which have been used worldwide as pesticides in veterinary medicine and field crops production since the 1990s and are licenced to 120 countries until today. There are 7 main compounds that belong in this family, and these are: imidacloprid (IMI), thiamethoxam (TMX), clothianidin (CLO), thiacloprid (THI), dinotefuran (DIN), acetamiprid (ACE), nitenpyram (NIT). Their similar structure with nicotine, which can act as a natural insecticide, allows them to act agonistically on nicotinic acetylcholine receptor (nAChR) of insects, causing dysfunction on their nervous system by disturbing the initiation of the signal in the post-synaptic neuron. Their broad-spectrum action and their relatively high selectivity as insecticides initially considered as an epitome option for pest control with

minimum impact on environment and on non-target wildlife. However, in 2008 mass poisoning of bees, a non-target species, was observed after the use of neonicotinoid during cultivation of maize in Germany (Pistorius et al., 2009).

Since then, several studies have investigated their occurrence in the environment and their effects on non-target species. Neonicotinoids have been detected in environmental matrices including water, soil, dust, sewage, and have been identified by several studies in different countries over the years (Goulson, 2013; Anderson et al., 2015; Abdel-Ghany et al., 2016; Bradley et al., 2017; Sadaria et al., 2017; Salis et al., 2017; MacDonald et al., 2018; Montiel-León et al., 2018; Sultana et al., 2018; Zhou et al., 2018; Berheim et al., 2019). Various studies have shown that neonicotinoid remains in soil for long periods and that they accumulate in the ground after repeated use (Bonmatin et al., 2005; Hladik et al., 2017). A study conducted in the UK revealed that, after treating seeds with IMI, there was a 10-fold increase of the imidacloprid levels in the soil which remained for six years (Goulson, 2013).

Several *in vivo* and *in vitro* studies have been conducted investigating the impact of neonicotinoids in mammals confirming their toxicity on non-target species. Adverse effects on hepatic, neurological and reproductive system have been reported in rats and mice after the *in vivo* exposure to IMI or CLO (Toor et al., 2013; Hirano et al., 2015; Han et al., 2018). Furthermore, *in vitro* studies revealed that insulin resistance is induced as well as impairment of early reproductive events after IMI and ACE exposure (Gu et al., 2013; Kim et al., 2013).

Human exposure to neonicotinoids is primarily through food and water consumption. Neonicotinoids are systemic insecticides meaning that they are absorbed by the plants which make their removal harder (Zhou et al., 2020). Inhalation of pollen and dust has been considered as route of exposure to neonicotinoids as well (Zhou et al., 2020). Their occurrence in human fluids, such as urine, serum, blood, breast milk, saliva has been confirmed and is region and specific- population depended (Tao et al., 2019). For instance, the detected concentrations are higher in populations from Asian countries compared to US or EU countries (Zhang & Lu, 2022), furthermore specific populations, such as farmers or rural populations, shows higher concentrations of neonicotinoids compared to urban ones (Tao et al., 2019). A comprehensive review of the neonicotinoid's appearance in the human fluids and other biological matrices can be found in the study conducted by (Zhang & Lu, 2022).

Based on the fact that neonicotinoids are circulated and metabolised by the human body, concerns have been raised regarding the impact on human health. Positive correlation of the neonicotinoids levels in human body and adverse effects on human health are summarised in a systematic review published in 2017 (Cimino et al., 2017). Neonicotinoid exposure has been correlated with adverse effects on oxidative stress, neurological dysfunction, osteoporosis, and liver cancer (Marfo et al., 2015; Li et al., 2020; Zhang, Zhang, et al., 2022). Metabolic and endocrine disrupting properties of the neonicotinoid's metabolites have been also reported recently. It seems that neonicotinoids negatively associated with insulin and glucose metabolism. (Vuong *et al.*, 2022; Wang *et al.*, 2022). In a recent study conducted in US population including individuals aged > 6 years old, the levels of testosterone in serum were negatively correlated with the levels of the neonicotinoid's metabolites in urine irrespective of the gender (Mendy & Pinney, 2022). Finally, presence of neonicotinoids in urine samples of infants have been correlated with low birth weight (Ichikawa et al., 2019).

Based on the risk assessment of European Food Safety Authority (EFSA) (EC European Commission, 2013) the restriction of CLO, TMX, and IMI on bee-attractive crops, was implemented by the European Commission in 2013 (EC European Commission, 2013) and their total restriction of the outdoor usage along with the restriction of THI was implemented in 2016 (EFSA, 2016; EFSA et al., 2019). Nowadays, the neonicotinoids are restricted as plant protection products in the EU, however ACE is excluded of this restriction until 2033 (EC European Commission, 2018). For this reason, ACE was considered as target of the environmental compounds that were investigated during this thesis.

1.5.2.1 Acetamiprid: Occurrence in the environment and Human exposure

Acetamiprid (ACE) is a chemical compound which belongs to the family of neonicotinoids insecticides. ACE, along with IMI, THI and NIT, belongs in the first generations of neonicotinoids and it was firstly introduced in global market in 1995. Its structure is that of a chloronicotinyl chemical and as the rest of neonicotinoids resembles the structure of nicotine. (fig 1.11)

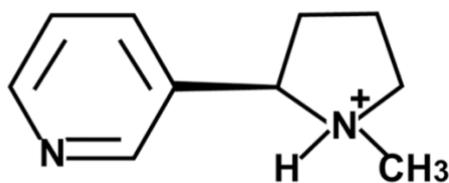
Data regarding the presence of 7 neonicotinoids in various food products collected from 1999 until 2015 by US Department of Agriculture's Pesticide Data Program (PDP) were summarised in a recently published review. The results revealed that ACE (along with IMI) commonly

detected in the food samples. The average detection frequency was 13.4% and 3.2% in fruits and vegetables, with mean concentration 0.0043mg/L (19.3nM) and 0.0011mg/L (4.9nM) respectively. The detection rate of ACE in some specific cases of fruits, such as those of strawberries, apples and pears, was over 20% and over 45% in the case of cherries (Craddock et al., 2019)

ACE has also been identified in water, many studies conducted over the years have reported the occurrence of ACE in aquatic environment such as in surface, ground water as well as in tap/drinking water. A recently published meta-analysis, with 43 studies included from 10 different countries revealed that the reported frequency of ACE in global surface water was 72% with a range of concentrations from 0.003 to 1370 ng/L and mean 23.36 ± 4.01 ng/L (Wang et al., 2023).

Due to the prevalence of ACE -and other neonicotinoids- in the environment, biomonitoring studies have investigated the appearance in human bodily fluids and matrices. ACE has been identified whole blood (Xu et al., 2021; Zhang, Zhang, et al., 2022), serum (Chen et al., 2021; Zhang et al., 2022), urine (Xu et al., 2021; Zhou et al., 2021) breast milk (Chen et al., 2020; Anand et al., 2021), as well as other matrices such as hair , saliva (Hua Zhang et al., 2021), teeth (Nan Zhang et al., 2021). The levels of ACE depend on the human bodily fluid, the country, and the examined population. For instance, in a study conducted in Southern China examining 6 different neonicotinoids and 2 metabolites, it was found that liver cancer patients showed elevated concentration of ACE (0.49ng/L) compared to healthy population (0.29 ng/L) in their blood (Zhang et al., 2022a). Moreover, the levels of ACE found in breast milk of lactating women were country dependent. ACE level in breast milk of women originate from India were, lower of detection limit (<LOD) (Anand et al., 2021), whilst those originate from China was 0.0014ng/L (Chen et al., 2020). Data showing the levels of ACE as well as other neonicotinoids in human bodily fluids from different countries, and different populations are summarised in a recently published review (Zhang & Lu, 2022).

A. Nicotine



B. Neonicotinoid ACE

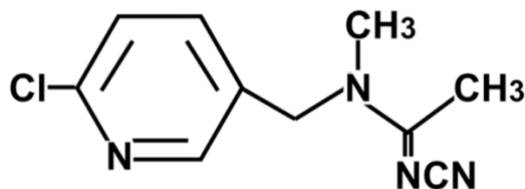


Figure 1.11: Chemical structure of a) Nicotine and b) Acetamiprid (ACE) (Kimura-Kuroda et al., 2012)

1.5.3 Environmental contaminants of interest and their effects on reproductive system

1.5.3.1 Bisphenol A and the effects on oogenesis, oocyte quality and fertilization

In an in vitro study conducted on human oocytes derived after culture of fetal ovaries for 7, 14, and 21 days in the presence of five different concentrations of BPA (1 μ M, 5 μ M, 10 μ M, 20 μ M, 30 μ M), oocyte survival was significantly decreased in all exposure groups (Bri  o-Enr  quez et al., 2011). Furthermore, it was concluded that meiotic progression was significantly delayed in oocytes exposed to BPA concentration higher than 10 μ M (Bri  o-Enr  quez et al., 2011). In subsequent work, exposure to 30 μ M of BPA was confirmed to alter the expression of genes related to oestrogen receptors, double-strand break generation, signalling and repair, markers of meiotic progression genes and genes involved in pairing and synapsis were significantly (Bri  o-Enr  quez et al., 2012). Impaired oogenesis because of modified meiotic genes expression has also been observed in in vivo mouse research ((Lawson et al., 2011; X. F. Zhang et al., 2012). Delayed meiosis during oogenesis and effects on follicles development have also been identified in in vivo mouse (Zhang et al., 2012) and rhesus macaque studies (Hunt et al., 2012).

In vitro studies on mouse early preantral follicles exposed to a range of BPA concentration (3 μ M – 30 μ M) showed that BPA causes meiotic arrest and impaired spindle alignment (Lenie

et al., 2008). Spindle abnormalities and chromosome misalignment along with a drop of maturation rates have also been confirmed in bovine oocytes exposed to 30 ng/ml BPA during in vitro maturation (Ferris et al., 2015). In an in vitro study conducted on porcine oocytes exposed to BPA (200 μ M and 250 μ M), apart from the observed spindle abnormalities, a disruption on polar body extrusion and delay on cell cycle was also observed. The proportion of oocytes on MII phase was significantly lower when oocytes exposed to higher dose of BPA. It seems that most of the oocytes exposed to BPA were stopped on the stage of metaphase I or anaphase/ telophase I (Wang et al., 2016). Impaired maturation and delayed on meiosis have also been reported in mouse oocyte exposed to 2 μ g/ml and 50 μ g/ml BPA respectively (Nakano et al., 2016). Increase of meiotic arrest and disruption of spindle formation along with spontaneous oocyte activation has also been confirmed by in vitro study in human oocyte exposed to BPA related to concentration that have been identified in follicular fluid (Machtinger et al., 2013).

These studies provide evidence that BPA can interfere with the oogenesis by disrupting the process of the meiosis across a range of concentrations. Impaired meiosis seems to be related with the presence of BPA during the maturation process, more specifically with abnormalities on spindle architecture and chromosome alignment. However, importantly, in all studies (except from (Machtinger et al., 2013) oocytes were exposed to concentration higher than that have been identified in human biological fluids (supraenvironmental concentrations).

The results of in vitro studies are strengthened by in vivo studies which also confirm drop on quality and quantity of the oocytes. An in vivo study conducted on mice (H. Q. Zhang et al., 2012) and macaques (Hunt et al., 2012) exposed to BPA from gestation determined that the number of multi-oocyte follicles, enclosed oocytes were significantly increased compare to control group. Furthermore, the primordial follicles reserve was decreased while the number of oocytes on secondary and antral follicles was increased at birth (Hunt et al., 2012). Neonatal lamb exposure to BPA revealed that the progression from primordial to primary follicles was increased however the total number of follicles remained unchanged (Rivera et al., 2011). In the same study an increase of granulosa and theca cells proliferation as well as increased multi-oocyte follicles was observed. Increased granulosa cells proliferation along with increased primordial follicular recruitment has also been confirmed in rats (Rodríguez et al., 2010).

Finally, negative correlation between urinary BPA concentrations and the number of retrieved oocytes, oocytes at metaphase II stage (MII) and fertilized eggs with two pronucleus have been confirmed in two prospective studies of 84 (Mok-Lin et al., 2010) and 174 (Ehrlich, Paige L Williams, et al., 2012) women undergoing fertility treatments. The association of increased BPA concentration in urine and decreased number of retrieved oocytes have also been confirmed in a more recent prospective cohort study of 351 women who were under IVF treatment because of tubal factor infertility (Shen et al., 2020). In the same cohort study urinary BPA was also negatively correlated with reduced implantation rates (Shen et al., 2020). However, it is important to mention that in this study the urinary BPA concentration was only measured once, on the day of the oocyte retrieval.

1.5.3.2 Bisphenol A and the effects on spermiogenesis and sperm quality

Several *in vivo* studies mainly conducted in rodents have confirmed that exposure to BPA through different routes (subcutaneous injection, oral gavage, drinking water) during different developmental periods (gestation, neonatal, postnatal) leads to a decrease in sperm count and impaired sperm motility (Salian et al., 2009; Tainaka et al., 2012; Dobrzyńska & Radzikowska, 2013; Tiwari & Vanage, 2013). Reductions in sperm motility and viability have also been observed after *in vitro* BPA exposure of bovine spermatozoa at a concentration >10 mg/ml when sperm were cultured in the presence of BPA for 6 and 24h respectively (Lukacova et al., 2015).

Reverse effects on spermiogenesis after BPA exposure have also been confirmed. In a *in vivo* study, adult male rats were exposed to an environmentally relevant dose of 20 µg/kg/day of BPA for 1 week. The findings of this study revealed an accumulation of chromosomal abnormalities as well as an increase in DNA double-strand breaks (DSBs) in sperm. Furthermore, a delay in the seminiferous epithelium cycle was observed, suggesting spermiogenesis disruption (Liu et al., 2013). Additionally, impaired spermatogenesis is correlated with alterations in the environment of the testis. Alterations in steroidogenesis and the testis cell morphology and function (Leydig and Sertoli cells) could have an impact on spermatogenesis and subsequently on sperm production. Indeed, alterations in the endocrine environment have been identified in *in vivo* studies on rodents exposed to BPA through different routes during different developmental stages, mainly during the postnatal and adult period. The common denominator of these studies was the decrease in levels of serum and/or intratesticular testosterone (Nakamura et

al., 2010; Xi et al., 2011; D'Cruz et al., 2012; Castro et al., 2013; El-Beshbishy et al., 2013). Furthermore, suppression of steroidogenic enzyme expression on Leydig cells (Nakamura et al., 2010; Xi et al., 2011; D'Cruz et al., 2012; Nanjappa et al., 2012) and increase on oxidative stress (Anjum et al., 2011) and the production of reactive oxygen species production (Minamiyama et al., 2010; D'Cruz et al., 2012) have been observed. Additionally, alteration on the total number and the morphology of the Sertoli (Tainaka et al., 2012) and the number and apoptosis rate of Leydig cells (Nakamura et al., 2010; Nanjappa et al., 2012) have been observed. Finally, cells viability of human primary Sertoli cells was significantly decreased after BPA exposure at a dose of 20 μ M. Decreased expression levels of occludin, ZO-1, β -catenin, was additionally observed at the same BPA dose (de Freitas et al., 2016).

However, some in vivo studies on mouse and rat models have failed to observe effects on sperm quality or hormones levels after BPA exposure. For example. Kobayashi et al., found that after dietary BPA exposure during gestational and prenatal period in rats, no effects were observed on sperm quality parameters (motility and total count), testosterone levels or on reproductive organ (testis, prostate) weight (Kobayashi et al., 2012). This latter finding was also observed in an in vivo study on rats after exposure to BPA from gestational to postnatal period (Howdeshell et al., 2008) which has also been confirmed on postnatal exposed -to -BPA male mouse (Tyl et al., 2008).

Finally, epidemiological studies on occupationally exposed to BPA or seeking fertility treatment adult men investigated the correlation of BPA concentration in the urine with sperm quality factors such as sperm concentration, total count, motility, viability, morphology and DNA damage and semen volume. In a prospective cohort study of 218 male workers on factories with or without BPA exposure, BPA was measured in urine twice, one pre- and post-sift measurement also sperm sample was collected the same day. The results showed that increased levels of BPA correlated with decreased sperm concentration, total sperm count, motility and viability. However no association was identified regarding morphology and semen volume (Li et al., 2011). Negative correlations between BPA levels in urine and sperm quality parameters such as sperm concentration and sperm motility were also confirmed in a study of 190 male patients recruited from fertility clinic. Furthermore, increased sperm DNA damage have been associated with high levels of urinary BPA (Meeker et al., 2010). On the contrary, in a research of fertile men the BPA identification on their urine samples was not associated with any alteration on sperm parameters (Mendiola et al., 2010). Moreover, a prospective

cohort study conducted in two states of USA taking as sample 501 American adults' men from general population found an association between higher level of urinary BPA with decreased sperm DNA fragmentation (Goldstone et al., 2015). However, in this study the geometric mean of BPA detected in the urine samples was lower (0.55 ng/ml) compare to the BPA mean (2.92 ng/ml and 1.4 ng/ml) that was found in the urine of men participating in NHANES research and the study of the male participants from the IVF clinic, respectively (Meeker et al., 2010).

1.5.3.3 Bisphenol A and the effects on Embryo development

Studies on the effects of BPA on early embryo development are limited. In two in vitro studies conducted on bovine models, exposure on BPA had an impact on embryo development rate quality (Choi et al., 2016; Ferris et al., 2016). and metabolism (Choi et al., 2016). More specifically, Ferris et al., studied the effects on embryos derived from bovine cumulus oocyte complexes exposed to BPA during maturation. After exposing the oocyte on BPA (15 ng/ml and 30 ng/ml) developmental rates of embryos were affected. The high dose (30 ng/ml) significantly decrease the cleavage rate and both doses had a great impact on blastocyst rates. However, no affect was reported on the total blastocysts cell number. Furthermore, embryos generated from oocytes exposed to high BPA dose resulted on an increase of DNA damage and apoptosis rates. However, no significant difference was identified on blastocyst gene expression (Ferris et al., 2016). In the second in vitro study, the embryos were exposed during the early stages of development on BPA (1 ng/ml and 10 ng/ml). Again, a significant decrease was observed on the blastocyst rates after exposure to high dose and no alteration on the total blastocyst cell number and the gene expression of oestrogen receptors. Furthermore, in this study the metabolic profile of embryos was investigated, and a significant increase of glucose consumption was observed on embryos exposed to high dose of BPA. However, no alteration was found on expression of genes related to glucose transport (glycolysis, the pentose phosphate pathway, Krebs cycle or mitochondrial biogenesis (Choi et al., 2016).

In vivo studies related to BPA effects on embryo development have been conducted only using mouse model showing impacts on implantation, embryo transport and blastocyst rate (Xiao et al., 2011)) as well as disruption on embryos imprinted gene regulation (Susiarjo et al., 2013). Xiao et al., group exposed mice to BPA (d 0.025, 0.5, 10, 40, and 100 mg/kg/day) during the embryonic period of gestation (Day 0.5 to 3.5). The results show that only the highest dose had a significant impact on implantation sites in the uterus, inhibited implantation and also delayed

embryo transport into the oviduct as well as decrease the blastocyst rate (Xiao et al., 2011). Another *in vivo* study demonstrated that exposure to 10 µg/ml/kg and 10 mg/ml/kg of BPA from the period breeding until pregnancy disturbed the expression of imprinted genes in midgestational embryos and alters DNA methylation levels (Susiarjo et al., 2013).

Finally, in a study of 174 women, urinary BPA concentration inversely correlated with oestradiol peak, and mean number of retrieved oocytes. Furthermore, a trend in decreased blastocyst rate was identified (Ehrlich, Paige L Williams, et al., 2012). In another prospective cohort study of 137 women undergoing IVF treatment conducted by the same group, a positive correlation was identified between urinary BPA concentration and implantation failure (Ehrlich, Paige L. Williams, et al., 2012). However, in both studies, the BPA was measured only twice, during the day of egg retrieval and the start of the cycle and male factor was not included as a possible co-factor regarding male exposure to BPA or semen quality.

1.5.3.4 Bisphenol S and the effects on reproductive system

Despite evidence of widespread bioaccumulation of BPS in the environment and the human exposure to this contaminant neither its metabolic nor its biological impacts on human organism have been examined in detail. Compared with the knowledge gathered for BPA impacts on human reproduction, our knowledge about BPS and how it interferes with the reproductive function is negligible.

Early *in vitro* studies using cells lines found that BPS binds on ERs (Delfosse et al., 2012; Rosenmai et al., 2014; Le Fol et al., 2015) causing estrogenic and antiandrogenic activity like bisphenol A (Molina-Molina et al., 2013; Rosenmai et al., 2014). However, in a series of *in vitro* studies, BPS demonstrated weaker estrogenic activity compared to estradiol (Kuruto-Niwa et al., 2005; Grignard et al., 2012; Molina-Molina et al., 2013). A systematic review published in 2015 found that BPS had a comparable affinity to ER to that of BPA (Rochester & Bolden, 2015). Furthermore, in a study using spectroscopic methods BPS like BPA has also the ability to bind on serum albumins (Mathew et al., 2014).

Regarding BPS effects on development and reproduction outcomes *in vivo*, zebrafish has been initially used as a model so far. In two studies where adults or embryo zebrafish were exposed to BPS dose of 0.5, 5, and 50 µg/L and 0.1, 1, 10, 100 µg/L for 75 and 21 days respectively

the results shows significantly differences on endocrine system and reproduction outcomes (Ji et al., 2013; Naderi et al., 2014). More specifically, egg production (Ji et al., 2013) and the number of sperm (Naderi et al., 2014) was decreased. Regarding embryo development the ratio of female to male (Naderi et al., 2014) along with embryo malfunctions (Ji et al., 2013) was increased. Finally changes on testosterone and estrogen levels after BPS exposure have been noticed with the E2 to be increased in both sexes (Naderi et al. 2014, Ji et al. 2013). A more recent study on zebrafish investigated the effects of BPS exposure during embryo development on the reproductive neuroendocrine system. The zebrafish embryos were exposed to BPS dose 100µg/L for 25 hours after fertilisation. The group investigated the potential association of exposure with the estrogen receptors, thyroid hormone receptors, and aromatase enzyme pathways. The results showed that low dose of BPS could increase the number of Gonadotropin -Releasing Hormones 3 (GnRH3) neurons in hypothalamus and the expression of genes related to reproduction such as kiss1, gnrh3, era (Qiu et al., 2016).

More recent in vitro studies show that oxidative stress is induced after BPS exposure. An increase of reactive oxidative species (ROS) was observed after acute in vitro BPS exposure at a dose of 250 ng/ml. However, compared to BPA the caused oxidative stress by BPS was not statistically significant (Maćczak et al., 2017). Induced reactive oxidative species along with lipid peroxidation after BPS exposure has also been confirmed in a in vivo study of adult rats where the animals were exposed to a range of concentrations (0, 1, 5, 25, 50 µg/kg/day) for 28 days. Along with the induced oxidative stress, a significant decrease of antioxidant and serum and intratesticular testosterone secretion was observed. Finally, alterations on the morphology of the seminiferous epithelium was confirmed however the population of different cell types in the seminiferous tubules on treated animals compared to control group remained unchanged (Ullah et al., 2016). This group by following the same approach they conducted a comparative study of the effects of BPA and its analogue BPS on sperm and testicular tissue of rats. The results confirmed the evidence of the previous study regarding the effects of BPS on rats' male reproductive system and also identify that the effects of BPS are similar of those of BPA (Ullah et al., 2018).

Impacts of BPS have also been confirmed in reproductive processes of females. Similar to BPA, it seems that BPS impairs with the meiotic maturation of mammal oocytes. In this study, pig oocytes were exposed to different concentrations of BPS (30pM, 3nM, 300nM, 30µM) for 24, 48 and 72 hours and maturation rate, ERa, ERb and aromatase expression and

distribution was examined. Results shows a significant decrease of oocytes reach MI and MII phase after BPS exposure at the dose of 3 nM and 300nM. Furthermore, the mRNA levels of ERs and aromatase expressed were significantly decreased in groups exposed to BPS compared to control. Apart from the decrease of the mRNA levels, an alteration on expression and distribution of ERs and aromatase was observed. Finally, cumulus cells expansion was disrupted (Žalmanová et al., 2017). Moreover, BPS had a negative impact on the process of oogenesis due to the fact that there are recent evidence suggesting that BPS interfere with the steroidogenesis in granulosa cells. In vitro culture of ovine granulosa cells treated for 48h with a range of BPS concentrations from 1 nM to 200 µM showed significant alterations on steroidogenesis of this cells. More specifically, BPS seemed to significantly decrease cell proliferation, progesterone secretion, increase oestradiol secretions and ER gene expression (Téteau et al., 2020). However, the alteration was identified in this study only observed after granulosa cells were exposed to the highest concentration (100 µM and 200 µM) which are considered as supraenvironmental doses.

Finally, regarding BPS metabolism, in an in vitro study using cell lines and yeast cells, it has been proposed that the dominant metabolic pathway of BPS is glucuronidation. The two main metabolites of BPS it seems to be BPS glucuronide and hydroxylated BPS 4-(4-hydroxy-benzenesulfonyl)-benzene-1,2-diol (BPSM1) (Gramec Skledar et al., 2015). In this study the estrogenic activity of the BPS metabolites was investigated, and the results indicated that BPSM1 act as a weak agonist of ER like BPS while BPS glucuronide has no affinity with estrogen, androgen or thyroid receptors (Gramec Skledar et al., 2015).

1.5.3.5 Triclosan and the effects on reproductive system

Triclosan has been categorized as a potential reproductive toxicant. However, knowledge of the effect of TCS on human reproduction is limited and, in some cases, conflicting. For instance, three studies have investigated whether there is an association between urinary TCS and male fertility (Cite them here). Two concluded that urinary TCS in urine is positively associated with reduced sperm concentration and the proportion of morphological normal sperms (Zhu et al., 2016; Jurewicz et al., 2018). By contrast, a study which conducted in China found no association between TCS exposure and male infertility (Chen et al., 2013).

Studies using animal models suggest that TCS has adverse impacts on reproductive function. Kumar et al (2008) reported a testes weights and sperm density following exposure of TCS in a rat model (Kumar et al., 2008). TCS exposure has also associated with a decrease of weights in female sex organs in rats (ovaries and uterus) (Rattan et al., 2017). It seems that TCS interferes with the reproductive function by disrupting the synthesis LH, FSH and testosterone (Kumar et al., 2009).

In vivo and in vitro studies in different animal models have confirmed that TCS can disrupt endocrine function, specifically as an antioestrogen (Stoker et al., 2010), and anti-androgen (Kumar et al., 2009) in female and male rats respectively. Furthermore, in a study conducted in a fish model, weak androgenic activity was detected. Additionally, many experiments have been conducted in order to identify if TCS interfere with thyroid function given its structural similarity to thyroid hormone thyroxine (T4) (Crofton et al., 2007),. Animal studies identified that after TCS exposure T4 levels in the serum were significantly reduced allowing researchers to conclude that thyroid homeostasis could be affected by TCS exposure (Crofton et al., 2007; Zorrilla et al., 2009; Paul et al., 2010, 2013; Stoker et al., 2010). These results were also confirmed in studies conducted in pregnant rats (Zorrilla et al., 2009; Paul et al., 2012; Axelstad et al., 2013).

In summary, TCS is an environmental contaminant to which the general population are regularly exposed. The studies' results have demonstrated that TCS is rapidly absorbed and metabolised by the organisms and distributed in the tissues. Due to the similar chemical structure to steroids and thyroid hormones, it displays the ability to interfere and disrupt the physiological function of the endocrine system. Potential reproductive toxicity has also been reported; although, the evidence is still limited and, in some cases, conflicting. However, given the firm evidence that TCS is widely distributed in the environment and human is daily exposed to this chemical is considered as a good target for studying the impacts on female reproductive function and the early stage of embryo development.

1.5.3.6 Acetamiprid and the effects on reproductive system

The toxicity of neonicotinoids on reproductive system has been confirmed by in vivo and in vitro studies using mainly rodents as a model. The results of these studies show detrimental effects on reproductive organs development such as on ovary and epididymis weight,

impairment with follicle morphology, effects on sperm parameters and decrease on steroids hormones levels such as testosterone, 17 β -oestradiol in serum of male and female rats respectively, and they are summarised in a review published in 2022 (Terayama et al., 2022) .

Among the neonicotinoids studied for their reproductive toxicity, ACE is the one least investigated. In vivo studies using rats or mice as model have shown adverse effects on male reproductive system. More specifically in a study conducted in 2017, male Sprague–Dawley rats aged 5-weeks were orally exposed to 10 or 30mg/kg for 35 days. The results showed that the testis weight was decreased and combined with decreased number of spermatids and sperm in epididymis irrespective of the administrated dose. Moreover, the cyclic adenosine monophosphate (cAMP) production in Leydig cells was decreased by the high dose, while the ATP was decreased in both experimental groups. In the same study, decreased levels of testosterone in serum as well as decrease production of enzymes essential for steroidogenesis, such as STAR, cytochrome P450, CYP11A1, were also observed irrespective of the dose. Finally, increased LH levels in the plasma was found after exposure to 30mg/kg (Kong et al., 2017) The increased levels of LH, as well as GnRH and FSH, in plasma were also confirmed in a study conducted later on in male rats exposed to similar doses (12.5mg/kg, 25mg/kg and 35mg/kg) for 90 days. Sperm concentration and the testosterone levels were also decreased in confirming the results of Kong et al., 2017. Furthermore, antioxidant parameters in serum and in testes were significantly reduced irrespective of the administrated dose. (Arıcan et al., 2020). Decreased levels of essential enzymes for steroidogenesis (STAR, Cyp11a1, HSD17b3) were also observed when mouse aged 3 weeks old were exposed to 21.4mg/kg for 180days (Terayama et al., 2018). Additionally, in a study investigating the protective effects of folic acid against ACE exposure in male rats it was found that sperm parameters (sperm count, viability, and motility) were significantly reduced when the animals were exposed to 10mg/kg ACE for 5 weeks. Moreover, testosterone and GnRH levels in serum was significantly decreased compared to the untreated group. Apart from the adverse effects on reproductive parameters, the results of this study showed effects on haematological and immunological parameters influencing the liver and kidney function. To sum up so far, the results of these studies show that the toxicity of ACE in male reproductive system is linked with endocrine impairment, oxidative stress, and cell apoptosis.

Studies on the effects of ACE in female reproductive system remains limited number, even though the effects of other neonicotinoids such as IMI (Kapoor et al., 2011) or the effects of

nicotine on mammalian female reproductive system have been described (Zenzes & Bielecki, 2004). At the moment, there is only one study investigating the effects of ACE on mammalian reproductive system published in 2015. In this study porcine oocytes cultured in a monocultured method were exposed to 10 particles per million (9.98mM), 30ppm (134.72mM) and 100ppm (98.85mM) during the in vitro maturation. The maturation rate of porcine oocytes was significantly decreased in a dose dependent way and chromosomal abnormalities were confirmed after exposure to 30ppm and 100ppm of ACE. However, the adverse effects of ACE on oocyte has also been confirmed in other model species such as xenopus (Cartereau et al., 2018).

Finally, regarding the effects of ACE on early embryo development a limited number of studies has been conducted using zebrafish and mouse as model. In a study published in 2019, the effects of 10 different ACE concentration with range from 0.25 mM to 4.5 mM had detrimental effects on embryo development. First of all, increased mortality and impaired hatchability were confirmed. Subsequently, for the embryos that survived, developmental landmarks such as decreased heartbeat and body weight as well as behavioural malfunctions such as alteration on spontaneous movement and the touch response were observed (Ma et al., 2019). ACE embryotoxicity on zebrafish is caused by oxidative stress, and impaired endocrine function (Araujo et al., 2022; Hu et al., 2022; Ma et al., 2022). Regarding the effects of ACE on mammalian embryo development, to the best of our knowledge only one study has been conducted so far. In this study, mouse zygotes, and 2-cell embryos were exposed to 500µM during the early stages of development until the blastocysts stage. The results showed that morula and blastocyst formation was significantly reduced and at the same time the fragmented embryos were significantly increase compared to untreated group (Gu et al., 2013).

To sum up, even though the number of studies investigating the effects of ACE in reproductive system and early reproductive events are limited, they present strong evidence of impairment after exposure to this pesticide. It seems that ACE induce a series of adverse effects by interfering with the steroid hormones, inducing oxidative stress, triggering morphological and developmental abnormalities in female and male gametes and embryos. However, it is essential to highlight at this point that all the studies mentioned above have been conducted using ACE doses and concentration excessively higher than those have been quantified in the environmental matrices or in the human bodily fluids

1.6 Metabolism in cellular level

1.6.1 Metabolism overview

The term metabolism is used to describe the total set of the chemical reactions that take place within a living organism to maintain life. In the cellular level, metabolism indicate the sequence of the chemical reactions that undertaken to generate energy for the maintenance of normal function of the cell. The generation of the energy in a cellular level is accomplished through three main metabolic pathways, glycolysis, Kerbs cycle (or TCA cycle) and oxidative phosphorylation (Berg et al., 2002).

Through glycolysis, molecules of glucose, that enter the cellular cytoplasm are broken down and produce 2 molecules of adenine triphosphate- ATP- for each molecule of glucose. However, through its final product pyruvate, glycolysis, also contribute to the generation of Acetyl-CoA molecule which in turn can enter the mitochondria where it takes part in TCA cycle. Acetyl-CoA may also be generated by fatty acid β -oxidation. Through the TCA cycle, FADH_2 and NADH are generated which subsequently act as electron donors in the electron transport chain reaction of the oxidative phosphorylation which produces up to 32 molecules of ATP. To generate the appropriate amount of ATP, which is considered as the “currency” of the processes that demand energy, the cooperation of all the metabolic pathways is required (Berg et al., 2002).

The metabolic pathways occur in different part of the cell, glycolysis is taking place in the cytoplasm of the cell and the TCA cycle, and the oxidative phosphorylation take place in the mitochondria of the cells which are considered as the “factories” of energy generation within the cell. Its metabolic pathway has multiple regulatory steps which allow to adjust the energy generation depending of the organ demands in response to the microenvironment and the conditions such as nutritional factors and external signals (Berg et al., 2002). A schematic presentation of metabolic pathways is presented in Figure 1.12.

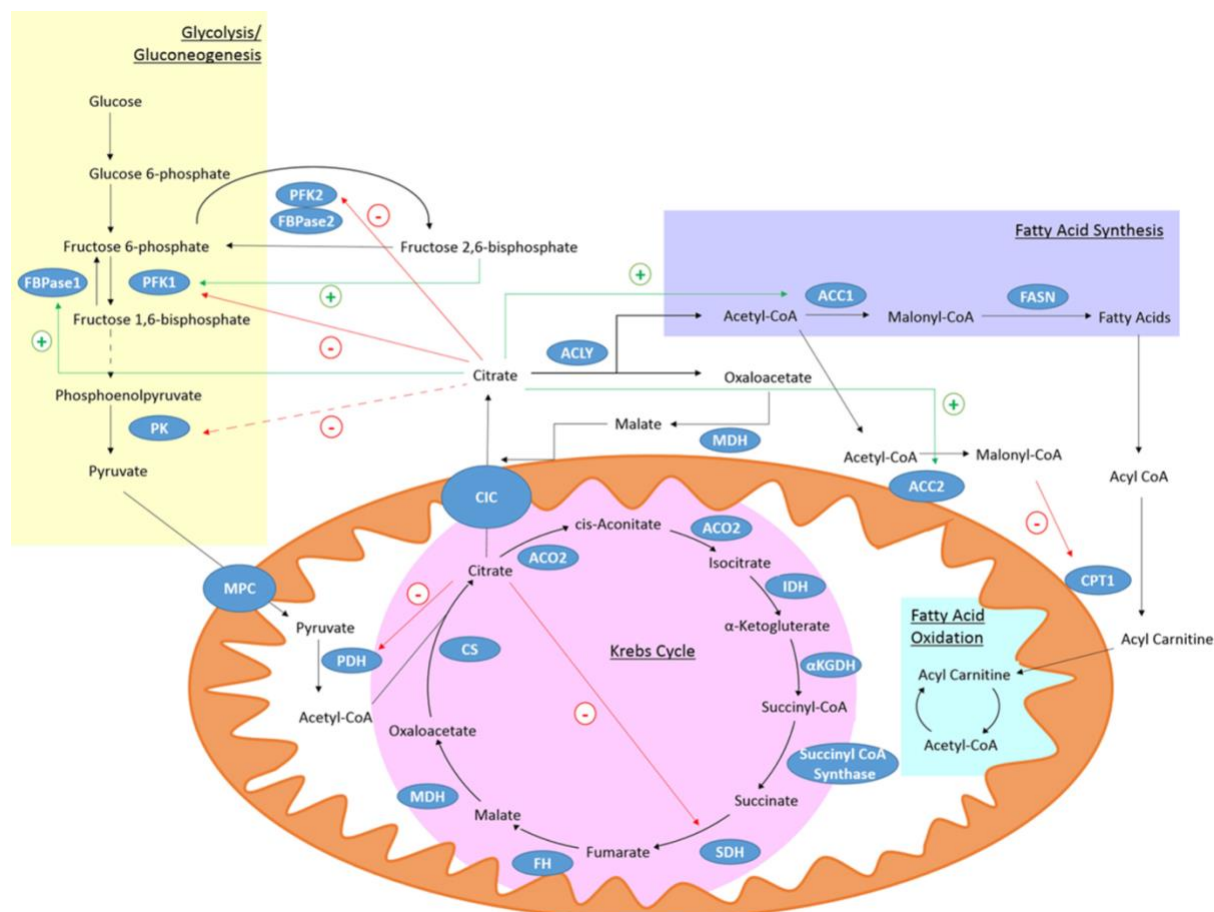


Figure 1.12: Metabolic pathways overview (Williams & O'Neill, 2018). Glucose enters the cell and through glycolysis a small amount of ATP is generated. The final product of glycolysis, pyruvate as well as fatty acids through β -oxidation are converted to Acetyl-CoA which enters the mitochondria and participate to TCA cycle. During glycolysis and TCA cycle electron carriers NADH and FADH are generated which in their turn participate to oxidative phosphorylation the pathway through which high amount of ATP is produced.

1.6.2 Metabolic pathways

1.6.2.1 Glycolysis

Glycolysis is the metabolic pathway through which glucose- a six carbon molecule- is oxidised and finally converted into 2 molecules of the three-carbon molecule, pyruvate. Initially, glucose, as a water-soluble solute, enters the cell cytoplasm through 2 different families of transporters, GLUTs and SGLTs. GLUT transporters are involved in facilitated diffusion whereas glucose is actively transported through the SGLTs, triggered by sodium flow (Deng & Yan, 2016). During glycolysis there is a net production of 2 ATP and 2 NADH molecules. NADH is later involved in oxidative phosphorylation acting as H^+ donor. The NADH action

in oxidative phosphorylation will be explained later in the following section (Chandel, 2021; Chaudhry & Varacallo, 2024).

The fate of the final glycolysis' product, pyruvate, depends on the presences (aerobic conditions) or absence of oxygen (anaerobic conditions). In anaerobic or very low oxygen conditions, pyruvate is converted to lactate, a reaction catalysed by LDH enzyme, which recycles NADH to NAD⁺ allowing glycolysis to continue and more ATP molecules to be produced. However, during aerobic conditions, glycolysis is the initial step of the cellular respiratory process, and the produced pyruvate is transported into the mitochondria and converted to acetyl-CoA. The conversion of pyruvate to acetyl-CoA is catalysed by PDH and with CO₂ produced as a by-product. Subsequently, acetyl-CoA enters the TCA cycle which is going to be discussed in the following section (Chaudhry & Varacallo, 2024).

The glycolysis pathway consists of 10 distinct biochemical reaction and each one is catalysed by a specific enzyme. Three of these enzymes, hexokinase, phosphofructokinase (PFK) and pyruvate kinase (PK), participate in the non-reversible biochemical reactions and contribute to the regulation of this pathway. Hexokinase catalyses the first step of glycolysis by phosphorylating glucose to glucose-6-phosphate. Hexokinase is regulated allosterically by its own product glucose-6-phosphate (Chandel, 2021). However, hexokinase can be hormonally regulated and is stimulated or inhibited by insulin or glucogen respectively. The second and more essential regulatory step of glycolysis is PFK. PFK catalyses the third step of glycolysis where fructose-6-phosphate is phosphorylated to fructose-1,6-bisphosphate by using an ATP molecule (Chandel, 2021). The regulation of this enzyme is quite complex and involves high levels of ATP and citrate which inhibit the enzyme allosterically. However, high levels of ADP stimulate this regulatory enzyme (J. Berg et al., 2002). Finally, PK, which is the enzyme participate in the final step of glycolysis is part of the regulatory mechanism of glycolysis (J. Berg et al., 2002).

1.6.2.2 Fatty Acid metabolism

Fatty acids are aliphatic hydrocarbons with carboxylic end, and they can be saturated or unsaturated. Fatty acids originate from three primary sources, de novo synthesis, hydrolysis of acylated proteins, phospholipids, and triglycerides and exogenous derived from blood circulation. They are transported in the blood circulation combined with albumin and they are

transferred into the cellular cytoplasm by specific transporters such as fatty acids transport proteins (FATPs) or caveolins. These transporters facilitate the transport of long-chain fatty acids (Houten & Wanders, 2010).

Irrespective of the origin, once they enter the cytoplasm, all fatty acids, are activated, a process which is catalysed by Fatty Acyl CoA Synthetase and resulting in the creation of acyl-CoA (Houten & Wanders, 2010). However, acyl-CoA are unable to reach the mitochondria matrix, which is the site where fatty acid oxidation take place, in this form. For this reason, are combined with L-carnitine by CPT-I enzyme and transported into to mitochondria via a specific transporter named CACT. Once they enter the mitochondria fatty acyl-carnitine is converted by CPT-II enzyme to acyl-CoA, which are the molecules used for the fatty acid oxidation (FAO) (Longo et al., 2016). FAO is a cyclic metabolic pathway consisting of 4 steps. The result of this process is the formation of a acyl-CoA, an acetyl-CoA, one NADH and one FADH₂ molecule. The acyl-CoA enters another FAO cycle, the acetyl-CoA is used for the TCA cycle and the FADH₂ and NADH are going to be used in oxidative phosphorylation pathway as electron donors (Houten & Wanders, 2010)

Fatty acid oxidation takes place under aerobic conditions with low ATP levels. During this process there the net production of ATP is higher than this of glycolysis, However, under normal conditions- when the organism is well-fed- glucose is the primary substrate for oxidation leaving fatty acids to become the main source of energy during fasting periods. The regulation of fatty acid oxidation relies allosterically on substrates and enzymes availability, Also, in regulatory mechanism of this pathway specific hormones are involved. For example, insulin, acts as inhibitor and adrenalin, noradrenalin or glucagon act as promoter of this pathway (Zhang, Wei, et al., 2022).

1.6.2.3 Amino acid metabolism

Amino acids are an organic class of molecules known as the building blocks of protein. It is now well established that apart from the protein synthesis they also participate in other biological processes as energy substrates, osmolytes, antioxidants, electron carriers and neurotransmitters (Wu, 2009). Their chemical structure consists of the main carbon (alpha carbon) which is bonded with a hydrogen (-H), a carboxylic group (-COOH), an amino group

(-NH₂), and an “R” group. The R group is different for each amino acid and determines the different chemical properties among them (Berg et al., 2002).

Amino acids classification depends on the R group properties and their de novo synthesis in the organism. Amino acids are divided into 4 groups: polar, non-polar, basic (positively charged) and acidic (negatively charged amino acids, based on the chemical properties of the R group (J. M. Berg et al., 2002). They are also categorised as non-essential and essential depending on if they can be synthesised in the body or they only can be acquired via diet respectively (Hou et al., 2015). Finally, amino acids are categorised as glucogenic, ketogenic or mixed. Glucogenic are the amino acids that are participate in glucose formation via gluconeogenesis and contribute to glycolysis. Ketogenic are those which converted to fatty acids and contribute to β -oxidation, and finally some may contribute to both pathways (D’Andrea, 2000).

Apart from these pathways, amino acids participate in TCA cycle. Through a reversible biochemical reaction, called transamination, amino acids transfer the amino group to a keto-acid. Through this process, new amino acids are formed, as are molecules that participate in TCA cycle. For instance, through alanine transamination pyruvate and glutamate are created. Pyruvate can be converted to acetyl-CoA and enter TCA cycle and glutamate can be converted to α -ketoglutarate, another TCA cycle molecule. Furthermore, aspartate, tyrosine, valine can be converted through transamination to oxaloacetate, fumarate, succinyl-CoA, respectively, all of which can participate in the TCA cycle (J. M. Berg et al., 2002).

1.6.2.4 TCA cycle

The TCA cycle (Tricarboxylic acid cycle) also known as Krebs’s or the Citric acid cycle is an essential metabolic pathways that takes place in the mitochondrial matrix (fig 1.12). This metabolic pathway can only occur under aerobic conditions, even though the reactions themselves have no direct use of oxygen during this pathway (Berg et al., 2002).

In the first step of the TCA cycle, acetyl-CoA is combined with oxaloacetate – a 4-carbon molecule- and form citrate – a 6-carbon molecule- in a reaction catalysed by citrate synthase. As already described, acetyl-CoA can be derived either from the final product of glycolysis, pyruvate, from fatty acids through β -oxidation pathway or via transamination of amino acids.

In the following steps, citrate undergoes a series of reactions resulting on the generation of seven products: one FADH_2 , three NADH , one GTP (guanine triphosphate) and two CO_2 (carbon dioxide) (Berg et al., 2002).

The TCA cycle is regulated allosterically in three points within the pathway. The enzymes, citrate synthase, isocitrate dehydrogenase, and α -ketoglutarate dehydrogenase are responsible for the regulation. High levels of ATP or the products from the reaction that each enzyme catalysed seems to inhibit, while high levels of ADP stimulate, the action of the enzymes. Meanwhile it has been found that Ca^{2+} act as a stimulator of isocitrate dehydrogenase and α -ketoglutarate (Berg et al., 2002; Arnold & Finley, 2022).

Crucially, the TCA cycle links almost all the metabolic pathways as it can contribute to anabolic and catabolic pathways. However, the integration of the TCA cycle with oxidative phosphorylation is key to synthesis of ATP .

1.6.2.5 Oxidative phosphorylation

Oxidative phosphorylation is the metabolic pathway where electron donors NADH and FADH_2 , produced during the TCA cycle, as well as O_2 through a series of reactions are used to produce cellular energy in the form of ATP . This metabolic process occurs in mitochondrial matrix of eukaryotic cells and is able to produce higher amounts of ATP per molecule input compared to other metabolic pathways such as anaerobic glycolysis (Berg et al., 2002).

All these reactions take place at the inner mitochondrial membrane (IMM). One of the key roles of the IMM is to act as a barrier to positively charged protons, thus allowing a concentration gradient to be maintained where the intermembrane space (IMS) has more protons than the mitochondrial matrix. This produces a pH and charge gradient, which is crucial for the phosphorylation of ADP to produce ATP through the action of a large complex called ATP synthase (or Complex V) which uses the proton gradient to drive the synthesis of ATP molecules. These ATP molecules ultimately provide the energy for the most of the cells reactions. The synthase makes ATP by using proton flow from one side of the inner membrane to the other and rotate protein subunits. If there is no proton gradient, the ATP synthase subunits stop rotating and the cell stop generate energy and die. Therefore, the protein complexes and

small molecules that establish and maintain this proton gradient. This system is composed of four protein complexes numbered I through IV. Complexes I, III, IV directly pump protons from the matrix into the IMS whereas complex II does not, but it does promote proton pumping in complexes III and IV (Guerra et al., 2002).

Proton pumping requires energy, and the four protein complexes get this energy by transferring electrons through a series of coupled reactions. This linked process of electron transport is why the four complexes are referred to as the electron transport chain (fig 1.13). NADH, derived from TCA cycle, deposits 2 electrons in Complex I where they are passed along a series of redox centres and a small amount of energy is released each time an electron passed between redox centres. Complex I harness this energy and uses it to pump protons into the IMS. The last redox centre in Complex I donates two electrons to a coenzyme Q molecule.

Complex II is similar to Complex I in two important ways. Firstly, high energy electrons also enter complex II via FADH₂, a second product of the TCA cycle. Complex II also transfers electrons between several redox centres before donating them to co-enzyme Q. One major difference however is that Complex II does not use the energy liberated to pump protons. Co-enzyme Q molecules from Complexes I and II donate the electrons to Complex III. Subsequently electrons are passed to cytochrome c which carries the electrons to Complex IV.

The electron transport chain ends in Complex IV, where a series of reactions involving four electrons converts a molecule of O₂ to two molecules of H₂O. The proton gradient is strengthened because four H⁺ from the matrix are incorporated into water molecules and another four H⁺ are pumped into the IMS.

As mentioned at the beginning of this section the proton gradient is used by ATP synthase for the production of the ATP molecules. The accumulation of protons in the IMS leads to depolarisation of this membrane which subsequently activates the F₀ region of the ATP synthase protein complex, and the protons move from the high concentration (from IMS) to the low concentration (into the mitochondrial matrix) through the pore of the alpha subunit located at the F₀ region of ATP synthase. Since H⁺ are transferred from high to low concentration it does not require energy, thus occurs via passive transport. This passive transport of H⁺ activates the rotation of the c structure of the F₀ region. This subunit is connected with catalytic subunits of F₁ region which sequentially also rotate and generate a

proton motive force and the whole ATP synthase absorbs energy as H^+ flows into the matrix. This absorbed energy is used by beta subunit of F1 region to alter the affinity for ATP and ADP, catalyse the synthesis and release of ATP (Jonckheere et al., 2012). It is estimated that approximately 32 molecules of ATP are generated per complete oxidation of glucose (J. M. Berg et al., 2002).

During respiration, reactive oxygen species (ROS) are produced as by-products. ROS molecules such as superoxide anion (O_2^-), hydroxyl radical (HO^*), and singlet oxygen (O^*) are generated because of the partial reduction of oxygen. Also, H_2O_2 can be formed which act as a ROS mediator because it can facilitate the production of ROS. Excessive accumulation of ROS in the cell leads to oxidative stress which sequentially leads to DNA, protein and lipid damage mediated by the reactive species. Pathological conditions, such as cancer, metabolic diseases, neurodegenerative conditions as well as aging has been linked with oxidative stress. However, it has been suggested that ROS have a significant role in healthy cell function by regulating physiological system such as differentiation, hypoxia immunity and longevity. It is possible that ROS response to a stress signal and act as signalling mediator to assist the adaptation of the system to this stressor. However, it remains unclear if ROS action is essential for homeostasis in the absence of the stressors (review by (Murphy, 2009; Sena & Chandel, 2012; Zhang, Pan, et al., 2022)

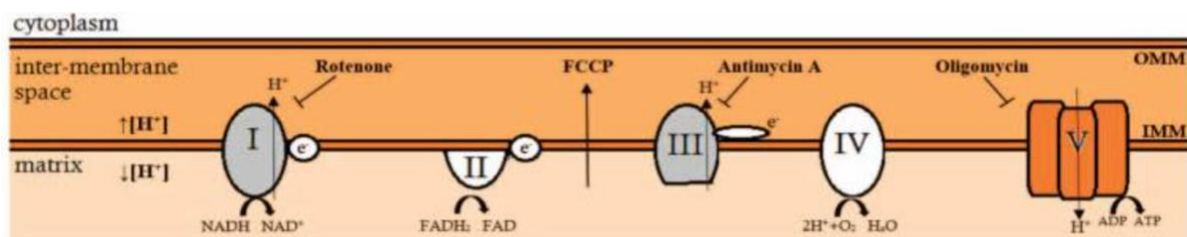


Figure 1.13: The electron transport chain (Muller et al., 2019). The process of the electron transport chain reaction is described in detail in the section above (section 1.6.2.5)

1.7 Developmental Origin of Health and Disease

According to the Developmental Origins of Health and Disease (DOHaD) hypothesis (Barker, 2007), there are critical periods during development in which the development and function of the offspring can be modified by environmental stressors that the mother is exposed during pregnancy, and this can subsequently have adverse health outcomes on the offspring in later stages of life as an adult. Poor nutrition, stress, and environmental chemicals are some examples of the stressors that the mother could be exposed to. The foundation of this work was the observation reported by David Barker which demonstrated an association between the low birth weight and the development of cardiovascular disease on adult offspring (Barker, 2007).

One of the most well-known historical events that provided proof of concept for the DOHaD hypothesis is the Dutch famine, during the winter of 1944-1945 when people were malnourished (500 to 600kcal per day) because of the Nazi's food embargo. This event was later linked with various metabolic diseases in the offspring (Roseboom et al 2006) and with low birthweights in the second generation of offspring (Lumey LH 1992). The DOHaD hypothesis has been also studied and confirmed in animal models; malnutrition during pregnancy has been linked with a wide range of metabolic conditions such as obesity, diabetes as well as inflammatory and cardiovascular disease (Gaillard et al., 2016; Godfrey et al., 2017; Fleming et al., 2018)

Apart from the impact of maternal nutrition in offspring's health status later in adult life, association between the offspring health and drugs or environmental contaminants exposure during pregnancy have been also confirmed. A typical example of this is the case of the drug diethylstilbestrol (DES) which was prescribed between 1940 and 1970 to women during pregnancy. Retrospective data analysis revealed that offspring exposed to DES in utero had a higher risk of rare cancer and adverse reproductive outcomes and reproductive malformations. Carcinogenic action of DES was later confirmed in animal studies. Moreover, mercury contamination in Japan in 1956 was linked with brain dysfunction on exposed-in-utero offsprings. The effect of maternal exposure to environmental chemicals on offspring's health was summarised in a review recently published by (Heindel et al., 2017). In this review, 425 papers were included and 60 different chemicals such as bisphenols, phthalates, pesticides were included and it was concluded that maternal chemical exposure led to offspring's adverse health outcomes such as neurological, respiratory, metabolic outcomes including obesity,

immune disorders, reproductive, endocrine and cardiovascular dysfunctions and in some cases, it was also linked with cancer.

1.7.1 Linking DOHaD to epigenetic remodelling of embryo

Precise copies of genome sequence are one of the fundamental traits of embryonic cells, however embryos go through epigenetic changes because DNA methylation level and the epigenetic marks at histone status undergo remarkable changes. Initially, the embryos repress the epigenetic and histone modification, however, as embryo forms the blastocyst this repression is decreased, and methylation status rises (Smith *et al.*, 2012; Guo *et al.*, 2014; Okamoto *et al.*, 2016). Even though the developmental stage in which the maximum level of methylation is observed varies among species, it is well known that wholesale epigenetic changes that the embryo encounters occur in the oviduct.

As described previously, the oviductal environment acts as co-factor of embryo development by providing nutrients, embryotropic and growth factors that embryos need. Furthermore, other molecules including glycosaminoglycans, proteoglycans, carbohydrates, ions, hormones, as well as the physiological characteristics of oviductal fluid (OF) including O₂, CO₂ concentrations, pH, temperature, electrolyte concentration is vital for the equilibrium of the oviductal system and subsequently in the early stage of embryo development. Consequently, any alteration that results in disturbance of the oviductal environment's balance could also have an impact on embryo development.

It has been suggested that OF composition varies and is influenced by maternal nutritional, metabolic and inflammation factors (Leese *et al.*, 2008). A preimplantation embryo is susceptible to agitation in its nutritional, organic, biochemical, and physical environment. The developmental program of embryo is modulated via epigenetic pathways which are influenced by oviductal environment alterations, resulting in a modified adult phenotype (Lane, *et al.*, 2014). For instance, in pregnant women the shortage of folate or other nutrients such as methionine, choline, and vitamin B-12, in the maternal nourishment could be responsible for DNA hypomethylation (reviewed by Jacob, 2000). In addition, low protein consumption during the initial 3 days of embryogenesis results on deceleration of cell cleavage and modify the equilibrium of cell differentiation of the blastocyst stage in the mouse (Fleming, Eckert and Denisenko, 2017). The outcome of nutritional disturbance during fertilisation process

influences nutrient transfer ability after birth; the newborn gains weight more sharply and exhibits higher systolic blood pressure combined with elevated anxiety (Sun *et al.*, 2014). Alteration in oviductal temperature or abnormal ROS level could also affect the methylation reprogramming and the DNA demethylation progress respectively with the subsequent influence on embryo phenotype (Pérez-Cerezales, *et al.*, 2018).

Combined, these data, along with the results of studies which suggest that environmental factor play a role for the correct formation of epigenetic marks (Barker, 2007), support the theory that oviductal microenvironment could affect the epigenetic features of the embryo (Pérez-Cerezales, *et al.*, 2018) and any changes in this microenvironment could have a long-lasting effect in later development as it has been suggested by the theory of Developmental Origins of Health and Disease (DOHaD) (Barker, 2007). This hypothesis is reinforced by data which claim that imprinting disorders are 3-4 times more common for ARTs-conceived children compared with natural conceived children (Owen, *et al.*, 2009; Lazaraviciute *et al.*, 2014).

The question that arises is which other factors apart from nutritional factors could potentially change the microenvironment of the oviduct in order to affect the epigenetic marks of embryos. Given the indication that oviductal environment is influenced by maternal nutritional, metabolic and inflammation factors, it is necessary to research the external factors which contribute to the alteration of oviductal microenvironment and the early reproductive events. Considering, the daily interaction and exposure of all organisms in environmental factors and given the intense changes in the environment because of the excessive release of chemical compounds, the target of interest for this thesis is chemical contaminants which are accumulating in the environment resulting on human daily or consistent exposure and their impact on the oviductal secretions as well as oocyte function and early stages of embryo development.

1.8 General Hypothesis and Aims

Given the modern, rising levels of a range of chemicals in the environment and the paucity of knowledge on how many of these compounds might influence reproductive events, this thesis will test the hypothesis that the preimplantation phase of mammalian development will be sensitive to a range of common environmental contaminants.

To test this hypothesis, the thesis has three core aims:

Aim 1: To examine the extent to which the physiology of the cells of the oviduct are modified by environmental contaminants

Aim 2: To explore the impact of exposing oocytes to environmental contaminants on subsequent developmental potential of embryos

Aim 3: To determine the extent to which the environmental contaminants influence the early stages of embryo development and metabolism.

To achieve this, the **bovine** system will be used. The cattle is a good model for early human embryo development, with similar tempo of many key preimplantation events. Moreover, the process of in vitro preparation of cattle embryos is well established and largely free of many of the ethical concerns associated with research on human gametes and embryos. Moreover, as a commercially valuable species, understanding how the reproductive events of cattle may be affected by common environmental contaminants may will be important for future livestock production and food security.

Chapter 2

Material and Methods

Statement of Ethics.

The work in this thesis received favourable ethical review from the Hull York Medical School Research Ethics Committee

2.1 Bovine Oviduct Epithelial Cell culture

2.1.1 Bovine Oviduct Epithelial Cell harvest and maintenance

Bovine reproductive tracts were collected from animals slaughtered for entry into the UK food chain from APB abattoir in York. From harvest, tracts were transported to the lab within 90 minutes of slaughter. The tissue was transported in a buffer consisting of 100mL HBSS with phenol red, without CaCl_2 , and MgCl_2 , 1mL HEPES and 200uL aprotinin from bovine lung. However, after COVID-19 lockdown and due to the restrictions applied in the APB abattoir (contactless approach), bovine reproductive tracts were transferred within plastic bags in warm environment.

On arrival at the laboratory, the oviducts were dissected from bovine reproductive tracts using the method described in Simintiras et al., (2016). Briefly, in each tract the connective tissue surrounding the oviduct was trimmed off using tweezers and scissors. The oviduct was then cut at the utero-tubal junction and placed into a petri dish containing HBSS.

When the desired number of oviducts were dissected, the Bovine Oviduct Epithelial Cells (BOEC) were mechanically isolated into another petri dish containing HBSS. This was done by slitting the oviduct longitudinally and the epithelial lining scraped off using a sterile glass slide. The cells were mixed and centrifuged twice at 2000rpm for 5 minutes in order to remove any blood cells and remaining debris from the solution. After the second centrifugation, the pellet was re-suspended in 10mL BOEC culture medium and cell count and viability were assessed using trypan blue exclusion in a haemocytometer. A sample of cells were mixed with trypan blue in 1:4 ratio to determine cell number and viability. Viable cells with an intact cell membrane do not take up Trypan Blue. A sample of this solution was added to the haemocytometer, in order to count the number of alive cells. All live cells included in squares A, B, C, and D (fig 2.1) were counted and added together and the number of cells per ml was determined.

BOECs were seeded at a density of 2×10^6 cells in T₇₅ flasks in a final volume of 10ml and cultured for 18 hours.

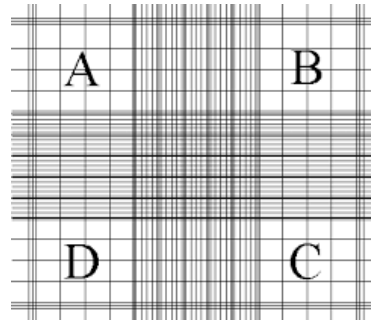


Figure 2.1: Layout of Improved Neubauer haemocytometer. Alive BOEC located in squares A, B, C, D were counted and added together

BOECs were purified based on the observation that fibroblast adhere to flasks faster compared to epithelial cells. Thus, 18 hours after the incubation of the isolated cells, the fibroblast were adhered while the epithelial cells remained in suspension. Thus, the epithelial cells were separated from fibroblasts, washed, and depending on the experiments seeded in conventional type of multi-well or in Transwells. The cell density for 6-well and 12-well conventional plates was 3.5×10^5 cells and 1.75×10^5 , and for Transwell was 0.5×10^6 cells per well. Finally, the media was replaced every 24 hours after the first seeding. Only primary BOECs were used during the experiments of this thesis.

2.1.2 In vitro culture of BOEC and confirmation of a confluent epithelial monolayer in Transwells

In experiments where BOEC were maintained in Transwells, the epithelial cells were seeded at the apical phase and maintained by supplying with BOEC culture media in both compartments (apical and basal) to a final volume of 2ml in each compartment (fig 2.2).

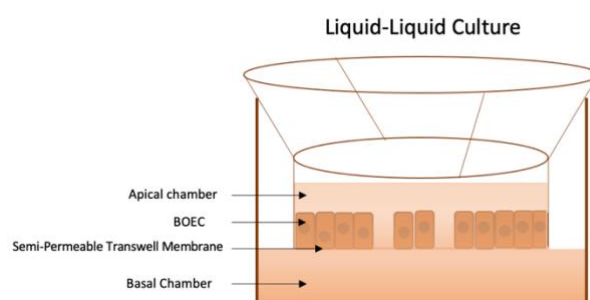


Figure 2.2: BOEC culture in Transwell. The Transwell consist of 2 compartments the apical and the basal compartment and they are separated by a semi-permeable membrane. On this semi-permeable membrane, the BOEC are seeded and maintained by supplementing each compartment with media (liquid-liquid interphase) until the BOEC became confluent.

The confirmation of a confluent epithelial monolayer was identified by measuring the TransEpithelial Electrical Resistance (TEER) by using an EVOM voltohmmeter. Electrical resistance was measured by immersing the electrode probe into the apical and basal compartment of the Transwells. The TEER was calculated using the mathematical formula below:

$$\text{TEER} = (\text{resistance of cells in transwell} - (\text{resistance of the transwell without cells} - \text{medium resistance})) * \text{surface area}$$

The resistance of the transwells containing only the medium was from 120 Ω to 150 Ω and the medium resistance was from 11 Ω to 13 Ω . The surface area of the 6-well and 12-well transwells were 4.52cm² and 1.13cm², respectively. Confluent epithelial monolayer was confirmed at >1500 Ω cm² using a Fluorescein rejection assay described in the following section.

2.1.3 Confirmation of epithelial monolayer integrity using Fluorescein rejection assay

The principle of this method is based on the fluorescein chemical characteristics, which is a non-permeable dye with high molecular weight (332.31g/mol). For this reason, this type of dye is not able to cross an intact epithelial monolayer.

The establishment of a confluent BOEC monolayer and the use of TEER as a non-invasive method to identify the epithelial monolayer integrity, was confirmed using a fluorescein rejection assay as previously has been described in Simintiras et al 2017. Briefly, standard medium was removed from the culture and Krebs-Ringer buffer (Table 2.1 and 2.2) was added in both compartments.

After the monolayer had achieved confluence according to visual assessment, the BOECs were incubated in Krebs-Ringer buffer for 30 minutes at 39°C, 5%CO₂, pH=5.0. Fluorescein was added to the basal compartment of the Transwell at a concentration of 200µM. Samples of 50µl were collected from both compartments every 30 minutes for 200 minutes. Every time a sample was collected TEER was measured as well. After the 120 minutes, 1% EDTA was added in the apical compartment of the Transwell to disrupt the intact monolayer of the epithelial cells. As it is known EDTA is a chelating compound which binds to Ca²⁺⁺ resulting on tight junctions' disruption. The EDTA was used as a positive control of fluorescein transport in the case on a non-intact epithelial monolayer.

The samples of medium retrieved from the apical and basal compartments were then placed in a 96 well plate and the fluorescence intensity was measured over the course of the 200minutes in a BMG plate reader, at excitation 485 nm and emission 520 nm.

The integrity of the BOECs monolayer was investigated at TEER>700Ω*cm² (Fig: 2.3a) (as has previously been suggested by Simintiras et al. 2017) and at TEER>1500Ω*cm² (Fig: 2.3b). BOECs confluence was confirmed by a TEER measurement >1500Ω*cm² (Fig 2.3b).

As shown in figure 2.3a, the fluorescence steadily increases over the course of the 200 minutes in the apical phase of the Transwell when the TEER of the BOECs fluctuate from 750 to 1250 Ω*cm² suggesting that there is fluoresceine transport to the apical compartment leading to the assumption that the BOECs are not fully confluent. However, when fluorescein rejection assay was performed at TEER>1500Ω*cm² (Fig: 2.3b), fluorescein was only observed in apical compartment only when the tight junctions were disrupted by the addition of the EDTA at 120 minutes. The differences noticed between Simintiras results, and the results of these observational experiments are probably based on the different Transwell supplier. For all the subsequent experiments of BOECs in Transwell, TEER>1500Ω*cm² was considered as the minimum threshold of confluency.

Table 2.1: Krebs - Ringer buffer stocks

<u>Stock 1</u>		<u>Stock 2</u>		<u>Stock 3</u>	
Compounds	Amount in grams	Compounds	Amount in grams	Compounds	Amount in grams
NaCl	6.896	NaHCO ₃	2.092	CaCl ₂ .H ₂ O	0.187
KCl	0.353	ddH ₂ O	Make up to volume 100ml	ddH ₂ O	Make up to volume 100ml
KH ₂ PO ₄	0.161				
ddH ₂ O	Make up to volume 100ml				

Table 2.2: Krebs - Ringer buffer composition

Final medium	
Component	Amount
Stock 1	10 ml
Stock 2	10 ml
Stock 3	10ml
Glucose	0.1 g
ddH ₂ O	Make up to volume 100ml

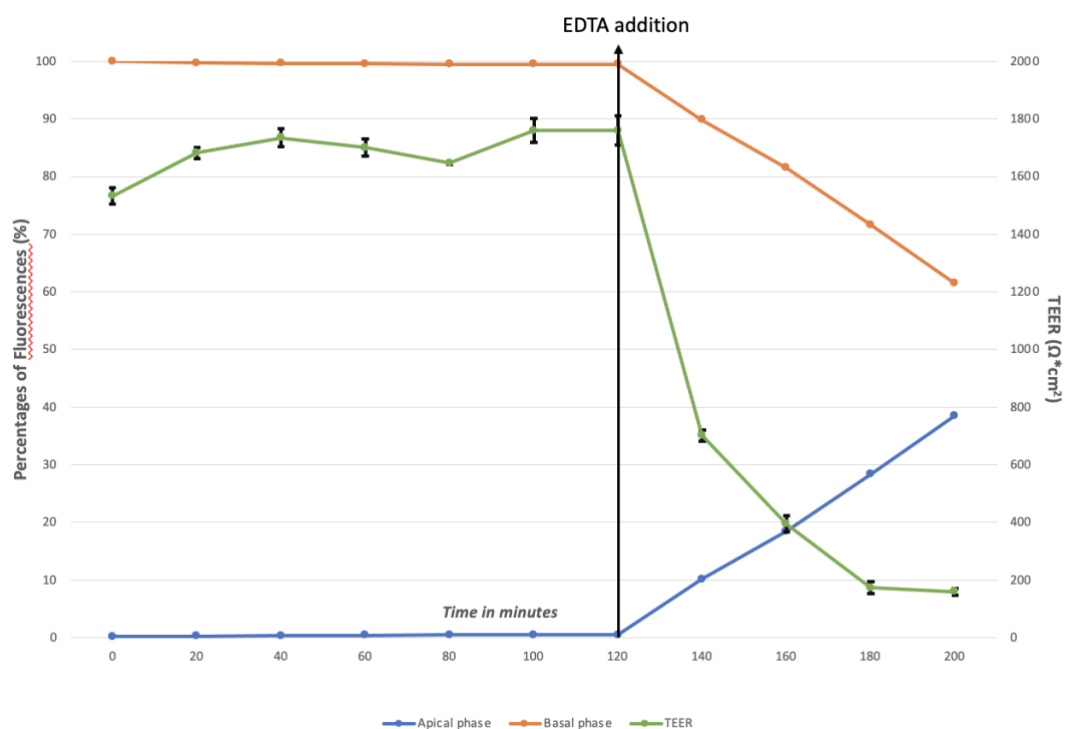
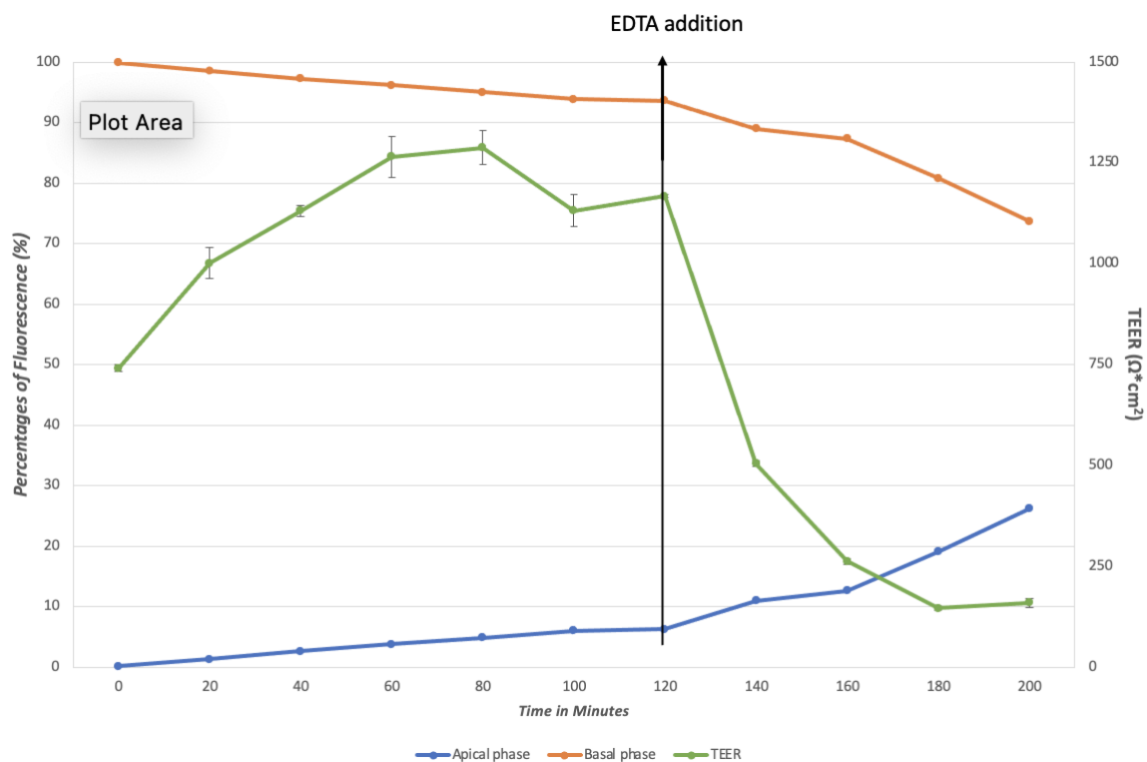


Figure 2.3. Fluorescein transport from the basal to apical compartment of BOECs over the course of 200 minutes when a) TEER > 700 Ω*cm² and b) TEER > 1500 Ω*cm². EDTA was added 120 minutes since the beginning of incubation. This assay performed as observational experiment and it is result of one replicate for each condition (n=1).

2.1.4 In Vitro Derived Oviduct Fluid formation

After the confirmation of an intact epithelial monolayer on the semi-permeable membrane of the Transwell (TEER>1500 ohms) the media used for the cell culture was aspirated from the apical compartment and was not replenished. At this point the BOEC were incubated in an apical-basal air-liquid interface in a moist air at 39°C and 5% CO₂ for 24 hours. After the 24 hours a thin film of fluid was formed on the apical compartment of the Transwell. This formed fluid known as In Vitro Derived Oviduct Fluid (Simintiras et al., 2016) was collected and stored at -80°C until analysis.

2.2 Proliferation assay

Cell proliferation was assessed using the MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay measures cellular metabolic activity as an indicator of cell viability and proliferation. MTT assay is used widely in cell biology and drug discovery research to assess the effects of various compounds, treatments, or experimental conditions on cell growth. The principle of this assay is based on the conversion of the yellow, water-soluble MTT compound into a purple formazan product by mitochondrial enzymes in viable cells. The MTT reagent freely enters the cells through viable cell plasma membranes and is reduced by mitochondrial dehydrogenases to produce formazan crystals, which are insoluble. The formazan accumulates in the cells and can be detected as a coloured precipitate. The amount of formazan produced is directly proportional to the number of metabolically active cells. The MTT assay cannot determine the exact number of cells per well, however based on the absorbance values obtained from treated samples compared to control group the relative cell viability and proliferation can be determined.

2.2.1 Cell Proliferation on BOEC

BOEC were isolated as described in section 2.1. The day after isolation, the cells were placed in 96 well plate at a density of 5000 cell per well, and treated as described in table 2.3 for 24, 48, 72, 96, 120, 144, or 168 hours. After each time point, the supplemented media with treatment was removed and the well washed carefully twice with PBS. Subsequently, media without treatments, but supplemented with 5 mg/ml MTT (MTT; Sigma, M2128) was added

into the 96 well plate and incubated in 5% CO₂, 37°C for 4 hours. The MTT stock was dissolved in 1xPBS and filtered with 0.22µm filters before use.

After the end of the incubation, a 10% sodium dodecyl sulphate buffer supplemented with 0.01M hydrochloric acid (SDS-HCl) was added to the wells and the formed formazan crystal were dissolved by pipetting up and down and incubated in a non- CO₂ incubator at 37 °C for 15minutes. The addition of the HCl into the SDS buffer was performed in order to avoid interference with the absorbance measurements as the media that was used for the assay contained phenol red (Truppo & Turner, 2010). Absorbance was measured in a BMG plate reader at 570 nm and the subtracted absorbance background at 650 nm.

Table 2.3: The final concentrations of the environmental contaminants used for the proliferation assay

<i>Environmental contaminant</i>	<i>High concentration</i>	<i>Low concentration</i>
<i>BPA</i>	10 µM	10 nM
<i>BPS</i>	1 µM	1 nM
<i>TCS</i>	10 µM	10 nM
<i>ACE</i>	1 µM	1 nM

2.3 Bovine oocyte maturation, sperm preparation and In vitro embryo production

2.3.1 Stocks, supplements, and additives preparation for in vitro production

A series of different stock solutions, supplements and additives were necessary to enable the production of culture media for oocyte and embryo culture. The stocks used for the media preparation were prepared under aseptic conditions using embryo graded water. Each stock was sterilised using 0.22 mm filters and osmolarity was checked where relevant using an Osmomat 030 (Gonotec) osmometer. All the stocks were stored at 4°C or -20°C for the appropriate period of time as shown in table 2.4 and table 2.5 respectively.

Table 2.4: Stocks necessary for the Media preparation. All the stocks are stored in 4°C for the appropriate period of time as indicated in the table.

Stock	Chemical	Molecular Weight	Concentration (mM)	Osmolarity (mOsm)	Storage period	Storage Temperature
Stock B	NaHCO ₃	84.01	230mM	430	2 weeks	4°C
Stock BSA	BSA		20% w/v	-	6 weeks	4°C
Stock C	Sodium Pyruvate	110.04	32.7mM	60	2 weeks	4°C
Stock D	CaCl ₂ ·2H ₂ O	147.01	171mM	405	6 weeks	4°C
Stock G	Glucose	180.16	60mM	60	6 weeks	4°C
Stock GLN	Glutamic Acid	146.14	20mM	3	2 weeks	4°C
Stock H	HEPES free acid HEPES sodium salt	238.30 260.30	126mM 125mM	375	6 weeks	4°C
Stock K	kanamycin	383.60	130mM	105	6 weeks	4°C
Stock L	Sodium Lactate syrup 60% (w/v)	-	4.7% w/v	-	6 weeks	4°C
Stock M	MgCl ₂ ·6H ₂ O	203.30	49mM	120	6 weeks	4°C
Stock S2	NaCl KCl KH ₂ PO ₄	58.44 74.55 136.09	1077mM 143mM 12mM	-	6 weeks	4°C
Stock TL	NaCl KCl NaH ₂ PO ₄ ·2H ₂ O Gentamycin	58.44 74.55 156.01	1141mM 32mM 5mM 5% v/v	-	6 weeks	4°C

Table 2.5: Supplements and additives for the Media preparation.

Stock	Chemical	Mass	Volume of ddH ₂ O	Storage period (months)	Storage temperature
EGF:FGF	EGF FGF	0.05 mg/ml	4ml EGF:185µl FGF	3 months	-20 °C
FHS:LH	Menopur	75 IU		3 months	-20 °C
Heparin	Heparin	0.2 g	20 ml	3 months	-20 °C
Pen/Hyp	Penicillamine Hypotaurine	6 mg 2.2 mg	10 ml	3 months	-20 °C
Maturation Additives	Apotransferrin Glutamax II Pyruvate B-mercaptoethanol PVA	25 mg 1 ml 0.11 g 7 µl 25 mg	24 ml	3 months	-20 °C
Percoll additives	CaCl ₂ .2H ₂ O MgCl ₂ .2H ₂ O NaCl KCL NaH ₂ PO ₄ HEPES free acid HEPES sodium salt Na-lactate Gentamycin	0.616 g 0.162 g 0.935 g 0.046 g 9 mg 0.238 g 0.260 g 0.74 ml 1 ml	20 ml	3 months	-20 °C
SPTL	CaCl ₂ .2H ₂ O MgCl ₂ .2H ₂ O NaCl KCL NaH ₂ PO ₄ HEPES free acid HEPES sodium salt Na-lactate Gentamycin Phenol Red	12.3 mg 3.2 mg 292 mg 14 mg 3 mg 74.5 mg 81.4 mg 0.231 ml 0.313 ml 0.25 ml	50.15 ml	3 months	-20 °C

Table 2.6: 50x Amino Acids supplements used in SOFaaBSA and SOFanalysis media for grouped and individual embryo culture respectively. This supplement was stored at -80°C for 3 months

<i>Amino Acids</i>	<i>Mass in 100ml ddH2O</i>	<i>Molecular Weight</i>	<i>Concentration in stock (mM)</i>	<i>Concentration in SOFanalysis (mM)</i>
Alanine	0.098 g	89.09	11	0.22
Arginine	0.4214 g	210.67	20	0.4
Asparagine	0.0751 g	150.1	5	0.1
Aspartate	0.0399 g	133.1	3	0.06
Cysteine	0.0315 g	157.61	2	0.04
D-ABA	0.0516 g	103.12	5	0.1
Glutamate	0.1471 g	147.1	9.5	0.19
Glutamine	0.2922 g	146.14	20	0.4
Glycine	0.03 g	75.06	4	0.08
Histidine	0.0838 g	209.6	4	0.08
Isoleucine	0.0262 g	131.17	2	0.04
Leucine	0.0787 g	131.17	6	0.12
Lysine	0.1096 g	182.6	6	0.12
Methionine	0.0298 g	149.2	2	0.04
Phenylalanine	0.033 g	165.19	2	0.04
Proline	0.023 g	115.1	2	0.04
Serine	0.042 g	105.1	4	0.08
Threonine	0.0596 g	119.1	5	0.1
Tryptophan	0.0408 g	204.2	2	0.04
Tyrosine	0.0725 g	181.2	4	0.08
Valine	0.0351 g	117.1	3	0.06

2.3.2 Media Preparation for the in vitro production

Stocks, supplements and additives listed in the (table 2.4) and (table 2.5) were used for the preparation of media necessary for oocyte collection and maturation, sperm preparation, fertilisation and embryo culture. Holding Media (HM) and Bovine maturation media (BMM) were made up the day of ovary collection as indicated by the (table 2.7). HEPES Tyrode's Albumin Lactate Pyruvate (TALP) and Fertilisation TALP (FERT TALP) were made on the day of fertilisation (table 2.8) and the following day, when embryo culture was taken place HEPES synthetic oviduct fluid (SOF) and SOF supplemented with amino acids (AA) (table 2.6) and bovine serum albumin (BSA) (SOFaaBSA) was prepared (table 2.9). All media were prepared in house under aseptic conditions the day of use and incubated for at least 2 hours in

a warming oven for HEPES-buffered media or gassed (CO₂) incubator where NaHCO₃ buffered. The variance of osmolarity were also checked; all media used were within the range of 270 to 300mOsm and less than <10mOsm between the media were used on the same day.

Table 2.7: Holding Media and Bovine Maturation Media. These media were prepared the day of ovary collection. The osmolarity should be in the range of 270 to 300 mOsm and the difference between these two media should be less that <10mOsm

Holding Media and Bovine Maturation Media Composition		
Stocks/Supplements	HM	BMM
Embryo graded water	80ml	8ml
Media 199	10ml	1ml
Stock B	2ml	1ml
Stock H	6ml	-
Stock BSA	0.2ml	0.1ml
Glutamax	-	0.1ml
Maturation Additives	-	0.222ml
EGF:FGF	-	0.1ml
FSH:LH	-	0.05ml

Table 2.8: HEPES TALP and FERT TALP Media Composition. These media were prepared the day of fertilisation. The osmolarity should be in the range of 270 to 300 mOsm and the difference between these two media should be less that <10mOsm.

HEPES TALP and FERT TALP Media Composition		
Stocks/Supplements	HEPES TALP	FERT TALP
Embryo graded water	15ml	14.4ml
Stock TL 10x	2ml	2ml
Stock B	0.16ml	2ml
Stock BSA	0.4ml	0.4ml
Stock C	0.16ml	0.16ml
Stock D	0.25ml	0.25ml
Stock H	1.2ml	-
Stock L	0.6ml	0.6ml
Stock M	0.2ml	0.2ml
Pen/Hypo	-	0.2ml
Heparin	-	0.02ml

Table 2.9: HEPES SOF and SOFaaBSA Media composition. These media were prepared the day of embryo culture. The osmolarity should be in the range of 270 to 300 mOsm and the difference between these two media should be less than <10mOsm

HEPES SOF and SOFaaBSA Media Composition		
Stocks/Supplements	HEPES SOF	SOFaaBSA
Embryo graded water	7.1ml	5.49ml
Stock S2	1ml	1ml
Stock B	0.2ml	1ml
Stock BSA	0.2ml	0.4ml
Stock C	0.1ml	0.1ml
Stock D	0.1ml	0.1ml
Stock H	0.8ml	-
Stock G	0.25ml	0.25ml
Stock GLN	-	1ml
Stock L	0.1ml	0.1ml
Stock M	0.1ml	0.1ml
Pen/Strep	0.06ml	0.06ml
50x Amino Acids supplement	-	0.4ml

2.3.3 Ovary harvest and preparation

Ovaries were collected from a local abattoir and transported in warmed PBS in a canister within 90 min of slaughter to in the laboratory. However, after COVID-19 lockdown and due to the restriction applied in the abattoir (contactless approach), bovine reproductive tracts were transferred within plastic bags in warm environment and the ovaries were isolated upon the tissue arrival in the lab.

Isolated ovaries were washed twice with warm PBS supplemented with antibiotic- antimycotic (Ab/Am) and placed in a pre-warmed beaker, immersed in warm PBS. Ovaries remained in a warming oven at 39°C during the course of the oocyte aspiration. Pre-warmed Holding Media supplemented with 0.4% of Heparin (HM+), 18G needles, 10ml syringe, and 15ml tubes were placed in the warming oven at least 2 hours before tissue arrival.

2.3.4 Aspiration and in vitro maturation of bovine oocytes

Wash dishes were prepared by adding 400µl of BMM into each of the four wells of a Nunc 4-well plate. Water (2ml) with antibiotic/antimycotic (20µl) was added to the intermediate space between the wells in order to minimise evaporation. *In vitro* maturation tubes were prepared by adding 400µl of BMM to an Eppendorf tube.

1ml of HM+ was aspirated into the syringe and the follicular fluid of the ovarian follicles between 2-8 mm in diameter were aspirated into the syringe. The follicular fluid aspirated from the follicles was dispensed into 15ml falcon tubes and allowed to sediment at 39°C for a minimum of 20 minutes. This allowed heavy materials to sediment at the bottom of the falcon tube. The supernatant was removed and discarded and the sedimented oocytes were resuspended in holding medium without heparin.

The contents of the falcon tube were then decanted onto a 12cm petri dish with a grid scored on the underside and oocytes were selected under a stereomicroscope fitted with a heated stage set at 39°C. The collected oocytes were washed initially in HM without heparin and then three more times in BMM dishes. Finally, the washed oocytes were transferred in groups of 30 to 50 in Eppendorf tubes contained 500µl of BMM at which they allowed to mature for 21-24 hours (39°C, 5% CO₂). Both BMM wash dishes and BMM maturation tubes were prepared the day of tissue arrival and pre-incubated at 39°C, 5%CO₂ for at least 2 hours before use to allow equilibration.

2.3.5 In vitro fertilisation (IVF)

HEPES TALP and FERT TALP solutions were prepared the morning of fertilisation as described in the table 2.8. Similar to BMM preparation, wash dishes and fertilisation dishes were prepared by adding 500µl of FERT TALP into each of the wells while the interwell space was flooded with embryo-tested water to minimise evaporation. The dishes were placed into the incubator at 39°C, 5% CO₂ at least 2 hours before fertilisation.

The sperm used for in vitro fertilisation during this research was cryopreserved from one single bull with proven fertility. Motile sperm was separated from immotile using discontinuous

density gradient method using Percoll. The Percoll gradient consisting of a 45% Percoll solution was layered on top of a 90% Percoll solution. The Percoll gradient was prepared as described in the Table 2.10. After 20-22 hours of oocyte maturation, the sperm was retrieved from liquid nitrogen and thawed in warm distilled water before being layered on top of discontinuous Percoll gradient.

Table 2.10: Percoll gradients preparation. The Percoll gradients were prepared the same morning of fertilisation and placed in warming oven at 39°C one hour before use.

<i>Percoll gradients preparation</i>	
90% Percoll	45% Percoll
4.5ml Percoll	2.0ml 90% Percoll
600µl Percoll additives	2.0ml 1x SPTL

Sperm layered on the top of the Percoll gradients (table 2.10) was centrifuged at 2200rpm for 30 minutes. After this, the supernatant was removed and the pelleted sperm was resuspended in 4ml of HEPES TALP solution, followed by another centrifugation at 1200rpm, for 5 minutes. The pelleted sperm was resuspended in 200µl of FERT TALP and a sperm count was performed using an Improved Neubauer haemocytometer. The sperm dilution for count was 1:20 in ddH₂O in order to render the sperm immotile during the sperm count.

During the sperm centrifugation, matured oocytes were washed three times through pre-equilibrated FERT TALP medium before being placed into groups of 50 in IVF droplets and placed back in the incubator at 39°C, 5% CO₂.until sperm preparation finished. After sperm count, final concentration of 1x10⁶ sperm/ml was added in each IVF well and the gametes co-incubated 20-22h at 39°C, 5% CO₂ and the volume of the well-adjusted to 500ul. The day of IVF was considered Day 0 (D0) of in vitro embryo culture.

2.3.6 In vitro embryo culture (IVC)

HEPES SOF and SOFaaBSA were prepared as described in the (Table 2.9) on the morning of embryo culture and placed in the warming oven and CO₂ incubator respectively for at least 2 hours before use. Wash dish and IVC dish prepared using SOFaaBSA in Nunc 4-well plates and 60 mm culture dishes respectively as pictured in figure 2.4.

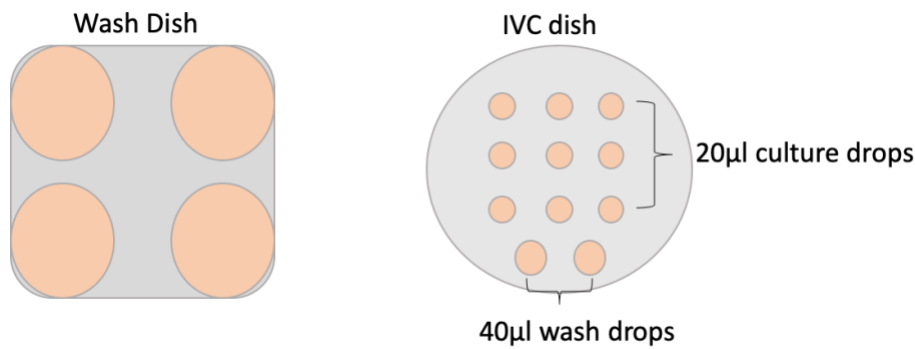


Figure 2.4: Layout of wash dish and IVC dish. Embryos were initially washed twice in the wash dish; the final wash was in 40µl drop in IVC dish. Finally, the embryos were placed in 20µl culture drops in groups of 20.

Approximately 21-22 h post fertilisation, presumptive embryos were retrieved from Fert TALP IVF droplets and vortexed in HEPES SOF to remove cumulus cells and bound spermatozoa. The contents of the tube were rinsed into a 35 mm petri dish. The zygotes were then washed twice through 2 wells of the 4-well wash dish and then once in the 40µl wash drops in IVC dish. Finally, the embryos were placed in 20 µl culture drops in the IVC dish in groups of 20-22. The embryos transferred and cultured for 8 days in an incubator at 39°C in 5% CO₂, 5%O₂, 90%N₂. The rate of cleavage was assessed visually on Day 2 (D2) to Day 3 (D3). Blastocyst rates were also monitored between Days 6 (D6) and Day 8 (D8) of grouped culture.

2.3.7 Individual embryo culture

Individual embryo culture was used to determine nutrient turnover and embryo metabolism at the cleavage and blastocyst stages. For this reason, modified SOF was used, with key metabolites being reduced to enable detection but remaining in the metabolic range. Glucose, pyruvate, and glutamine concentration were adjusted in a detectable range and the lactate was removed from the media in the modified SOF used for the metabolic analysis. This media was referred to as SOFanalysis and was prepared and stored in -80°C for 3 months in 1ml aliquots. When the individual embryo culture was carried out, embryos were retrieved from the grouped culture during the appropriate time for its stage (D2 for 2-4 cells and D7 or D8 for blastocysts) and placed in the SOFanalysis individual droplets. The selected embryos initially were washed twice in a 10µl wash drops, subsequently in a 5µl wash drop and finally single embryos were placed in the 5µl culture drop for 21-24hours (Fig 2.5). The embryo stage and the specific time

of moving into and out of the media was monitored. After embryo removal from the individual embryo culture the dishes were sealed with parafilm and placed in -80°C until metabolic analysis.

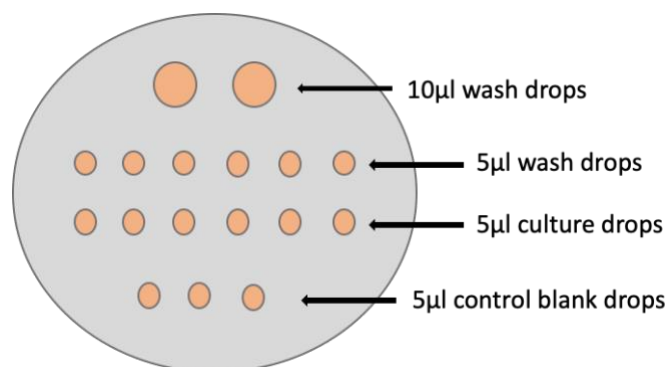


Figure 2.5: Layout of individual embryo culture dish. Embryos selected for individual embryo culture were removed from the group culture and washed twice in SOFanalysis media and subsequently were

2.3.8 Optimisation of embryo production

Low cleavage (40-50%) and blastocyst formation rate (0-12%) was observed initially and for this reason optimisation of the IVP protocol was performed. More specifically, it was noticed that the final sperm preparation and the blastocyst development was poor due to the sperm-clumps formation in the final solution and the low quality respectively. The optimisation of the IVP required a significant amount of time.

During the first step of optimisation, all the stocks were prepared from new chemicals and recalibration of the hypoxic incubator was performed in order to eliminate the option of the external environment (media and incubators) negative effects. However, the low cleavage and blastocyst formation rate was remained.

In the second phase of optimisation, new sperm was acquired and a test on Cumulus-oocyte-complexes (COCs) after in vitro maturation using Hoechst staining was performed in order to confirm that the maturation status of the COCs after IVM was not negatively affecting the IVP. The profile of sperm preparation remained the same after the use of the new sperm indicating that the Percoll Gradients protocol used was a limitation. The Hoechst staining revealed that

22-23 hours of IVM was the optimum time for the COCs to reach the stage of Metaphase I (MI) and Metaphase II (MII).

In the third stage of the optimisation process, the in-house media was compared in every different step (IVM, IVF, IVC) with the commercial media of IVF Bioscience. The comparison revealed the IVM was not significantly different after the use of the commercial media. Cleavage rate was increased (75-80%) after the use of commercial sperm- preparation media, further investigation revealed that the same cleavage rate was obtained after the use of commercial sperm-preparation media combined with either commercial IVF media or in-house IVF media (FERT TALP). This observation led us to replace the Percoll Gradients with the commercial media for semen-preparation from IVF Bioscience. However, in none on the cases, the blastocyst formation rate was improved significantly.

The fact that the blastocyst formation rate remained low after the improvement of the cleavage rate and at the same time possible negative effect of chemicals used for in-house media or/and incubators environment had been eliminated during the first stage of optimisation led on the plasticware replacement used during IVC and also to completely replace EtOH used in the embryology room for standard cell culture with the use of Oosafe disinfectant purchased from IVFSynergy. After the final changes an increase on blastocyst rate was observed.

Finally, an observational experiment (n=1) was performed combining all the positive features noticed in the different stage of the optimisation. The use of new chemicals for the preparation of the media stock, the incubation of COCs during IVM for 22 hours, the use of commercial media (BO-SEMENPREP) for sperm preparation combined with the in-house IVF media (FERT TALP), the use of different plasticware (NUNC 4-well plates) and finally the use of Oosafe disinfectant led us to increase the cleavage rate to 75% - 85% and the blastocyst formation rate to 30%.

2.4 Metabolic assays

By using a microfluorometric assay (Guerif et al., 2013) and high-performance liquid chromatography (Houghton et al., 2002), the quantification of energy substrates and the

turnover of amino acids were determined respectively in BOEC ivDOF, spent cell culture media as well as embryo spent media (SOFanalysis).

The principle of the microfluorometric assays is based on the enzymatic conversion of NADH to NAD on substrate level reactions and measuring the resulting change in fluorescence. This allowed the quantification of glucose, lactate, and pyruvate within spent culture media. By contrast, the detection of the amino acids using High Performance Liquid chromatography is based on size and hydrophobicity differences among the amino acids.

For the determination of the mitochondria activity on bovine oocytes and embryos Oxygen Consumption Rate assay was performed using Seahorse XFp (Agilent Technologies). The principle of this assay is based on the detection of O₂ levels changes in the media by a O₂ sensor-probe when the oocyte or embryos are exposed to mitochondrial inhibitors (Muller et al., 2019).

2.4.1 Principles of Microfluorometric assays

The principle behind the glucose assay (Fig. 2.6) is based on the conversion of glucose to glucose-6-phosphate, a reaction facilitated by enzyme hexokinase (Leese & Barton, 1984). Glucose-6-phosphate is then converted to 6-phosphogluconate through the activity of Glucose-6-phosphate dehydrogenase (G6PD) enzyme. During the second reaction, NADP⁺ is converted to the fluorophore, NADPH. NADPH may be detected when excited with light at 355nm, giving a signal at 460nm. The level of fluorescence is directly proportional to the amount of NADPH formed, which in turn is stoichiometrically linked to the amount of glucose present in the sample. The fluorescence was detected using a BMG plate reader

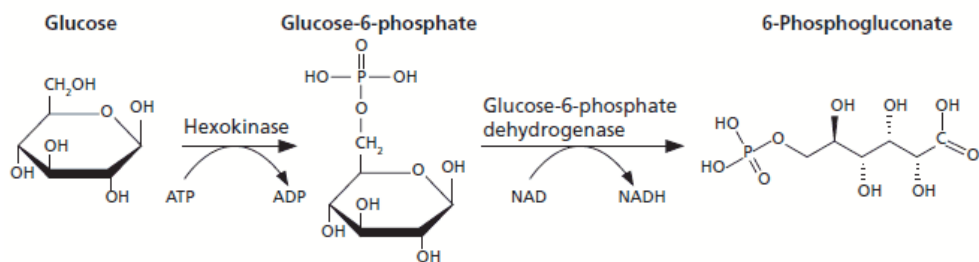


Figure 2.6: Glucose conversion to 6-Phosphogluconate. Initially, glucose is converted to glucose-6-phosphate, in a reaction catalysed by enzyme hexokinase. Glucose-6-phosphate is then converted to 6-phosphogluconate through the activity of Glucose-6-phosphate dehydrogenase (G6PD) enzyme. During the second reaction, NADP⁺ is converted to NADPH and the detected fluorescence is emitted by the produced NADPH, consequently, the higher the levels of glucose which are present in the solution, the higher the levels of fluorescence produced

The lactate assay relies on the conversion of lactate to pyruvate, a reaction catalysed by LDH (Fig 2.7). During this reaction NAD⁺ is converted to NADH. As in glucose assay the fluorescence is emitted by the produced NADH. For this reason, the higher the levels of lactate which are present in the solution, the higher the levels of fluorescence produced. The fluorescence was detected at excitation/emission of 355 nm /460 nm.

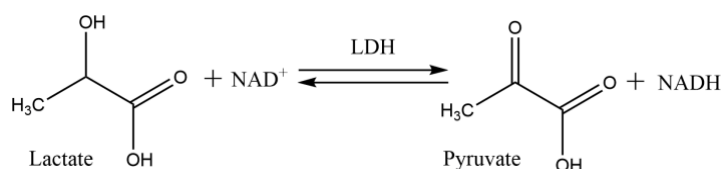


Figure 2.7: Lactate conversion to pyruvate catalysed by Lactate Dehydrogenase (LDH) enzyme. During this reaction NAD⁺ is converted to NADH and the fluorescence emitted by the produced NADH assisting on the quantification of the lactate in the media. the higher the levels of lactate which are present in the solution, the higher the levels of fluorescence produced

The determination of pyruvate is based on the same reaction catalysed by LDH but driven in reverse. (Fig 2.8). When lactate is converted to pyruvate, NADH is oxidised to NAD⁺. However, as previously mentioned the fluorescence detection relies on the NADH concentration in the mixture. For this reason, the higher the levels of pyruvate which are present in the solution, the lower the levels of fluorescence emitted. The fluorescence was detected at excitation/emission of 355 nm /460 nm, using the BMG plate reader.

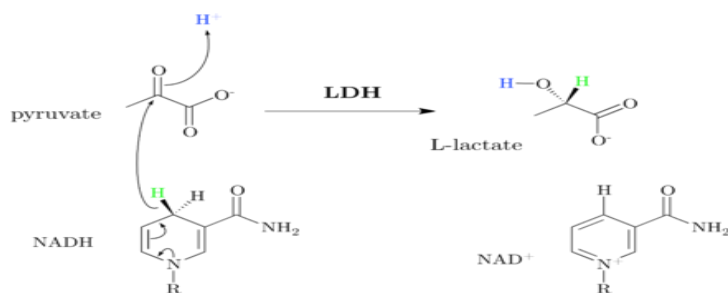


Figure 2.8: Pyruvate conversion to lactate catalysed by LDH enzyme. During this reaction NADH is oxidised to NAD⁺. The fluorescence detection relies on the NADH concentration in the mixture. For this reason, the higher the levels of pyruvate which are present in the solution, the lower the levels of fluorescence emitted.

2.4.2 Quantification of Glucose, Lactate and Pyruvate production or depletion using microfluorometric assays

The amount of glucose, lactate, pyruvate (GLP) present in ivDOF and/or spent cell culture media and/or spent embryo media was quantified by adding 1 µL of sample into 9 µL of the relevant reaction mixture. The stock preparation and the composition of relevant reaction cocktail has been described in table 2.11 and 2.12.

Initially 9 µL of the cocktail was added to the well of 96-well plate, after a short spin the fluorescence was measured. After the first fluorescence measurement, 1 µL of sample was added in the same plate and the plate was incubated at 37°C for a specific time (10 minutes for glucose, 30 minutes for lactate, 3 minutes for pyruvate). In order to avoid evaporation during the incubation time the plate was covered with parafilm. After the incubation a final spin occurred, and the final fluorescence measurement was taken.

Table 2.11: Buffers and stock solutions necessary for glucose, pyruvate and lactate cocktails preparation.

STOCK PREPARATION			
Stock	Chemical	Concentration (mM)	pH
	Glycine	1 M	
Glycine Buffer	Hydrazine Sulphate	400mM	9.4
	EDTA	6.8mM	
EPPS Buffer	EPPS	66.5mM	8
Dithiothreitol	Dithiothreitol	5mM	n/a
Magnesium sulphate (MgSO ₄)	Magnesium sulphate (MgSO ₄)	75mM	n/a
Stock NADP	NADP	10mM	n/a
Stock ATP	ATP	11mM	n/a
Stock NADH	NADH	5mM	n/a
Stock NAD	NAD	60.3mM	n/a

Table 2.12: Composition of glucose, pyruvate, and lactate cocktail for the microfluorometric assays. The cocktails were aliquoted and stored in -20°C for a maximum of two months.

Cocktail Solutions					
Glucose Cocktail		Pyruvate Cocktail		Lactate Cocktail	
Stock	Volume (ml)	Stock	Volume (ml)	Stock	Volume (ml)
EPPS Buffer	15	EPPS Buffer	14	Glycine Buffer	9
NADP	3	NADH	0.3	NAD	1.5
Dithiothreitol	2	LDH enzyme	0.4	LDH enzyme	0.5
MgSO ₄	2			ddH ₂ O	8
ATP	1				
Hexokinase	1				
G6PD enzyme					

The depletion and production of glucose, lactate or pyruvate was calculated by subtracting the difference between the first and second fluorescence measurement. The concentrations of the samples were determined by comparing the fluorescent signal against a six- point standard curve in the range of 0-1 mM for Glucose (Fig 2.9a), 0-2,5 mM for Lactate (Fig 2.9b) and 0-0.45 mM for Pyruvate (Fig 2.9c). Standard curves were prepared prior to each assay. Any standard curve with an $R^2 < 0.98$ was rejected and the respective samples run with it were excluded.

Conditioned media derived from BOEC cultures was analysed in triplicates unless otherwise is mentioned. However, due to the limited volume of the conditioned media after the individual embryo culture (5µl) these samples were analysed once per metabolic assay.

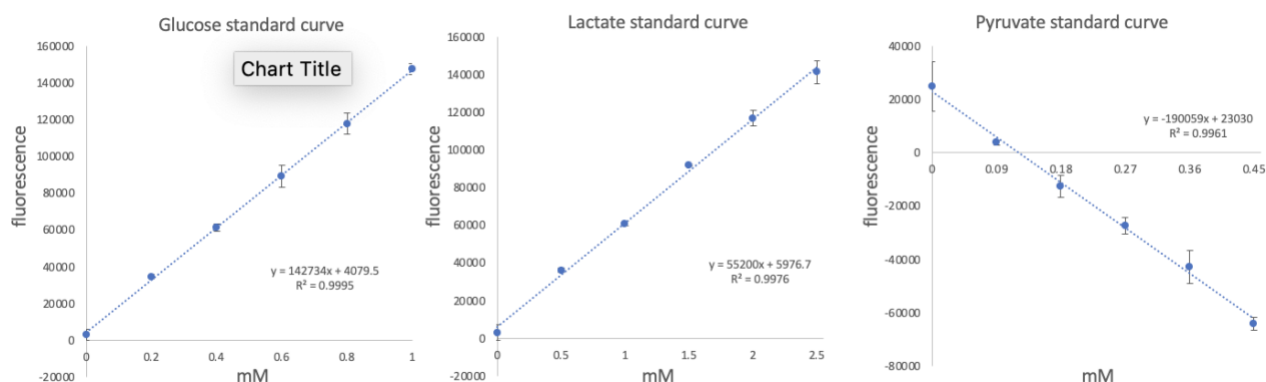


Figure 2.9: Representative standard curves of a) glucose, b) lactate and c) pyruvate used for microfluorometric assays. The concentrations of the samples were determined by using a six- point standard curve, 0-1 mM for Glucose, 0-2,5 mM for lactate and 0-0.45 mM for pyruvate. Any standard curve with an $R^2 < 0.98$ was rejected and the respective samples run with it were excluded.

2.4.3 High Performance Liquid Chromatography (HPLC) principle

The detection of the amino acids in spent media or in ivDOF relied on different size and hydrophobicity of the amino acids. Briefly, mixtures containing the amino acids underwent pre-column derivatisation with O-phthaldialdehyde (OPA), supplemented with 1mg/ml 2-mercaptoethanol, which resulted in the formation of amino acid conjugates with OPA. During this step the amino acids are labelled in order to generate a fluorescent signal, detectable at 450nm, which may be detected by a fluorescence detector attached to the HPLC system. Derivatisation and sample loading were performed using an Autosampling Injecting Unit connected to the HPLC system. Following derivatisation, samples were injected onto a HyperClone™ 5 µm ODS (C18) 250 x 4.6 mm column (Phenomenex, UK). Individual amino acids were eluted from the column using a gradient method comprising two buffers: Buffer A (80% 83 mM sodium acetate: 20% methanol: 0.5% tetrahydrofuran) and Buffer B (80% methanol, 20% 83 mM sodium acetate), both at pH 5.9 (table 2.13). Due to the different proportions of Buffers A and B, the organic phase of the solvent increased, and the aqueous phase decreased. The buffer gradient ensured amino acid separation at different retention times, based on the interaction with the solid phase of the column. Both Buffers were de-gassed using a 0.45 µm membrane filter, before the addition of the tetrahydrofuran in Buffer A.

Table 2.13: Buffer A and Buffer B composition, used for Amino Acid quantification by HPLC.

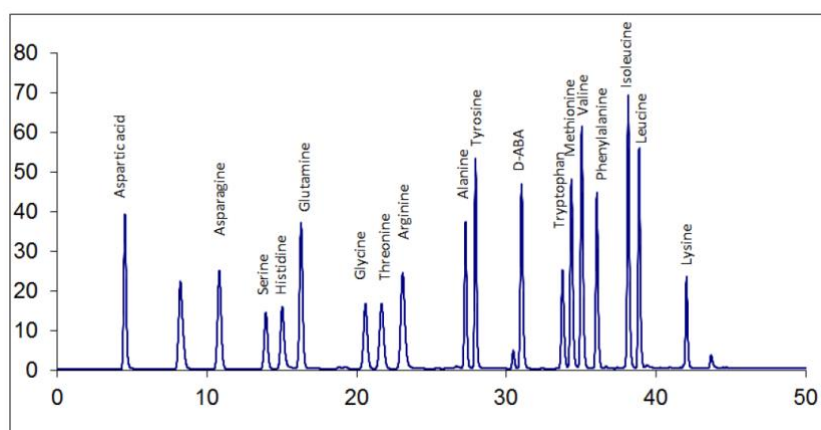
<i>Buffer A</i>	<i>Buffer B</i>
80% Sodium Acetate (83 mM)	20% Sodium Acetate (83 mM)
20% Methanol	80% Methanol
0.5% THF	

2.4.4 Quantification of Amino Acids turnover using HPLC

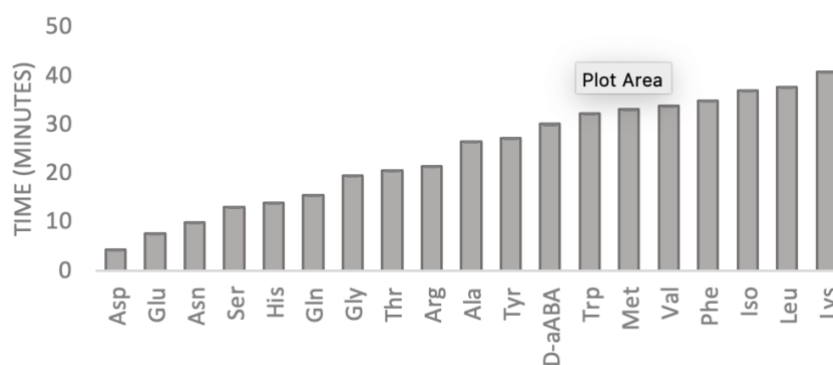
The samples were diluted to 1:12.5 in ddH₂O and analysed with single injection per vial. Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) was used to determine the composition of 18 amino acids in the BOEC IVDOF, BOEC basal media and embryo spent media (SOFanalysis). Using RP-HPLC, the amino acids detected were aspartic acid, glutamic acid, asparagine, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine and lysine (Fig:2.10a). The identification and subsequent quantification of the amino acids in the samples was calculated using the retention time (RT) and the area under the curve (AUC) respectively compared to standards (Fig: 2.10). Commercial, validated amino acids standards were used (AAS18, Sigma Aldrich) supplemented with asparagine, glutamine, tryptophan, and 2-(Diethylomino)-N-(3-phenylmethoxyphenyl) acetamide (D-ABA) which were made in-house. D-ABA served as a non-metabolisable internal standard. The final concentration of the amino acids in the standard solution was 12.5 μ M.

The derivatisation of the sample was executed using an Autosampling Injecting Unit contained in the HPLC system. After the derivatisation the samples were loaded onto a HyperClone™ 5 μ m ODS (C18) 250 x 4.6 mm column (Phenomenex, UK). Over the course of the run, the system run at 1.3ml/min flow rate and the proportion of the Buffer A to Buffer B was decreased, as showing in the following table, resulting on the increase of the organic phase (table 2.14). The composition of Buffer A and Buffer B is described in table 2.13.

a)



b)



c)

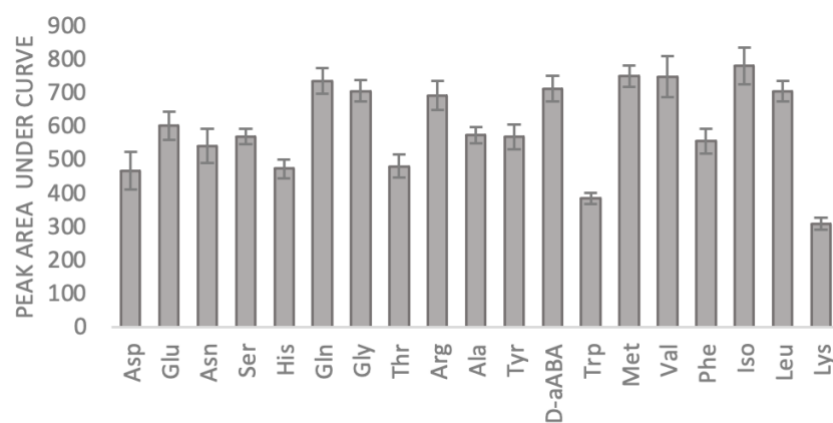


Figure 2.10: Example of amino acids standards as detected using HPLC, a) separation of the amino acids as appeared in the HPLC, its peak represents a different amino acid, b) the retention time of each amino acid and c) the area under the curve of each amino acid.

Table 2.14: Buffer A and Buffer B ratio during the course of one run in the HPLC. The buffer gradient ensured amino acid separation at different retention times, based on the interaction with the solid phase of the column.

Buffer Gradient during HPLC run

Minutes	Buffer A	Buffer B
0	100%	0%
14.5	86%	14%
26.5	50%	50%
42.5	0%	100%

2.4.5 Oxygen consumption rate assay

For the determination of the OCR and its components, on bovine oocytes and embryos, Extracellular Flux Analysis (EFA) was performed by adjusting the protocol previously developed in the lab (Muller et al., 2019). The components of OCR were determined using a Seahorse-XFp analyser (Agilent, Santa Clara, California) using a sensor-including Seahorse fluxpak. The fluxpak (Fig: 2.11a) was hydrated overnight, before the use, using a non-CO₂ incubator at 37°C. After the hydration step, the fluxpak was calibrated for 15 minutes in the Seahorse-XFp analyser according to manufacturer instructions. The protocol included 12 minutes of equilibration step before oocyte or embryos loading, followed by a series of measurements which lasted 3-minute and a re-equilibration period that lasted 1-minute. During the measurement period the sensor probe sank into the well and created an air-tight environment and the changes in the pressure are measured in millimetres of mercury (mmHg) for 3 minutes. In the following step during re-equilibration the sensor was raised for 1 minute (Fig: 2.11b)

For the needs of the experiments performed during this thesis a protocol of 17 points of measurements followed by re-equilibration step was developed (Fig: 2.11c). During the 17 points of measurements, 4 injections took place, the first one was the environmental contaminant of interest in two different concentrations and subsequently 3 injections of 4 different mitochondrial inhibitors followed. These were oligomycin, FCCP, and a mixture of Rotenone/Antimycin A. The final concentration of the environmental contaminants used during the first injection are presented in table 2.15 and the concentration of the mitochondrial inhibitors are presented in table 2.16 The results of OCR assay which is expressed as pmol/min/well were normalised against the number of oocyte or embryos per well.

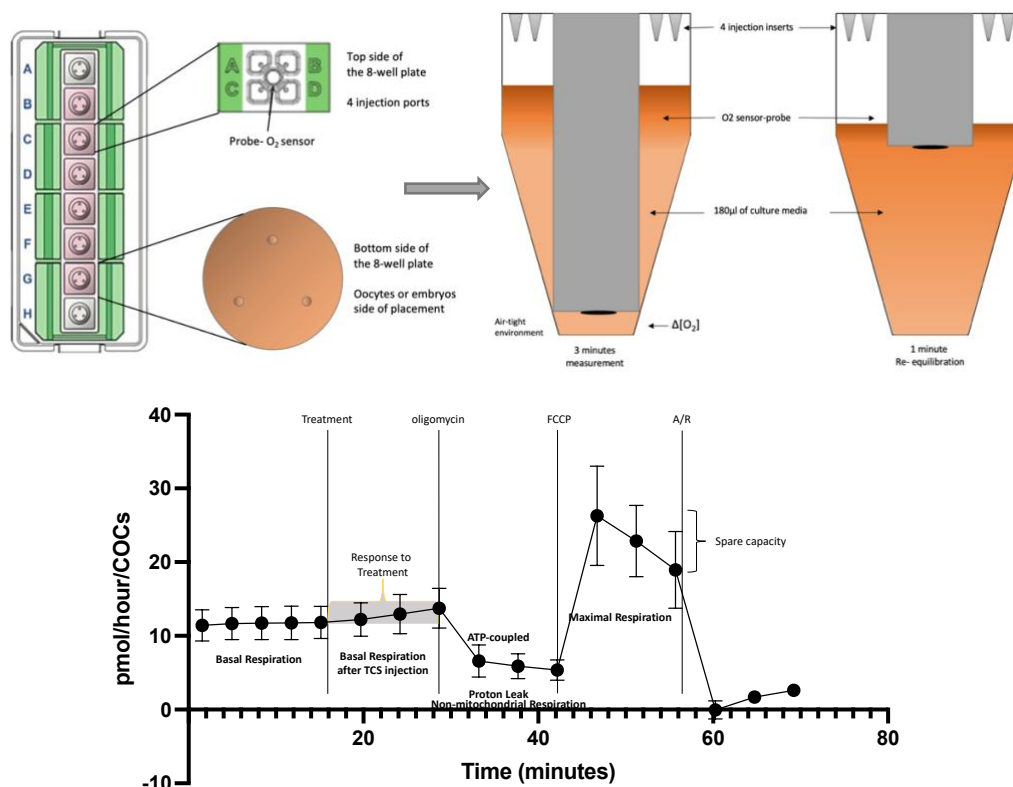


Figure 2.11: The measurement of Oxygen Consumption Rate using Seahorse XFp (Agilent Technologies).

a) The Seahorse XFp consists of a culture plate with 8 well and a cartridge on the top of the plate which contains 4 ports of injection per well connected with a probe containing a O_2 sensor, the 4 ports are used for the treatment, and the 3 mitochondrial inhibitors injections during the assay. b) The O_2 sensor-probe lower into the well creating a microenvironment where the O_2 is measured in mmHg for 3 minutes every 15s. Once the measurement period ends the probe upraise allowing 1 minute of re-equilibration. c) The timeline of the OCR assay, the results created by the Seahorse XFp are in the form of line gram which used for the determination of the OCR and its components as presented above.

Table 2.15: The final concentrations of the environmental contaminants used for the OCR assay

<i>Environmental contaminant</i>	<i>High concentration</i>	<i>Low concentration</i>
<i>BPA</i>	10nM	1nM
<i>BPS</i>	10nM	1nM
<i>TCS</i>	10nM	1nM
<i>ACE</i>	10nM	1nM

Table 2.16: The final concentrations of the mitochondrial inhibitors used for the OCR measure on bovine oocytes and embryos

<i>Mitochondrial inhibitor</i>	<i>Oocytes exposure</i>	<i>Blastocyst exposure</i>
<i>Oligomycin</i>	1 μ M	1 μ M
<i>FCCP</i>	5 μ M	3.75 μ M
<i>Antimycin/Rotenone</i>	2.5 μ M	2.5 μ M

2.5 Relative gene expression

2.5.1 RNA extraction

RNA was extracted from Bovine Oviduct Epithelial Cells cultured in Transwells and 12-well plates as described in Chapter 2 section 2.1.2. After the collection of the media or/and the ivDOF from the 12-well plate and/or the Transwells respectively the cells were gently washed twice with ice cold PBS. Following the wash, 0.5 ml of Trizol was added directly to each well and incubated at room temperature for 10 minutes. Following this, the lysates were transferred to sterile 1.5ml eppendorfs and 200 μ l chloroform was added following which the samples were vortexed for 15 seconds and incubated at room temperature for 20 minutes. Subsequently, the samples were centrifuged at 12,000 $\times g$ for 10 minutes, at 4°C. Phase separation was achieved after this centrifugation where the aqueous layer (top layer) contained the RNA, the interphase and organic layer (bottom) contain the DNA and proteins, respectively. The aqueous phase was then carefully removed without disturbing the interphase and the organic phase, precipitated with 1ml isopropanol for molecular biology and incubated at room temperature for 15 minutes.

After isopropanol precipitation step, samples were centrifuged at 12,000 $\times g$, 4°C for 40 minutes, and the pellet was resuspended in 70% (v/v) EtOH in cell graded H₂O and centrifuged again at

12,000xg, at 4°C for 10 minutes. This step was repeated for three times and any remaining organic solution was removed by pulse centrifugation. Finally, the RNA was re-suspended in 50µl of DEPC water. Using a Nanodrop spectrophotometer the purity and concentration of the RNA isolated was assessed by calculating the ratio of A_{260}/A_{280} , and A_{260}/A_{230} which is indicative of protein and the phenol red contamination of the sample respectively. Any samples with $A_{260}/A_{280} < 1.9$ was discarded. The absorbance was measured, and samples of known concentration and purity were immediately stored at -80°C.

2.5.2 Optimisation of RNA extraction

Applying the above protocol on BOECs cultured in Transwell resulted in low RNA purity or/and low RNA yield. For this reason, optimisation of the above protocol was performed. Initially, old isopropanol and chloroform stocks were replaced with new reagents of molecular grade. However, the yield of the RNA remained low. In the second phase of the optimisation different combinations of Trizol, chloroform, isopropanol, and ethanol was applied in different samples. Finally, the overnight incubation in -20°C against the room temperature incubation after the addition of isopropanol was also investigated. The combination which gave the purest and highest RNA yield combination was when cells were treated with 0.5ml Trizol combined with 0.2ml chloroform, after the isolation of the aqueous phase 0.5ml of 100% molecular graded isopropanol was added and incubated at room temperature for 1 hour. Finally, after the centrifugation the yield was washed with 75% Ethanol for 3 times.

2.5.3 Complementary DNA (cDNA) synthesis

High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368814) was used for the generation of cDNA libraries from the RNA extracted samples. Proper dilutions in H₂O were performed to have a final concentration of ng/µl to a final volume of 10µl.

A master mix containing necessary components for cDNA synthesis was prepared fresh (table 2.17), prior to each cDNA synthesis experiment. The master mix was then mixed with the diluted RNA, the samples were gently mixed and loaded onto an Applied Biosystems 2720

Thermal Cycler for cDNA synthesis. The conditions are described in table 2.18. cDNA samples were stored at -20°C for short term use or at -80°C for long term storage.

Table 2.17: Master Mix for cDNA libraries preparation

<i>Master Mix</i>	
Component	Volume (ul)
10xRT buffer	2
25xdNTP mix (100mM)	0.8
10xRT random primers	2
MultiScribe™ Reverse Transcriptase	1
ddH ₂ O H ₂ O	4.2
Total volume	10

Table 2.18: Conditions for cDNA synthesis

<i>Conditions for cDNA synthesis</i>				
	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2.5.4 Quantitative Polymerase Chain Reaction (q-PCR)

The efficiency of the primers was tested using the reaction conditions and qPCR thermocycler conditions presented in table 2.19 and table 2.20, respectively. The C_t values were plotted against the log of the cDNA concentration. The determination of the PCR reaction efficiency was calculated using the following formula:

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

Two copies of the target sequence per PCR cycle are produced when the primers bind to the target gene sequence in efficient and exclusive way resulting in 100% efficiency. Any efficiency less than 90% suggests lack of specificity to the sequence target whilst high efficiency -more than 110%- implies amplification of multiple target sequences, beyond the target sequence. Any efficiency values between 90% and 110% were considered acceptable. An example of this, as well as the efficiency of each primer set is presented in Fig: 2.12. Primers with efficiency lower than 90% or higher than 110% were excluded from the analysis. The sequences of primers used for qPCR analysis are presented in table 2.21. The primers of SLC7A3 gene have been excluded from the analysis due to low efficiency.

Table 2.19: Reaction conditions

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

Table 2.20: qPCR thermocycler conditions

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

Table 2.21: Primers' sequences

Primer name	Primer sequence	Gene
RPL19 Forward	GAAAGGCAGGCATATGGGTA	Reference gene
RPL19 Reverse	TCATCCTCCTCATCCAGGTT	
b-Actin Forward	CGC CAT GGA TGA TAT TGC	Reference gene
b-Actin Reverse	AAG CCG GCC TTG CAC AT	
SLC1A4 Forward	GAG GGG TCC TCA CCA TCG CCA	Amino acid transporter
SLC1A4 Reverse	TGG TGG TGC GGT CCA CAA TCC	
SLC1A5 Forward	AAC GGC ACT CTG GTG AAG GTG	Amino acid transporter
SLC1A5 Reverse	CTT CCG CAG GGC CAC ACC AA	
SLC7A1 Forward	GAC AAG GGC ACG TGG GTC CG	Amino acid transporter
SLC7A1 Reverse	AGT CCT TGA GGG GTC GGC GG	
SLC7A2 Forward	CTG CAA GTG CCA GGG ACC CAC	Amino acid transporter
SLC7A2 Reverse	GGT TGC AGC CCC AGC CAA AGT	
SLC7A3 Forward*	GTA GCC CCA ACC CAA CTC GGC	Amino acid transporter
SLC7A3 Reverse*	TGC TAG GAA GGA TCG AGG AGC TGT	
SLC7A4 Forward	CAC CTT CCA GAT GCC CCT GGT G	Amino acid transporter
SLC7A4 Reverse	TGA AGC GCA CCC AGG TCA GG	

SLC2A1 Forward	TAACCGCAACGAGGAGAACC	Glucose transporter
SLC2A1 Reverse	AGAAAACAGCGTTGATGCCG	

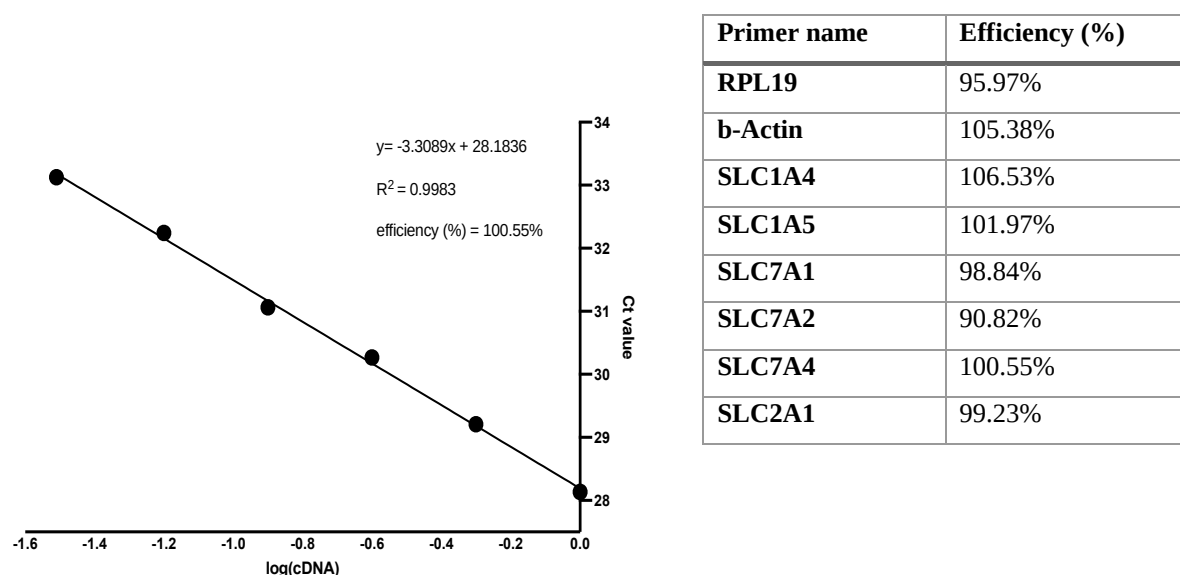


Figure 2.12: Primer's efficiency. a) Example of efficiency curve, presented as Ct values against log (cDNA), b) the efficiency of each primer.

Following the determination of the primer's efficiency, the same reaction and thermocycler conditions using 25ng of cDNA per reaction used for the determination of the relative expression of these genes in the experimental samples (presented in Chapter 3). The relative gene expression analysis was performed using $\Delta\Delta Q_c$ method.

2.6 Enzyme-linked Immunosorbent Assay (ELISA)

2.6.1 General principles of ELISA

ELISA is an immunoassay widely used to detect and quantify a range of different molecules such as peptides, proteins, hormones, antibodies. Elisa is a colorimetric 96-well plate assay through which we can determine and quantify a specific target antigen in a mixture based on the colour change of the substrate which modified by the enzyme. Different combinations of

antigen-antibody are used and depending on this combination ELISA method is divided on 4 different types, direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA.

Competitive immunoassay is the type of ELISA use for this research. This type of ELISA is based on the competition between the antigen in the sample and the reference antigen for binding to a specific amount of antibody which is immobilised on the surface of the microplate. The reference antigen is conjugated to alkaline phosphatase which catalysed the substrate and resulting on colour change in the well. Depending on the amount of the antigen in the sample more or less of the conjugate antigen will be able to bind on the primary antibody. Practically this means that the more antigen is present in the sample the less reference antigen will be bind the weaker the signal will be.

2.6.2 17b-estradiol and progesterone quantification using ELISA assay

The detection and quantification of 17b-estradiol and progesterone produced by cumulus-oocyte-complexes (COCs) during in vitro maturation was determined by using the ENZO 17b-estradiol ELISA kit (ADI-900-008, ADI-900-011) following the protocol as suggested by the manufacturer.

Briefly, after COCs harvest and in vitro maturation as described in 2.2.3.1 the bovine maturation media was stored in -80°C until analysis. For the ELISA assay standards and samples were added to microplate coated with IgG antibody. 17b-estradiol or progesterone conjugated with alkaline phosphatase as well as an antibody to 17b-estradiol or to progesterone was added in the microplate for the detection of 17b-estradiol or progesterone respectively. After a room temperature incubation, the plate was washed in order to remove any unbound antigen. Finally, pNpp substrate was added in the wells which forms a yellow colour as it catalysed by the alkaline phosphatase.

The absorbance was measured in 405 nm using a BMG plate reader. The levels of the 17b-estradiol or progesterone in the sample was quantified by using a 4-parameter logistic curve as suggested by the manufacturer.

The 17 β -estradiol ELISA kit (ADI-900-008) detection limit is 28.5 pg/ml with the inter-assay coefficient of variation ranged from 5.2 to 7.4% and intra-assay coefficient is and 8.4 to 9.2%. The testified sensitivity of the progesterone ELISA kit (ADI-900-01) is 8.57 pg/ml and the inter-assay and intra-assay coefficient of variation was varied from 2.7 to 6.8% and 4.9 to 7.6%, respectively.

Chapter 3:
The impact of environmental contaminants of interest on BOECs

3.1 Introduction

3.1.1 Bovine epithelial cells

The tubal lumen of the oviduct is lined with a layer of epithelial cells along its entirety, that mainly consists of two types of cells: ciliated and the secretory cells (Leese, 1988). Each cell type performs different activities of the oviduct; the ciliated cells participating in processes associated with reproduction (e.g. sperm capacitation, sperm and embryo transfer) and the secretory cells are involved with the production and secretion of the oviductal substances, (Gonella-Díaz *et al.*, 2017) which create the microenvironment for reproductive procedures such as the sperm selection and preimplantation embryo development (Pérez-Cerezales, *et al.*, 2018). In vivo, the epithelial cells create tight junctions between them, allowing the formation of a polarised monolayer. The basal side of the epithelial cells are connected with the stroma cells whereas the apical phase is exposed to the lumen of the oviduct.

This architecture of the cells cannot be mimicked accurately by conventional cell culture (flasks and multi-well plates), however, the so-called Transwell system has been used in different types of epithelial cells in order to mimic the in vivo epithelial arrangement (Chen *et al.*, 2013b; Simintiras *et al.*, 2016). This system has been used previously to study nutrient transport across the bovine oviduct epithelial layer (Simintiras *et al.*, 2016). Using such an approach, Simintiras *et al.* were able to demonstrate that the transport of key nutrients, including glucose, lactate, pyruvate and amino acids into the luminal compartment could be significantly affected by pathophysiological concentrations of reproductive hormones. These data indicated that the oviductal concentrations of compounds that play key roles in early embryonic development can be modelled and modified by endocrine disruption. Furthermore, this prior work established a pipeline to enable studies into the way that extraneous factors can influence nutrient transport properties of the oviduct.

3.1.2 Transwell system

The Transwell system consists of multiple-well plates and each well contains an insert with a semipermeable membrane, creating a basal and an apical compartment. Epithelial cells are seeded in the apical compartment on the semi-permeable membrane allowing at the same time to stay connected with the basal compartment of the Transwell. When the epithelial cells are growing in this arrangement, they can form a polarised monolayer imitating the *in vivo* condition. Importantly, the existence of a functional intact monolayer in a Transwell system can be confirmed by measuring TransEpithelial Electrical Resistance (TEER) (Chen *et al.*, 2015).

The *in vitro* model of BOEC had been previously established in the laboratory (Simintiras *et al.*, 2016); and further been confirmed by Roussi (2020), confirming the successful isolation and culture of the cells as per the previous model and the successful long term culture of BOEC in the Transwell system. For this reason, further confirmatory experiments were not conducted in this project.

Experiments of this chapter were initially performed using conventional culture system, as a pilot study, and subsequently the Transwell system were used to identify any potential impact of the environmental contaminants of interest on the BOEC physiological and metabolic function.

3.2 Hypothesis and Aims

The environmental contaminants of interest, Bisphenol A, Bisphenol S, Triclosan and Acetamiprid, have been identified in human bodily fluids and circulate into the human body through the blood stream (Chapter 1, section 1.5). We hypothesise that the nutrient profile of the oviduct could be affected by such exposure. To test this hypothesis bovine was used as a model. The aims of this project were to determine any alteration on metabolic function of BOEC and subsequently if the BOEC secretions were impaired after long exposure to the chosen panel of environmental contaminants by investigating the following aims:

- ⇒ The impact on BOECs energy substrates uptake and release
- ⇒ The Impact on BOECs proliferation
- ⇒ The effects on BOECs tight junctions' formation by using TEER as measurement
- ⇒ The effects on BOECs secretions after long exposure using air-liquid interphase system
- ⇒ The effects on gene expression of transporters related to BOECs secretion

3.3 Methods

3.3.1 Identification of the suitable solvent for the environmental contaminants stock preparation

3.3.1.1 Identify the suitable solvent for the metabolic experiments

The environmental compounds of interest (BPA, BPS, TCS, ACE) were not water soluble, thus an organic compound solvent needed to be chosen. To identify the solvent with minimal impact on BOECs function, the effects of ethanol and DMSO were initially investigated against untreated BOECs in terms of their metabolic activity. BOECs were exposed to ethanol and DMSO (0.1%) for 7 days and the release of energy substrates (glucose, pyruvate, and lactate) were quantified in the spent media in 24 hours intervals as described in Section 2.4.2. Lactate and pyruvate release was not significantly influenced by the presence of any of the two solvents (fig 3.1b, c). However, glucose uptake was significantly decreased when the BOECs were exposed to ethanol for 96 and 120 hours whilst DMSO did not cause any statistically significant difference (fig 3.1a). For this reason, DMSO was chosen as the solvent vehicle of the environmental contaminants for all subsequent experiments

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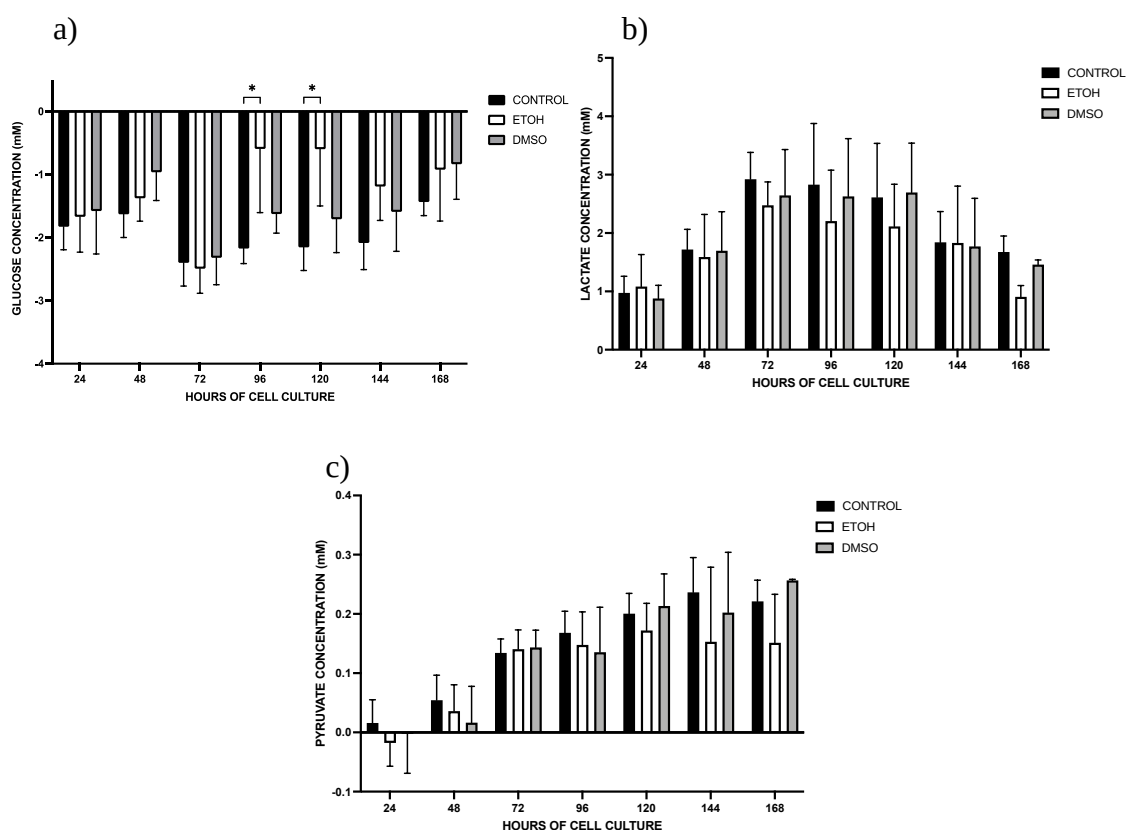


Figure 3.1: a) Glucose uptake, b) pyruvate and c) lactate release after BOECs were exposed to ethanol (EtOH) or DMSO. The energy substrates were measured in conditioned over the course of 7 days in 24 hours intervals. The exposure started immediately after the BOECs were attached on the wells and the media supplemented with the solvents was replaced every 24hours. Each graph is result of 4 biological replicates (n=4). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

3.3.1.2 Environmental contaminants stock preparation and storage

Based on the results in Figure 3.1, DMSO was chosen as the solvent of the environmental contaminants BPA, BPS, TCS, and ACE, the effects of which were investigated during these set of experiments. The effects of two different concentrations of each environmental contaminant were investigated (table 3.1). Each environmental contaminant was diluted into 100% DMSO (CAS: 67-68-5) creating a 1000x stock of each final concentration and stored in aliquots at -20°C in glass vials for no longer than 3 months to prevent degradation. The day before use, the vial was thawed and stored at 4°C for up to 5 days. Finally, the stocks were

further diluted into BOEC media immediately prior to the supplementation in the cell culture medium.

Table 3.1: Final concentration of the environmental contaminants, BOECs were exposed to

<i>Environmental contaminant</i>	<i>'High' concentration</i>	<i>'Low' concentration</i>
<i>BPA</i>	10 μ M	10nM
<i>BPS</i>	1 μ M	1nM
<i>TCS</i>	10 μ M	10nM
<i>ACE</i>	1 μ M	1nM

3.3.2 Experimental design overview

All the techniques mentioned in the experimental design were conducted as described in detail in Materials and Methods (Chapter 2).

To examine the impact of environmental contaminants on BOECs metabolic status, epithelial cells were isolated and seeded as described in Chapter 2, section 2.1.1. Cells were treated with environmental contaminants 24 hours after seeding (table 3.1). The BOECs were exposed to the BPA, BPS, TCS, ACE over 7 days, with media replenished every 24 hours. At media change, spent media was isolated and stored in -20°C for 4 weeks or -80°C for longer period, until further analysis. Subsequently, glucose, pyruvate and lactate concentration were measured in spent media using fluorometric assays and the energy substrates uptake and release was calculated as described in Chapter 2, section 2.4.2 and 2.4.4. The results of this set of experiments are presented in Figure 3.3 to Figure 3.5

To explore the effect of environmental compounds' on BOEC proliferation, epithelial cells from bovine oviduct were isolated and seeded in 96 well plates at a seeding density of 5000 cells per well. Cells were treated with environmental contaminants as described above. Every 24 hours an MTT cell proliferation assay was performed as described in Chapter 2, section 2.2.1 The proliferation of each group was calculated compared to the untreated cells as a reference. The proliferation rates of the BOECs treated with environmental contaminants are presented in Figure 3.4.

In the next set of experiments, BOECs were cultured in Transwells as described in section 2.1.2 to investigate the impact of the environmental contaminants on epithelial integrity of BOEC's as well as on the composition of the in vitro derived oviduct fluid (ivDOF).

For measures of epithelial integrity in response to BPA, BPS, TCS and ACE, BOECs were placed in Transwell system and cultured in liquid-liquid interphase for a period of seven days. During this period, media supplemented with the treatments (table 3.1) was administrated to BOECs in 24 hours intervals and the transepithelial electrical resistance (TEER) was measured as described in Chapter 2, section 2.1.2 The results are presented in Figure 3.6.

After seven days of exposure to the environmental contaminants of interest in liquid-liquid interphase, the BOECs were transferred in air-liquid interphase culture (Chapter 2, section 2.1.4) and the exposure continued through the basal compartment for a further 7 days. After 24 hours in air-liquid interphase, BOECs generated a thin film of fluid known as ivDOF (Chapter 2, section 2.1.4 and Simintiras *et al.*, 2017). The ivDOF generated in the apical compartment of the Transwell was isolated every 24 hours and stored in -80°C for up to 3 months until further analysis. The ivDOF was used to identify the glucose, pyruvate and lactate and the amino acids composition as described in Chapter 2, section 2.4.2 and 2.4.4, respectively. Graphical presentation of this experiment is presented in Figure 3.2. The results of the environmental contaminants' impact on BOECS secretions are presented in Figure 3.8 to Figure 3.14.

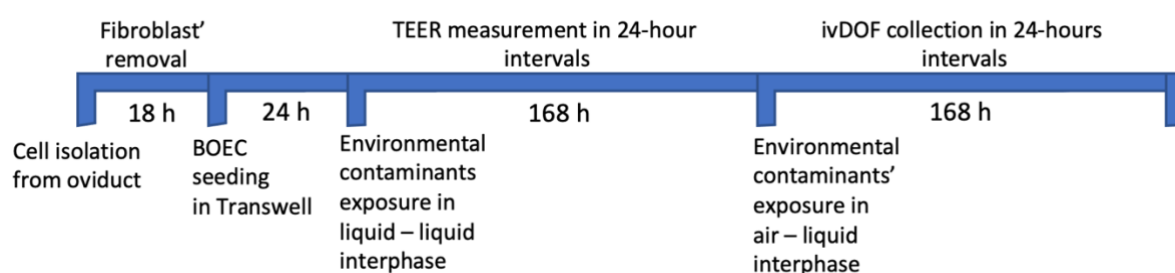


Figure 3.2: Graphical presentation of the experiment investigating the impact of environmental contaminants exposure on epithelial secretions (ivDOF). After the fibroblast removal from the culture the BOEC were seeded in the semipermeable membrane of the Transwell in liquid – liquid interphase. 24 hours later, the media was replaced with media supplemented with the treatments. The cells were exposed to the treatments for 7 days (168 hours) and the TEER was measured in 24 hours intervals. After the course of the 7 days in liquid – liquid interphase the cells were transferred in air-liquid interphase and the exposure to the environmental contaminants continued through the basal compartment for another 168 hours. During this period the ivDOF secreted by the BOEC in the apical compartment was collected at 24-hour intervals. During the whole period of exposure (14 days), the media was replaced every 24 hours.

Finally, based on the results of the impact on energy substrates and amino acids composition in ivDOF of BOEC exposed to BPA, BPS, TCS and ACE, the gene expression of a panel of five amino acids transporters and one glucose transporter were chosen in order to investigate the origin of the detected changes. Since, the major impact on ivDOF composition was observed during the first 24 hours of exposure in air-liquid interphase, the above experiment was repeated (fig 3.2) with the only difference that after 24 hours of exposure to air-liquid interphase the cells were isolated and RNA extraction, synthesis of cDNA libraries and finally qPCR analysis was performed as described in Chapter 2 section 2.5.1 to section 2.5.4.

3.4 Statistical Analysis

GraphPad Prism 9 was used for all statistical analysis. Shapiro-Wilk test was used to define whether a parametric or non-parametric analysis is required. When the data was normally distributed, One-Way ANOVA was used followed by Tukey's *post-hoc test*. However, when normal distribution was not followed, log transformation was performed and in the case that was not possible a Kruskal-Wallis test was performed followed by Dunn's *post-hoc test*. Amino acid data analysis was performed using Two-Way ANOVA with Tukey's *post-hoc test* after log transformation was performed.

3.5 Results

3.5.1 Pilot study: Impact of environmental contaminants on BOEC function

3.5.1.1 Impact on BOECs energy substrate uptake and release

The graphs in figure 3.4 represent the glucose concentration taken up by BOECs exposed to BPA, BPS, TCS, and ACE (table 3.1) every 24 hours over the course of seven days. Despite small fluctuations between the control and the treated groups, the depletion of glucose was not significantly impacted by the presence of any environmental contaminant at any administrated dose.

Pyruvate released by the BOEC after exposure to each environmental contaminants is illustrated in figure 3.4. Pyruvate concentration was significantly decreased after 96 hours of exposure to the high concentration of BPA (10 μ M) compared to control group (0.075 ± 0.011 mM vs 0.17 ± 0.018 mM $p=0.007$), (fig: 3.4a). Moreover, it seems that pyruvate concentration in spent medium from the untreated BOECs (control group) increased every day of cell culture; a trend not observed by the cells exposed to the 10 nM of BPA. However, when the BOECs exposed to 10 μ M of BPA the pyruvate concentration remained steady over the course of 7 days. These results indicate that 10 μ M BPA has a chronic effect on pyruvate release by BOEC.

In contrast, an acute effect on pyruvate release was seen when the BOECs were exposed to BPS (fig: 3.4b), TCS (fig3.4c), and ACE (fig: 3.4d). The low dose of BPS (1nM) significantly increased the concentration of pyruvate in the spend medium compared to that of untreated group the first 24 hours of exposure (0.062 ± 0.02 vs -0.027 ± 0.022 mM $p=0.011$). However, this difference vanished after 48 hours of exposure. Similarly, TCS and ACE induced a significant increase in the concentration of increased the pyruvate release after 24 and 48 hours of exposure irrespective of the administrative concentration in both cases. After 96 hours of exposure to TCS or ACE the pyruvate release returned to levels similar to those of control group.

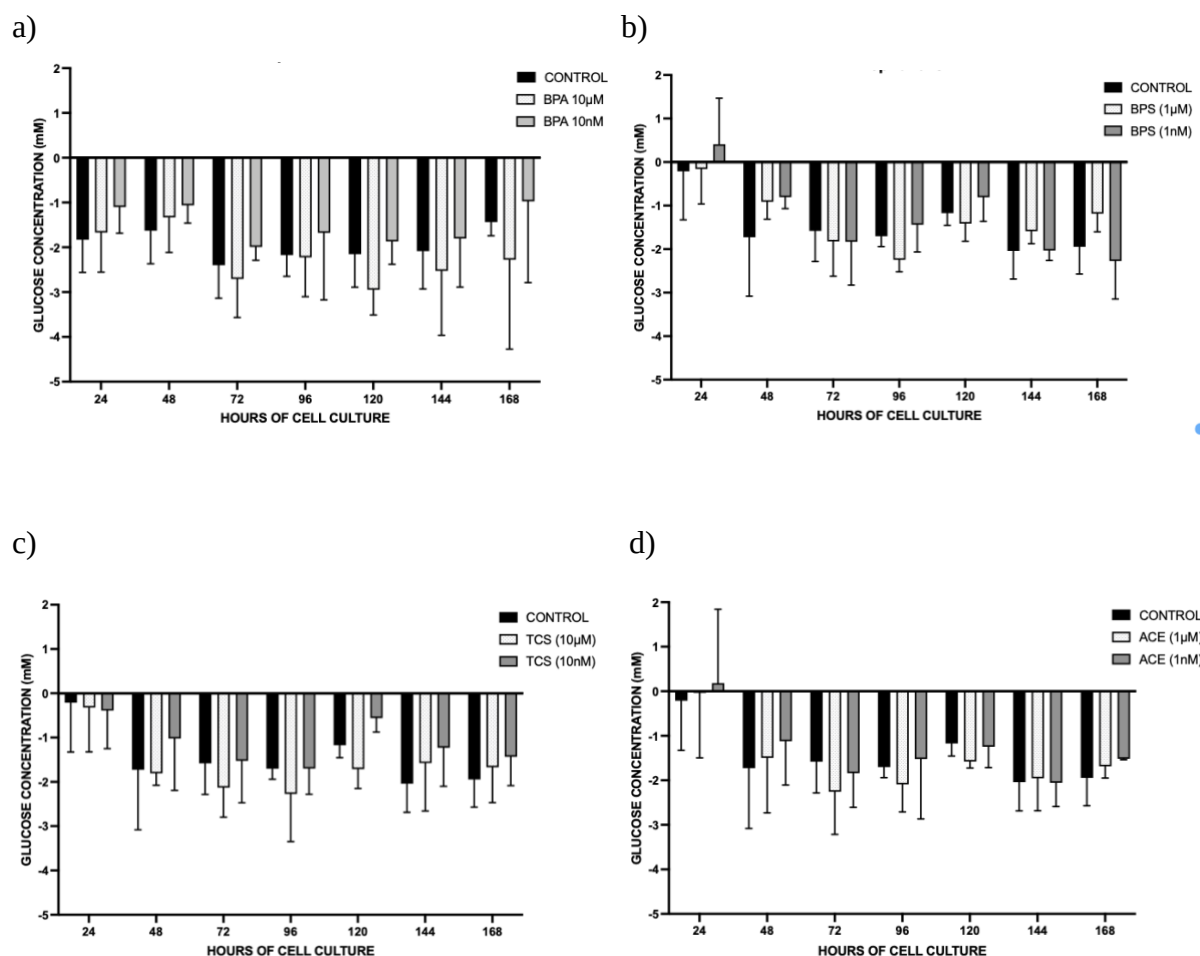


Figure 3.3: Glucose uptake after BOECs exposed to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamidiprid. BOECs glucose was measured in conditioned media over the course of 7 days in 24 hours intervals. The exposure started immediately after the BOECs were attached on the wells and the media supplemented with the chemicals was replaced every 24hours. Each graph is result of 4 biological replicates for BPA and 3 biological replicates for BPS, TCS and ACE. Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

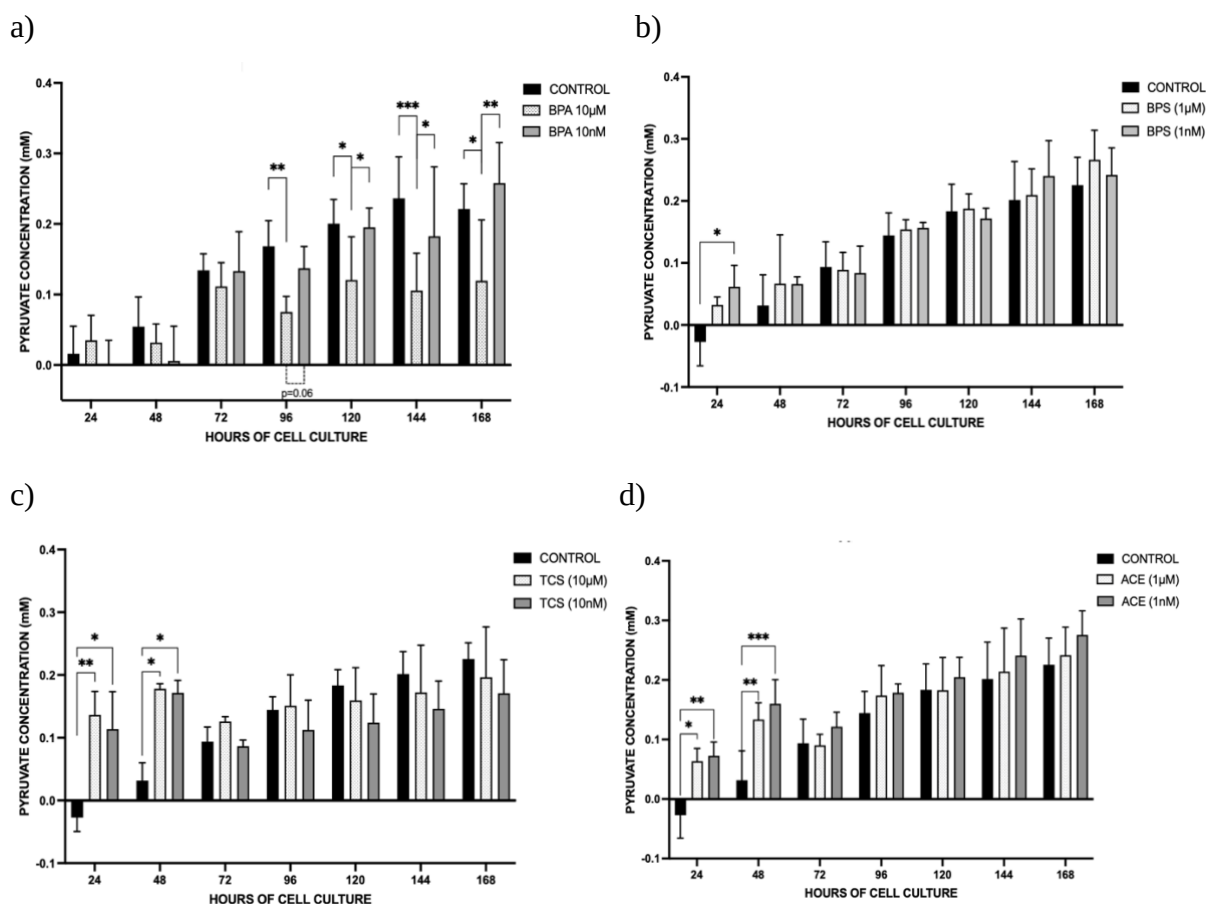


Figure 3.4: Concentration of Pyruvate in spent medium after BOEC exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid. The concentration of pyruvate was measured in conditioned media over the course of 7 days, collected at 24-hours intervals. The exposure started immediately after the BOECs attached on the culture wells and the media supplemented with the chemicals was replaced every 24 hours. Each graph represents 4 biological replicates for BPA and 3 biological replicates for BPS, TCS and ACE. Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Figure 3.5 illustrates the concentration of lactate in spent medium after BOECs were exposed to BPA, BPS, TCS and ACE respectively. Lactate concentration was not significantly impacted by the presence of BPS, TCS or ACE at any time point of the cell culture. The lactate release of the untreated BOECs follows an upward trend until the 96 hours and subsequently starting to decrease during the 120 to 168 hours of cell culture. The same trend on lactate release seems to be followed when the BOECs are exposed to BPS (fig 3.5b), TCS (fig 3.5c) and ACE (fig 3.5d). However, the lactate concentration released by BOECs exposed to 10µM BPA (fig 3.5a) follow an upward trend during the whole period of cell culture leading to significantly higher lactate concentration (3.56 ± 0.36 mM and 3.35 ± 0.27 mM) compared to control (1.84 ± 0.26

mM and 1.67 ± 0.19 mM) and the low dose of BPA (2.12 ± 0.29 mM and 1.78 ± 0.09 mM) after 144 and 168 hours of exposure, respectively.

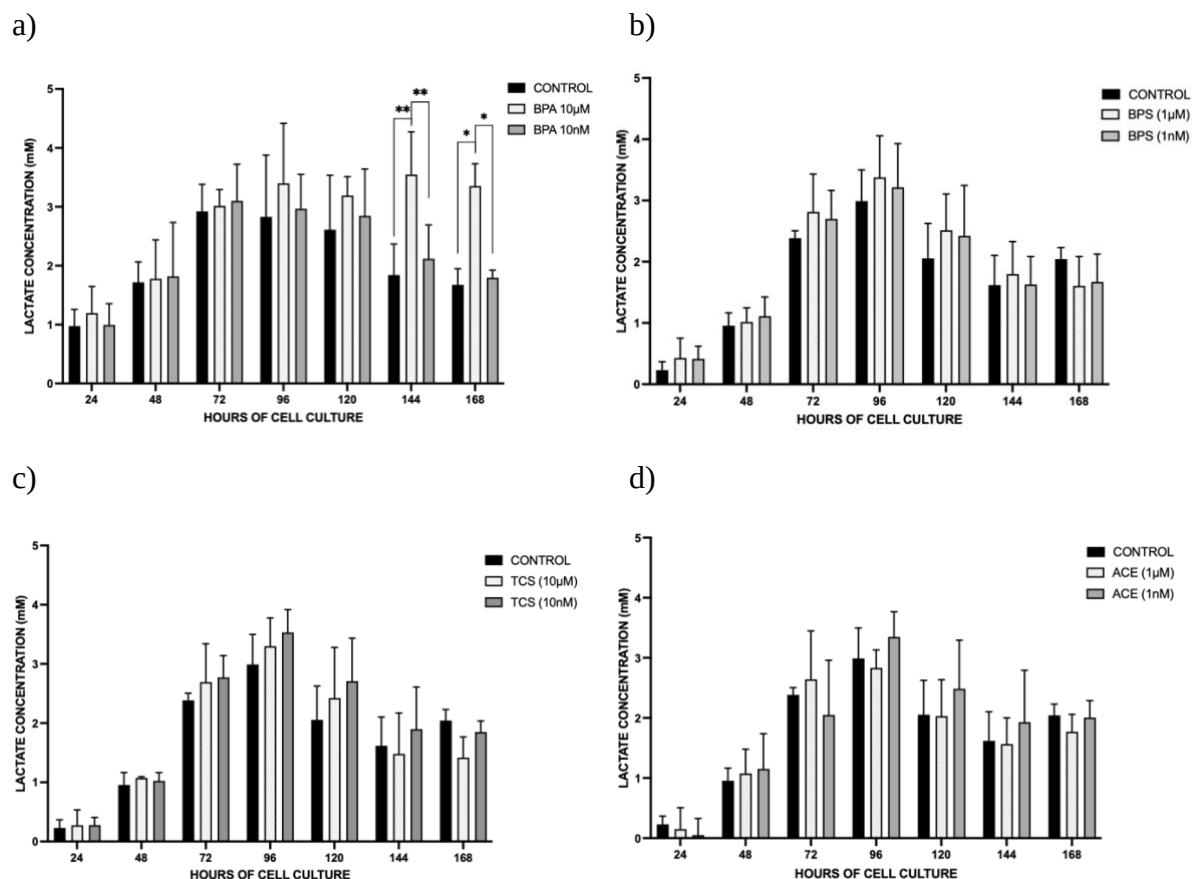


Figure 3.5: Concentration of lactate in spent culture medium after BOECs treatment with a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamidiprid. Lactate was measured in conditioned media over the course of 7 days in 24hours intervals. The exposure started immediately after the BOECs were attached on the culture wells and the media supplemented with the chemicals was replaced every 24hours. Each graph is result of four biological replicates for BPA (n=4) and three biological replicates for BPS, TCS and ACE (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Because the BOECs were exposed to the environmental contaminants from the beginning of cell culture, while they were actively proliferating (24 to 72 hours of cell culture) and continued once they reached confluency (from 96 to 168 hours of cell culture), the differences on energy substrates concentrations noticed above could also be result of the different number of cells. For this reason, the rate of proliferation BOEC exposed to BPA, BPS, TCS and ACE was investigated.

3.5.1.1 Effects on BOECs proliferation

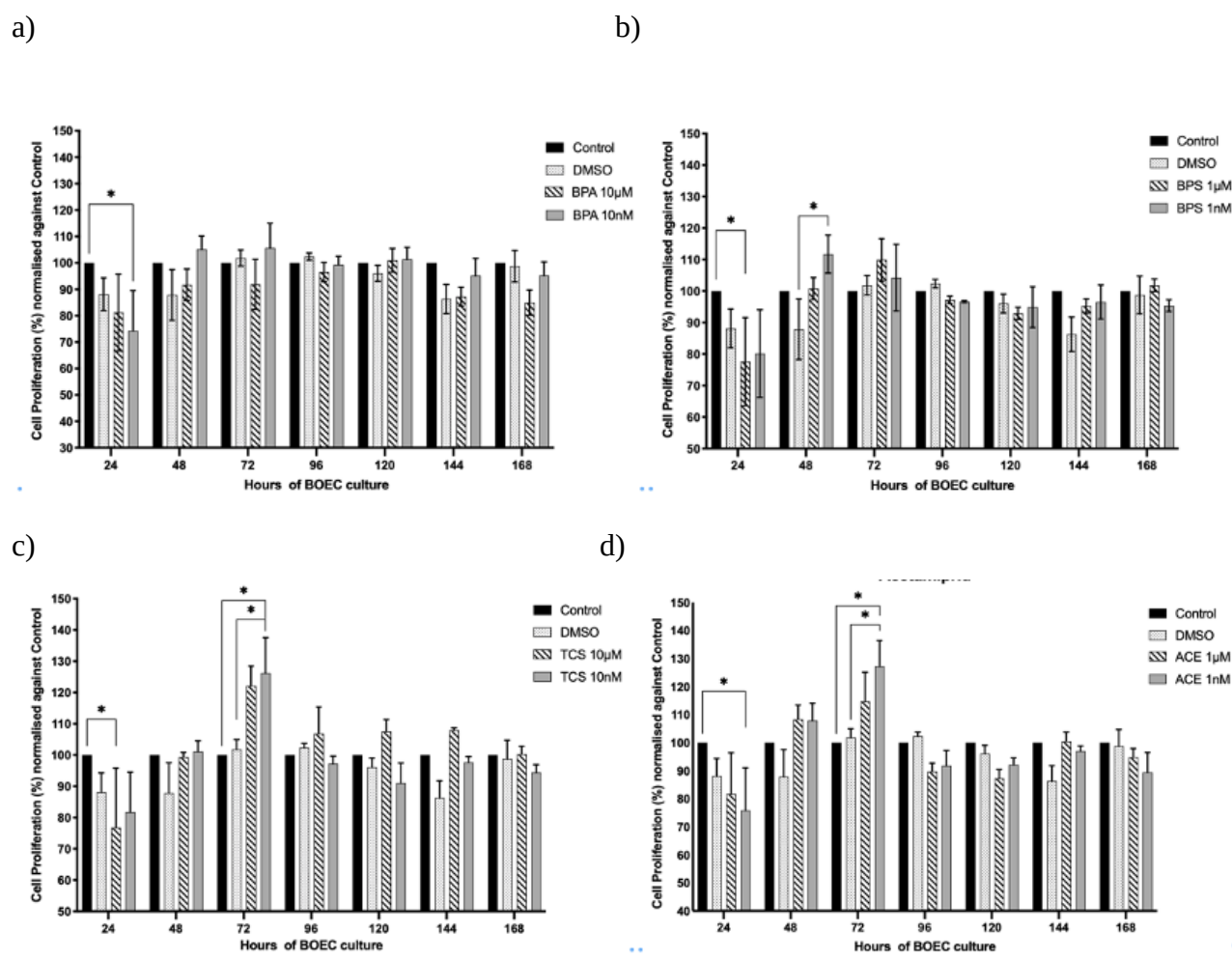


Figure 3.6: Cell proliferation of BOECs exposed to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid over the course of 7 days of culture. The percentage of cell proliferations of each experimental group was calculated compared to Control group which was defined as 100% for each day. Each graph represents data of three independent biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

The effect of environmental contaminants on proliferation of BOECs was investigated using MTT assay (see Methods section 2.2.1). BOECs were exposed to BPA, BPS, TCS and ACE for 7 days, proliferation assessed at 24-hours intervals compared to untreated BOECs (control group) at equivalent time points.

As shown in Fig 3.6, the proliferation levels were significantly influenced by the presence of all environmental contaminants during the first 24 hours of culture. All four of the treatments reduced proliferation of BOECs compared to control group the first 24 hours of culture; when BOECs were exposed to 10nM-BPA (Fig 3.6a), 1nM-BPS (Fig 3.6b), 10 μ M TCS (Fig 3.6c) and 10nM-ACE (Fig 3.6d) the proliferation level was significantly lower compared to control groups. However, in the subsequent 6 days of exposure no statistically significant difference was identified when the BOECs were exposed to BPA. However, increased proliferation was observed after 48 hours of exposure to 1 nM-BPS compared to control group. These differences disappeared after 72 hours of exposure. In the case of TCS and ACE, a significant increase of the proliferation level was observed at 72 hours of BOECs exposed to the low concentration, 10 nM and 1 nM, respectively. However, the proliferation rate of the TCS-treated and ACE-treated BOECs were similar to control group after the 96-hours of exposure.

3.5.2 Impact of environmental contaminants on BOECs secretions

For this experiment BOECs were grown in a Transwell system which as is described in Methods section 2.1.2, is an arrangement that more closely imitates the physiology of epithelial cells compared to conventional cell culture. This experiment consists of two different phases. During the first phase of this experiment the cells were cultured in liquid- liquid interface and exposed to the environmental contaminants for 7 days. TEER was measured at 24-hours interval (Fig 3.8). In the second phase of the experiment the BOECs were transferred in air-liquid interphase after attaining cellular confluence, and they were subsequently exposed to BPA, BPS, TCS and ACE for 7 days. After 24 hours in air-liquid, a clear fluid began to form in the apical compartment of the cells; this is referred to as in vitro derived oviduct fluid (ivDOF). The energy metabolites glucose, pyruvate, lactate and 18 amino acids were measured in ivDOF, and the data are presented in Fig. 3.8 to 3.14

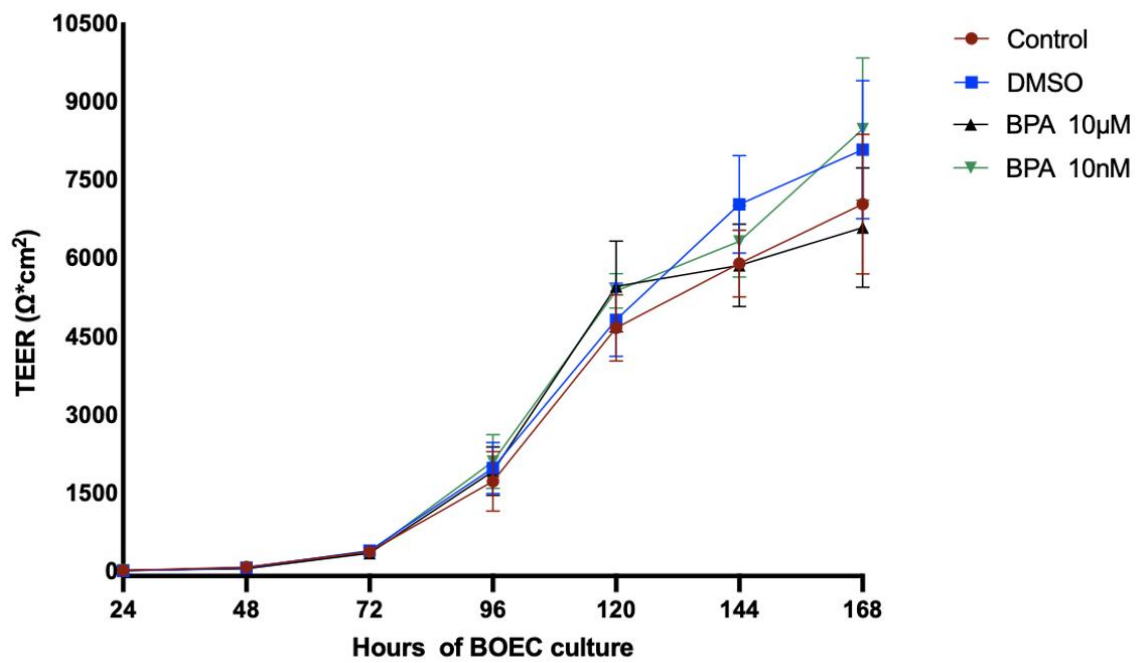
3.5.2.1 Effects on BOECs TEER

The effects of environmental contaminants on TEER were first determined (Fig 3.7). By measuring TEER during BOECs exposure, we were able to assess the integrity of tight junction dynamics. This allows us to understand if the presence of the environmental contaminants influence the generation of tight junctions.

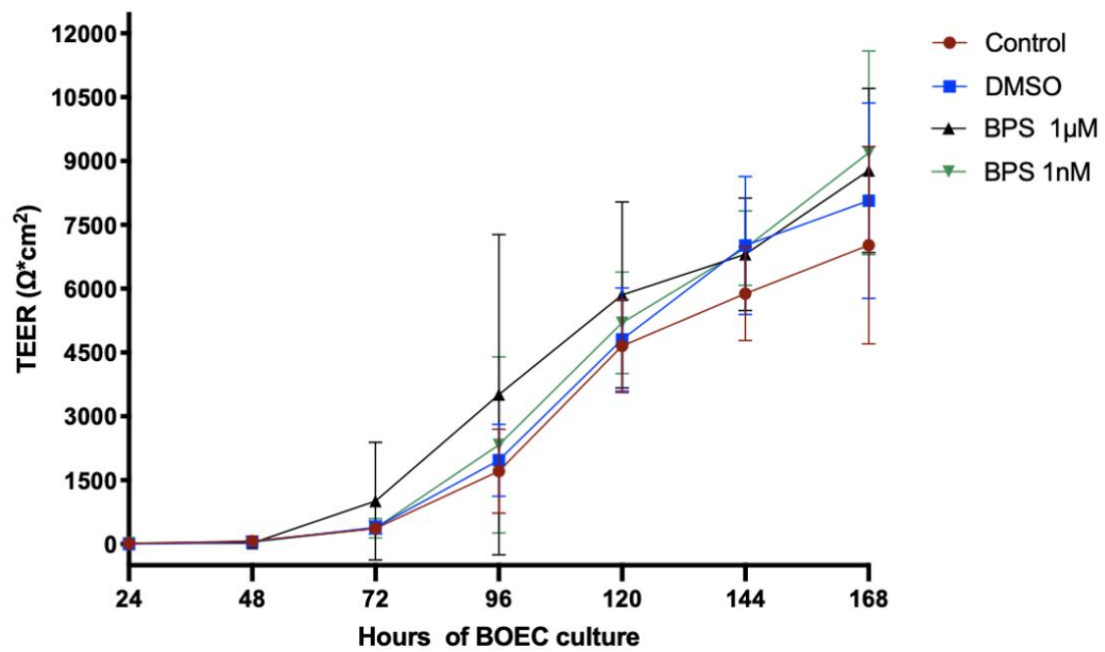
Figure 3.7 illustrates that TEER reflects the BOECs developmental curve. From 24 to 48 hours of culture there is the lag phase where the cells adjust to the new environment and the TEER is very low; over the next 24 hours- to 72 hours of cell culture- the acceleration phase takes place where the TEER starts to increase, reflecting the initiation of the cell growth. Subsequently, in the next 48 hours – between 72 and 120 hours of cell culture - the TEER increase exponentially reflecting the establishment of epithelial barrier integrity brought about by junctional complexes between neighbouring cells. By the final 48 hours -from 120 to 168 hours, the increase rate of TEER starts to decline showing that the BOECs indicating maintenance of a stable intact resistant epithelial monolayer.

Taking into consideration the data shown on Methods section 2.1.3 where intact BOECs monolayer is formed at $1500 \Omega \cdot \text{cm}^2$, epithelial cells attain confluency after 96 hours of culture (Figure 3.7 a, b, c). An exception to this was seen when BOEC were exposed to $1\mu\text{M}$ -ACE (Fig 3.7 d), where the entry on each developmental phase, as described above, was delayed by 24 hours and the BOECs became confluent after 120hours of culture. No statistically significant difference was noticed on TEER measurement among treated and untreated groups at any point of the exposure suggesting that epithelial integrity and the formation of the tight junctions is not influenced by the presence of any of the environmental contaminants during the growing period of the BOECs and also, they were able to form an intact epithelial monolayer.

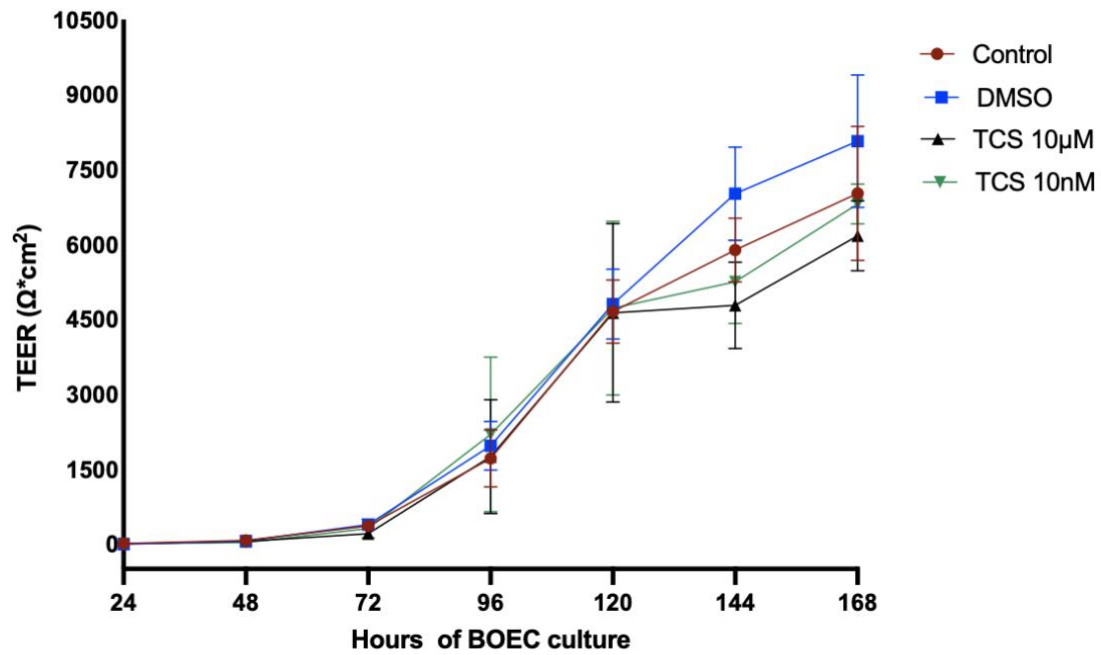
a)



b)



c)



d)

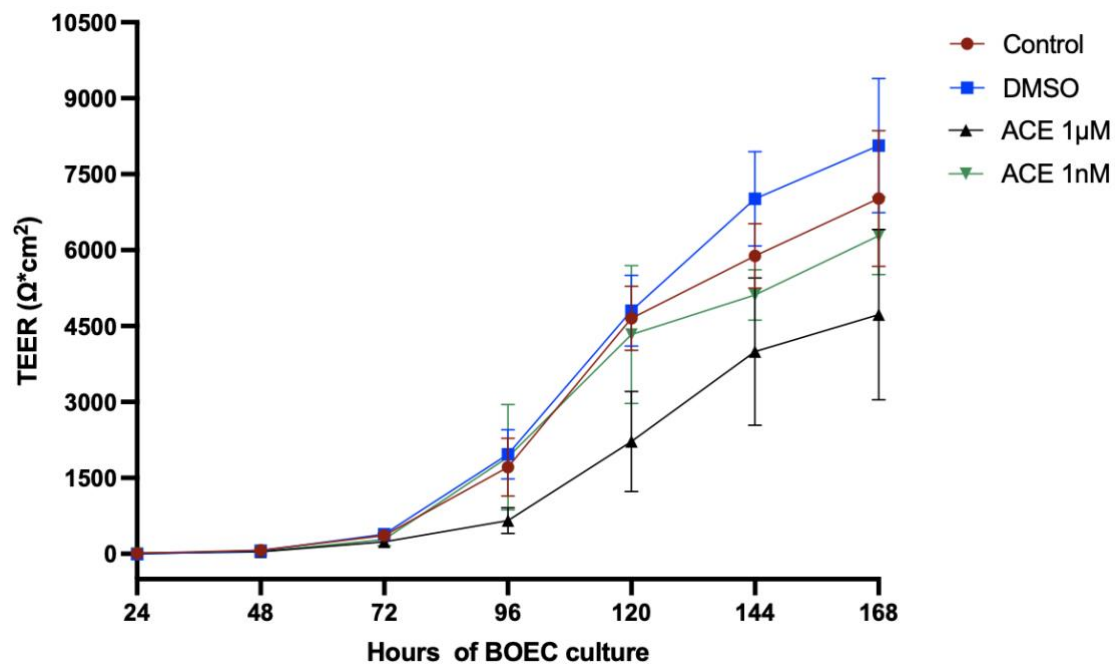


Figure 3.7: TransEpithelial Electrical Resistance (TEER) of BOECs cultured in Transwell system and exposed to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamiprid over the course of 7 days of culture. TEER was measured every 24 hours. Each graph represent data of three independent biological replicates (n=3),. Data are expressed as Mean and error $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test.

3.5.2.2 Impact on energy substrate transport after chronic exposure

After BOECs were exposed to environmental compounds for 7 days in liquid-liquid interphase, the cell culture was switched to air-liquid interphase, and the BOECs exposed to the chemicals through the basal compartment for a further 7 days. The BOECs air-liquid interphase system of culture represents the oviduct environment *in vivo* where the epithelial cells line the inner surface of the lumen, which is represented by the apical Transwell compartment. In an air-liquid interface, BOECs create a neat fluid *de novo* after 24h of culture. The cells are nourished from the basal surface, representing the blood stream (basal Transwell compartment). The ivDOF is a mixture of molecules secreted and/or transported by the BOECs from the basal to apical compartment. IvDOF was collected at 24-hour intervals and glucose, lactate, pyruvate and 18 amino acids were quantified as described in Methods section 2.4.2 and 2.4.4. The data presented from Fig.3.8 to Fig.3.14 are the product of 3 biological replicates with 1 technical replicate for each experimental group.

The glucose concentration within ivDOF after BOECs were exposed to BPA, BPS, TCS and ACE is shown in Figure 3.8 a, b, c, and d respectively. The glucose concentration in ivDOF after BPS (Fig:3.8 b) or ACE (Fig: 3.8 d) exposure was not significantly different compared to control and DMSO group at any point of the exposure. However, there was a strong trend of reduced glucose in ivDOF when BOECs exposed to 10 μ M of BPA the first 24 hours in air-liquid interphase system compared to control ($p=0.066$) and 1nM-BPA ($p=0.051$, Fig 3.8a). This difference vanished in the following days of exposure. TCS presence in the basal compartment of the culture system seemed to have a long effect on glucose concentration in the ivDOF. During the first 24 hours of exposure in 10 nM TCS, the glucose concentration was significantly higher compared to every other group with almost double glucose concentration detected in 10nM TCS group compared to control (1.13 ± 0.009 mM vs 0.608 ± 0.03 mM $p=0.0137$, Fig: 3.8c). Furthermore, there was a strong trend toward an increase in glucose concentration in ivDOF from BOECs exposed to 10 μ M and 10 nM TCS between 72 and 120hours of exposure compared to control group. The glucose concentration in ivDOF increased in both TCS groups after 144, control-vehicle group, and 168 hours compared to control and control vehicle group suggesting a deregulation of glucose transport in ivDOF after long exposure to TCS.

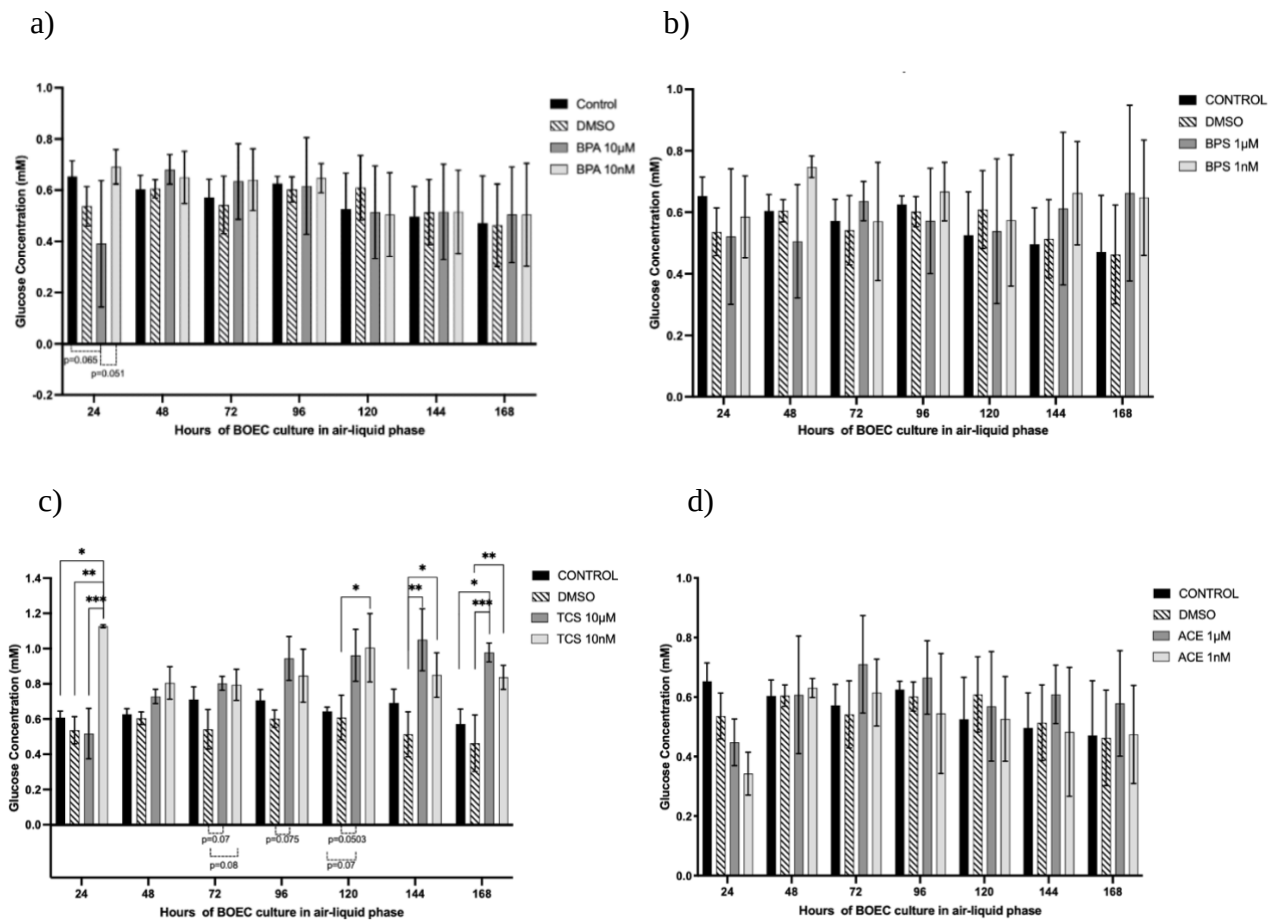


Figure 3.8: Glucose concentration in the in vitro derived oviduct fluid (ivDOF) after BOECs chronic exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamidiprid. The exposure started immediately after the BOECs were attached on the wells and the media supplemented with the chemicals was replaced every 24hours. The BOECs were cultured in liquid-liquid phase for 7 days. After the first week and once an intact monolayer was confirmed by TEER, BOECs transferred into air-liquid phase. Glucose was measured in ivDOF generated in the apical compartment when BOEC were cultured in air-liquid phase over the course of 7 days in 24 hours intervals. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p<0.05$; ** $p<0.01$; *** $p<0.001$

Next the lactate concentration was quantified (Chapter 2, section 2.4.2) in ivDOF of the treated and untreated BOECs. Lactate concentration was elevated in ivDOF of BPS exposed cells especially from 120 to 168 hour of culture (Fig: 3.9b), compared to controls. In addition, higher lactate concentrations were observed in ivDOF from ACE-treated BOECs during 72 and 96 hours of culture. However, none of these differences on lactate concentration were statistically

significant at any point of exposure as there was a high variability in the levels of lactate quantified in each group among the biological replicates.

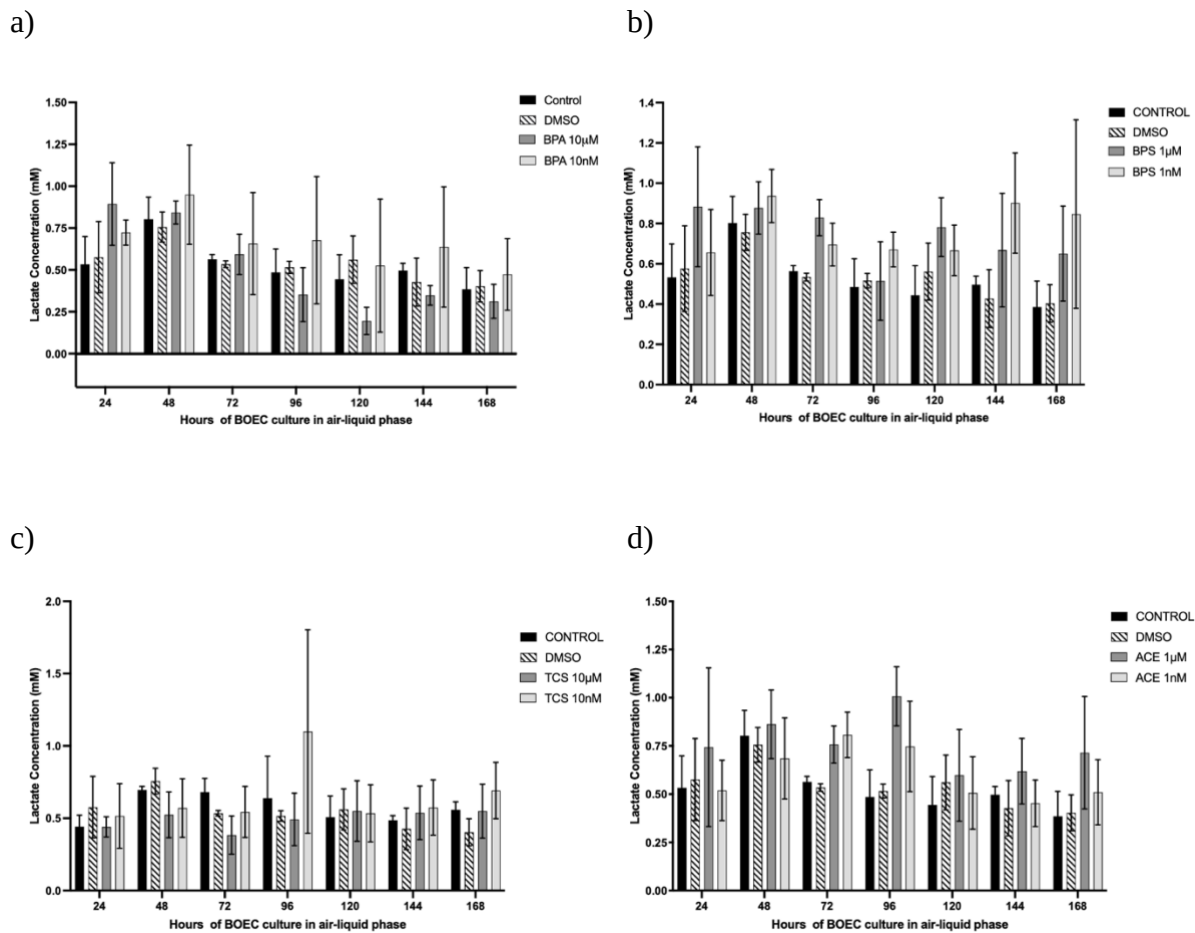


Figure 3.9: Lactate concentration in the in vitro derived oviduct fluid (ivDOF) after BOECs chronic exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamidiprid. The exposure started immediately after the BOECs were attached on the wells and the media supplemented with the chemicals was replaced every 24hours. The BOECs were cultured in liquid-liquid phase for a week. After the first week and once an intact monolayer was confirmed by TEER. Lactate was measured in ivDOF generated in the apical compartment when BOEC were cultured in air-liquid phase over the course of 7 days in 24 hours intervals. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Next, the concentration of pyruvate in ivDOF was measured. Treatment with TCS and ACE had an acute effect in pyruvate concentrations in ivDOF, whilst bisphenols did not have an influence. After 24 hours of ivDOF significantly enriched for pyruvate in BOECs exposed to 10 μ M TCS (0.036 ± 0.01 mM vs 0.099 ± 0.02 mM $p = 0.04$) and 10 nM TCS (0.036 ± 0.01 mM vs 0.145 ± 0.06 mM $p = 0.015$), when compared to controls. However, these differences were

normalised after 48 hours and for the rest of exposure period. BOECs treated with 1nM-ACE BOECs secreted higher concentration of pyruvate in ivDOF after 24 hours of exposure compared to control-vehicle group (0.029 ± 0.01 mM vs 0.106 ± 0.025 mM $p=0.045$). The pyruvate concentration on the ACE treated groups remained relatively low compared to control and control-vehicle group (Fig: 3.10d) over the course of the 7 days. However no statistically significant difference was identified.

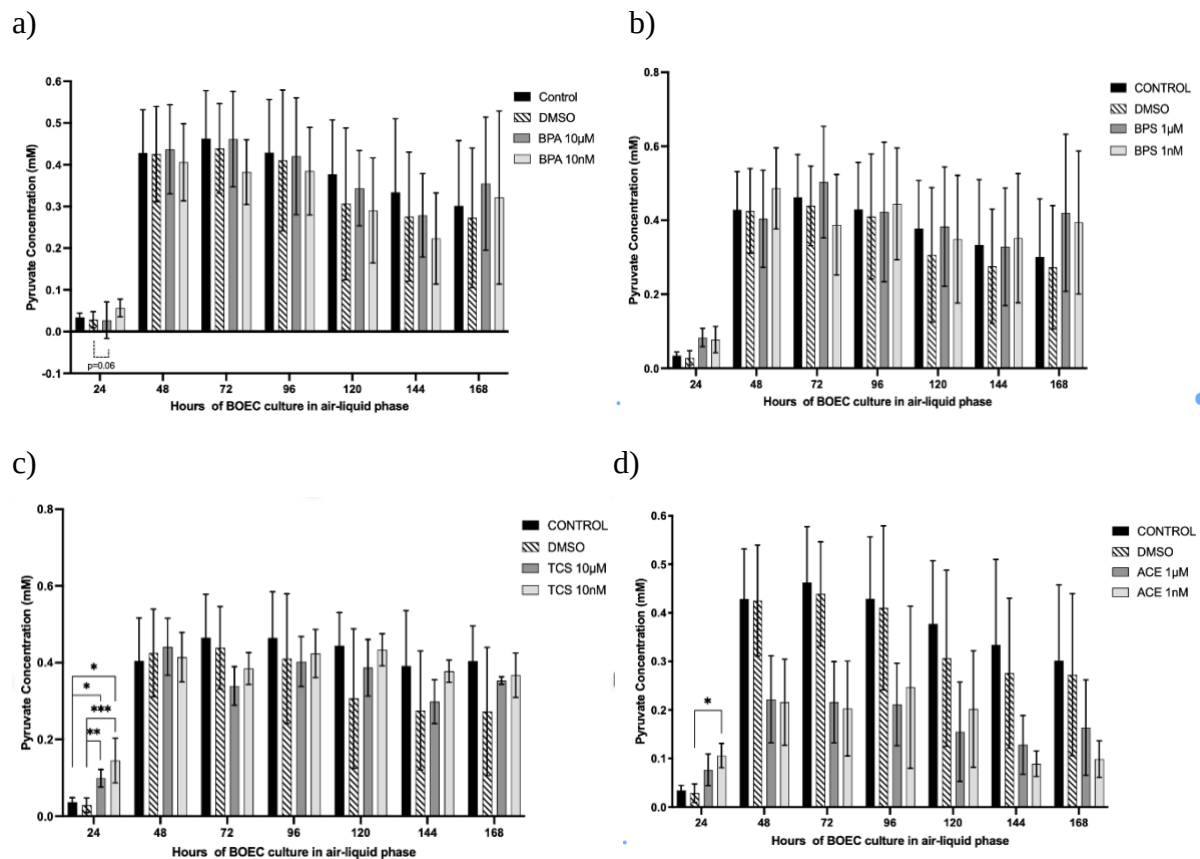


Figure 3.10: Pyruvate concentration in the in vitro derived oviduct fluid (ivDOF) after BOECs chronic exposure to a) Bisphenol A, b) Bisphenol S, c) Triclosan, d) Acetamidiprid. The exposure started immediately after the BOECs were attached on the wells and the media supplemented with the chemicals was replaced every 24hours. The BOECs were cultured in liquid-liquid phase for 7 days. After the first week and once an intact monolayer was confirmed by TEER, BOECs transferred into air-liquid phase. Pyruvate was measured in ivDOF generated in the apical compartment when BOEC were cultured in air-liquid phase over the course of 7 days in 24 hours intervals. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p<0.05$; ** $p<0.01$; *** $p<0.001$

The concentrations of 18 amino acids were quantified in the ivDOF of BOECs exposed to our panel of environmental contaminants using HPLC (Chapter 2, section 2.4.4) over the course of the 7 days exposure. The data of the first 24 hours of exposure are presented in Figures 3.11 to 3.14. and are results of three independent biological replicates. The amino acids composition between 48 and 168 hours of exposure are included in the Appendices section (from Figure 3.21_Appendix to Figure 3.24_Appendix).

The amino acid composition in ivDOF was significantly influenced by the presence of all environmental contaminants after 24 hours of exposure. BPA-treatment induced the most marked impact on amino acid composition of ivDOF, where 15 out of 18 amino acids were significantly different compared to control or control-vehicle group. Essential amino acids such as histidine, tryptophan, valine, leucine, isoleucine, (fig: 3.11a) threonine and lysine (fig 3.11b) were significantly reduced after exposure to 10 μ M and 10 nM BPA. Furthermore, the non-essential amino acids, glutamine, glycine, arginine, alanine, tyrosine (fig: 3.11c) and aspartate, asparagine, and serine (Fig 3.11d) were significantly reduced. The impact of BPA on amino acids composition was dose dependent as the 10 μ M -BPA had a stronger effect compared to 10nM-BPA in most of the cases. During the subsequent period of exposure these differences in amino acids composition in ivDOF arising from BOEC exposure to BPA were normalised and the results of each day can be found in Figure 3.21_Appendix.

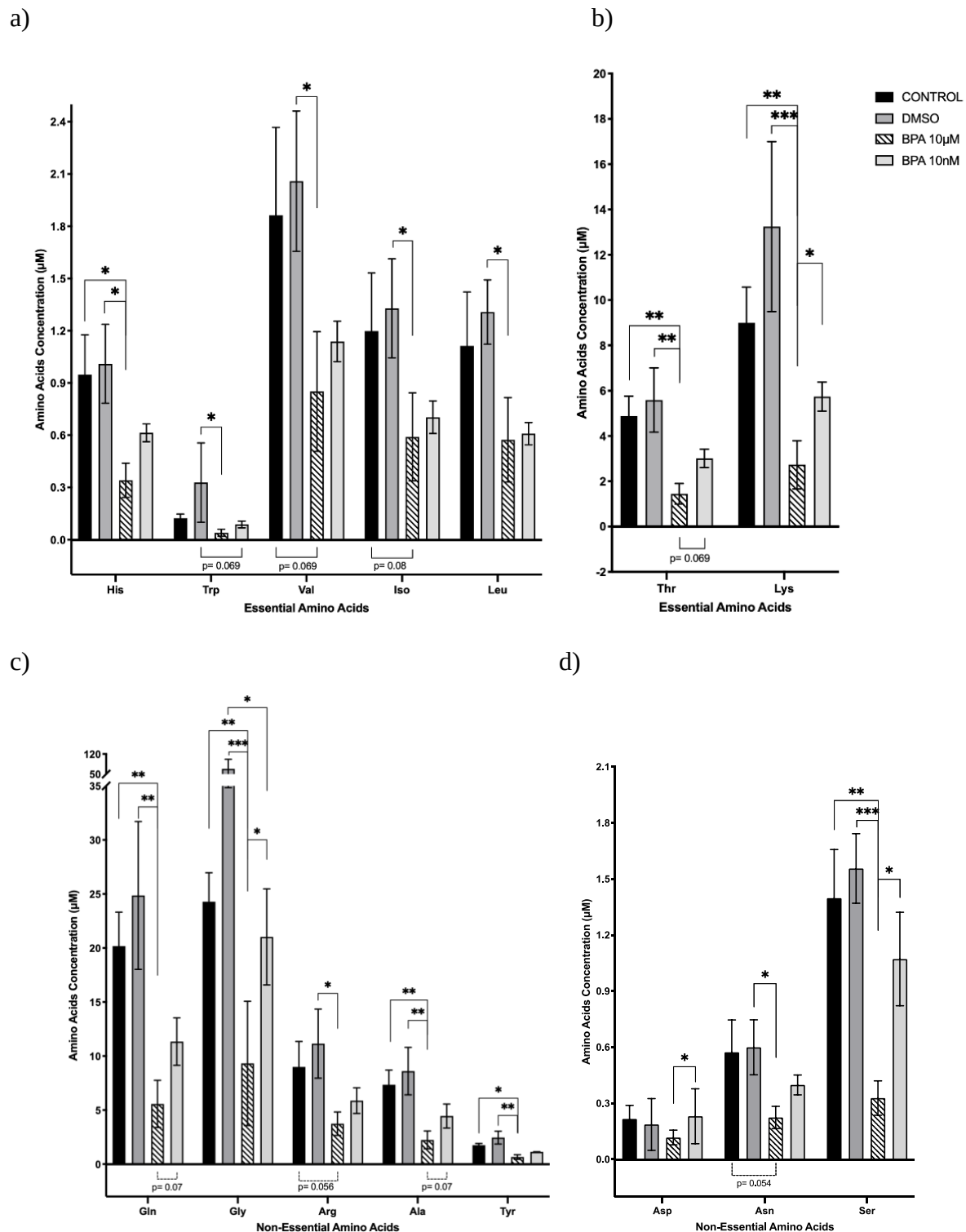


Figure 3.11: Bisphenol A impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after 24-hours of BOECs cultured in air-liquid phase. Essential and Non-essential amino acids with statistically significant difference are presented on a), b) and c), d) graphs respectively. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

BPS had also an influence on amino acid composition in ivDOF as 6 out of 18 amino acids were significantly different compared to control or/and control-vehicle group after 24 hours of BOECs cultured in air-liquid interphase (fig: 3.12). More specifically, two essential, lysine and tryptophan (fig 3.12a) and in four non-essential amino acids, aspartate, glycine, arginine, and alanine (fig 3.12b) were significantly different to the controls. The high concentration of BPS (1 μ M) significantly reduced the concentration of lysine, arginine and alanine compared to control and control vehicle group. In addition, lysine, and arginine concentration in ivDOF was significantly lower when the BOECs were exposed to the 1nM-BPS, whereas the same dose of BPS significantly increased the aspartate concentration in ivDOF compared to control-vehicle group. Finally, DMSO exposure prompted an increase in the concentration of tryptophan and glycine in ivDOF, compared to 1 μ M-BPS group. Similarities between the BPA and BPS impact on amino acids composition were observed, such as the reduction on lysine, arginine, and alanine concentration. Also, the influence of the bisphenols seems to be dose dependent in the majority of the amino acids influence by their presence.

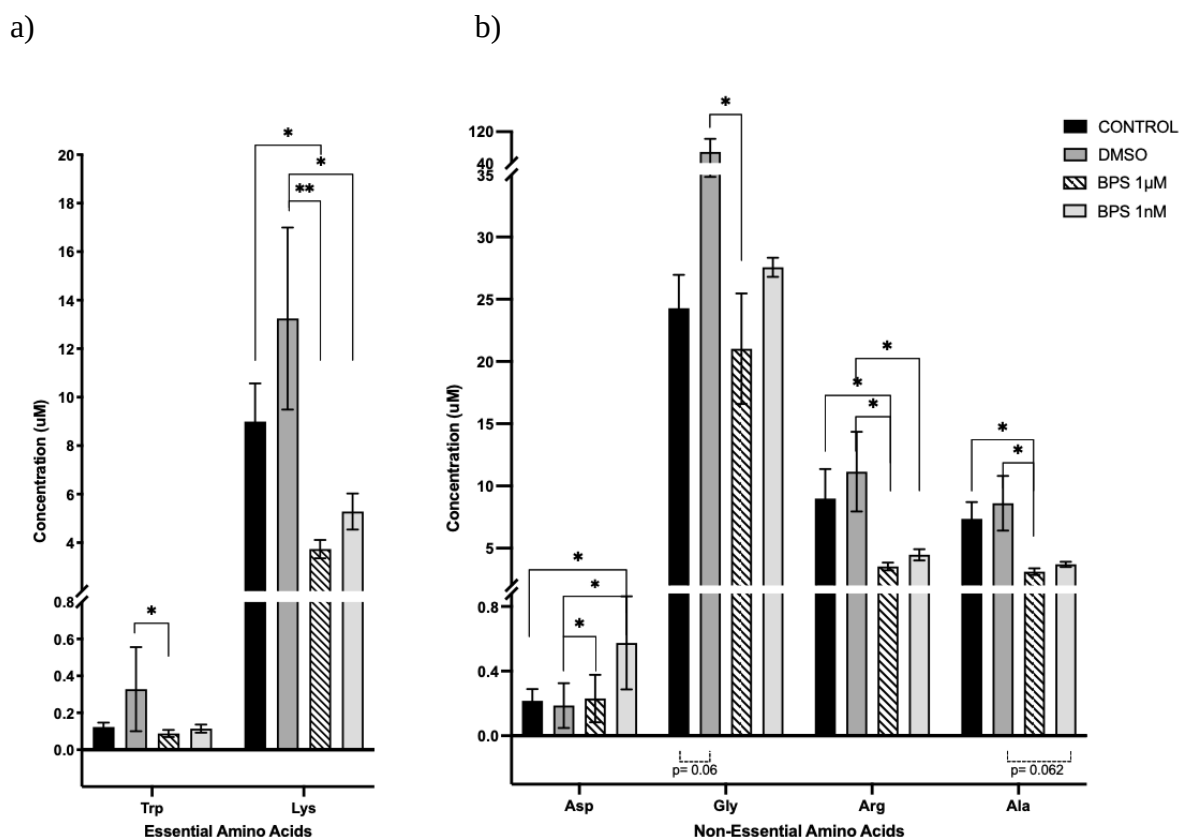


Figure 3.12: The impact of Bisphenol S on amino acid concentration in BOEC ivDOF after 24-hours culture in air-liquid phase. Essential and Non-essential amino acids with statistically significant difference are presented on a) and b) graphs respectively. Each graph represent data of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

The effect of 24h treatment of TCS on amino acids composition in ivDOF is shown in figure 3.13. Arginine and lysine were significantly impacted by the presence of 10 nM dose of TCS compared to control whereas DMSO had an impact on glycine concentration compared to all the other groups. Interestingly, the amino acid composition of ivDOF seemed to be most significantly affected by the lower concentration, 10 nM concentration of TCS, compared to a higher 10 μM . For example, arginine was significantly increased compared to control group ($20 \pm 0.042 \mu\text{M}$ vs $8.99 \pm 2.36 \mu\text{M}$ $p=0.045$) and a strong trend of difference ($p=0.06$) revealed between the 10 nM and 10 μM TCS groups. On the other hand, lysine concentration in ivDOF was significantly decreased when the BOECs exposed to 10nM-TCS compared to all the other groups. It appears that TCS has a weaker effect on the composition of ivDOF compared to those of bisphenols and the impact is not dose dependent. However, the impact on arginine and lysine concentration in ivDOF was observed in all these three cases of exposure.

Finally, TCS effects on amino acids compositions were investigated also during 48 and 168 hours of culture. During this period, the amino acids composition in ivDOF was not significantly influenced by the exposure of BOEC to TCS, the results are included in Figure 3.23_Appendix

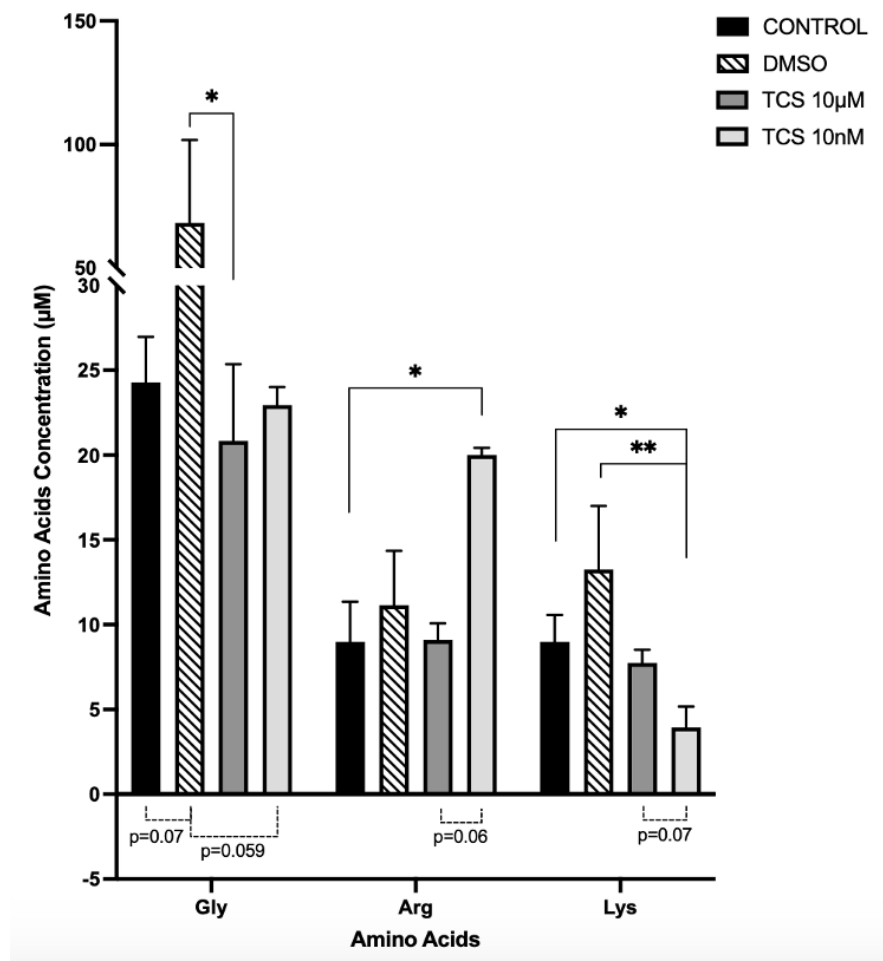


Figure 3.13: The effect of Triclosan exposure on the amino acid composition of ivDOF after 24-hours of BOECs culture in air-liquid phase. The graph represents data of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, p<0.05; ** p<0.01; *** p,0.001. Trends are illustrated by a dashed line, with p-value presented.

The effect of ACE on the amino acid composition in ivDOF was assessed. The differences in amino acid composition after 24 hours of exposure to ACE is presented in Fig: 3.14. Statistically significant differences between treated and control samples were observed in 3 out of 18 amino acids: aspartate, glutamate, and arginine. When compared to the control group,

aspartate and glutamate concentration were significantly enriched in ivDOF irrespective of the administered concentration of ACE. By contrast, the concentration of arginine was significantly lower in ivDOF after exposure to both doses of ACE. This was similar to the pattern observed during BPS exposure (fig 3.12b). The results of the ACE influence on amino acids composition for the next days of exposure (48 hours to 168 hours) were also investigated. BOEC exposure to ACE did not influence the composition of amino acids during this period., the results are presented in the Figure 3.24_Appendix.

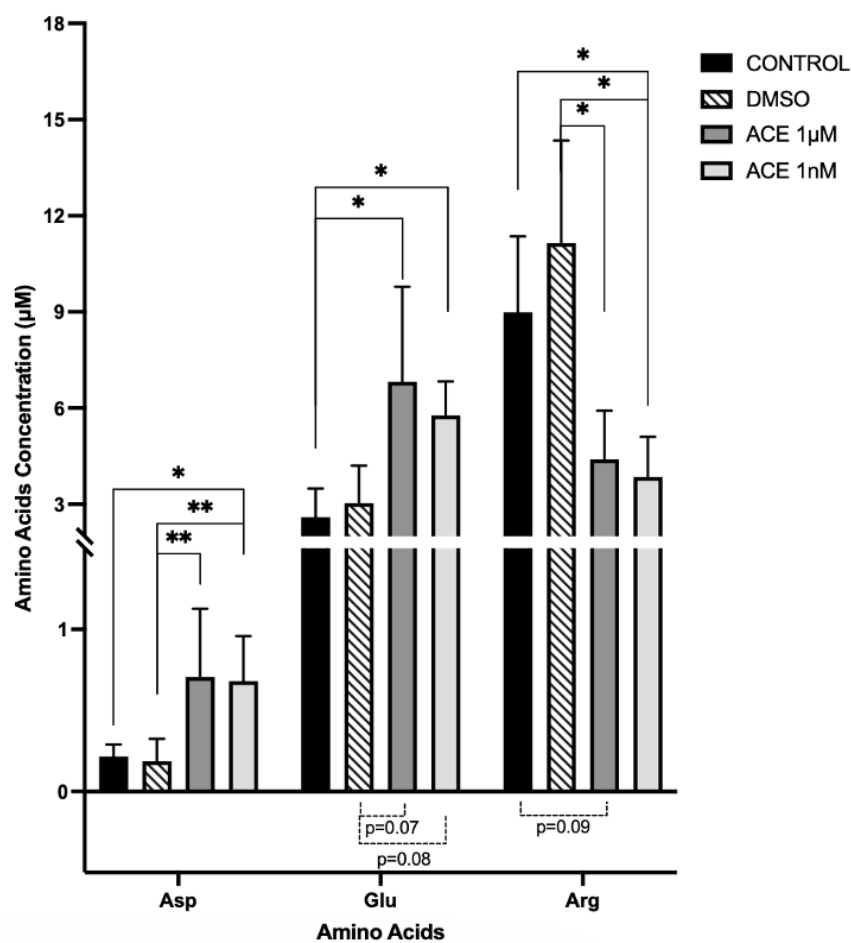


Figure 3.14: Acetamidiprid impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after 24-hours of BOECs cultured in air-liquid phase. The graph represent data of 3 biological replicates (n=3) Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

3.5.2.3 Impact of environmental contaminant exposure on expression of amino acid transporters

Having observed differences in the amino acid composition of ivDOF, the relative expression levels of genes encoding for relevant amino acid transporters was assessed. BOECs were cultured in the presence of BPA, BPS, TCS and ACE during the establishment of confluence, before being switched to air-liquid interface culture. Cells were maintained for an additional 24h in the presence of the environmental contaminant, before they were lysed and underwent RNA extraction to assess relative expression of mRNA for nutrient transporters. The expression levels of key amino acid transporters were determined, as well as the glucose transporter SLC2A1.

Based on the results of the effects on amino acids concentrations in ivDOF, the following amino acids transporters were selected: SLC1A4, SLC1A5, SLC7A1, SLC7A2, SLC7A4. The data presented in the following graphs are results of 3 biological replicates with 2 technical replicates for each group of BPA and BPS and 4 biological replicates with 1 technical replicate per group for TCS and ACE. In the following figures from fig 3.15 to 3.19 the Control group refers to the group of BOEC cultured in media without the addition of any treatment. Comparison between the control group and the DMSO group (which is the group of cells treated only with DMSO, the solvent of the treatments is presented in Appendix Figure 3.25 and no significant statistical difference was identified.

Figure 3.15 indicates a mild increase in the expression of SLC1A4, which approached statistical significance ($p=0.07$) after BOECs were exposed to 10 μ M BPA compared the control group (fig: 3.15a). By contrast, treatment with 1 nM ACE seemed to reduce expression of SLC1A4, although this did not reach statistical significance ($p=0.08$; fig: 3.15d). Beyond these, no statistically significant differences in the expression of SLC1A4 in response to exposure to environmental contaminants were identified among the experimental groups.

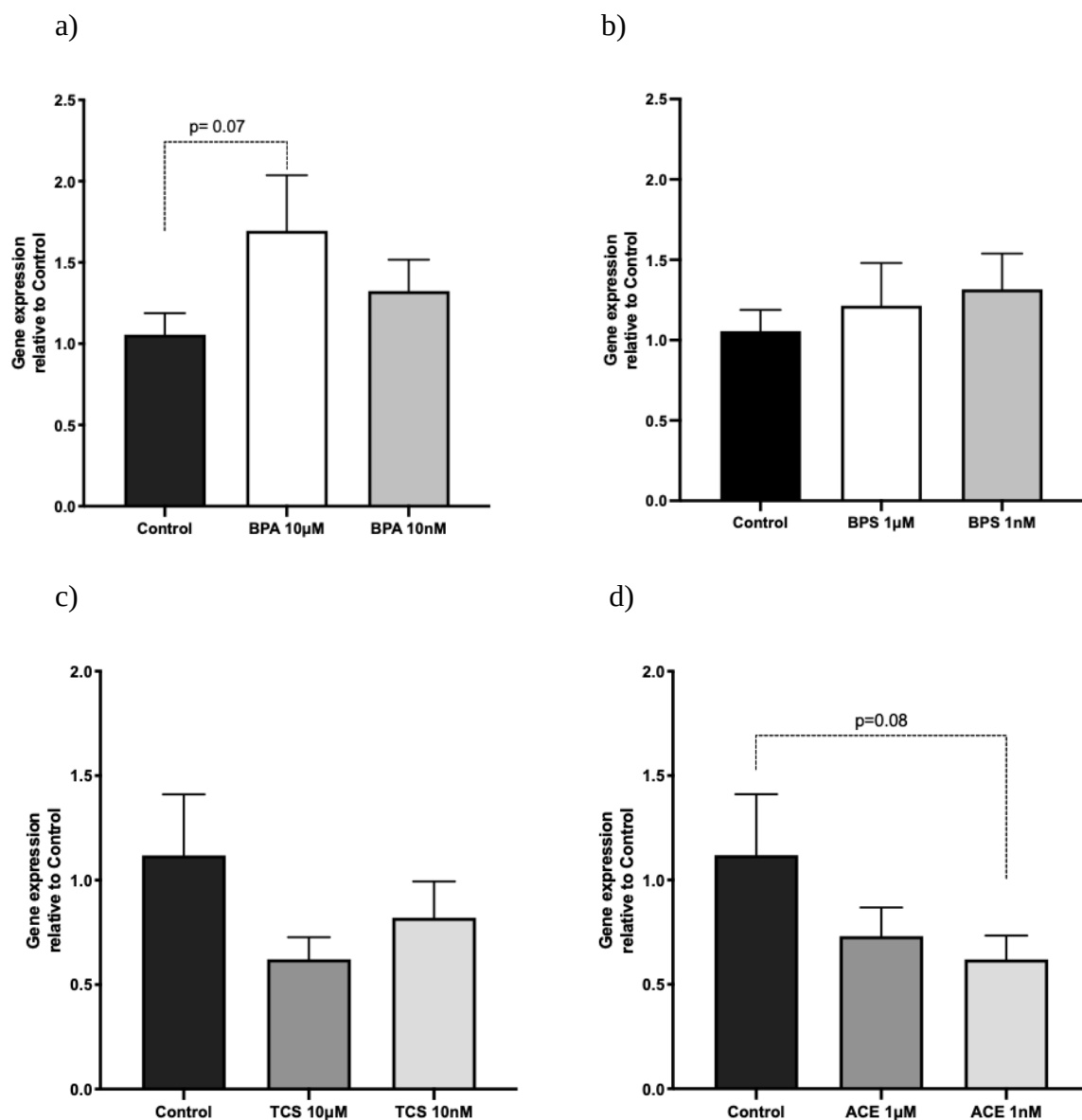


Figure 3.15: Relative gene expression of SLC1A4 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase. These graphs represent data of three biological replicates (n=3) for BPA and BPS and four (n=4) for TCS and ACE. Each biological replicate consists of two technical replicate for BPA and BPS and one technical replicate for TCS and ACE per experimental group. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

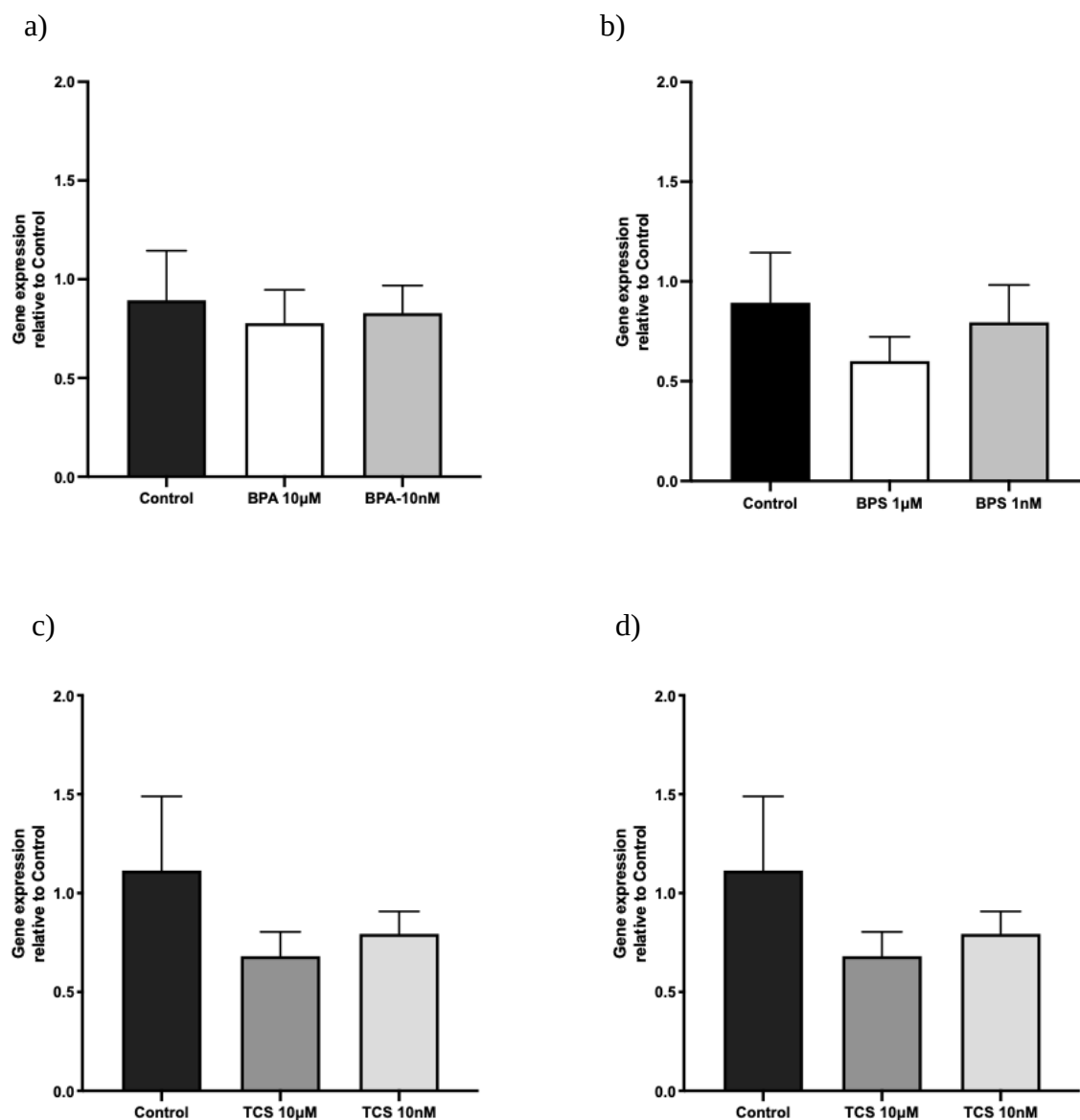


Figure 3.16: Relative gene expression of SLC1A5 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamiprid for 24 hours in air-liquid phase. These graphs represent data of three biological replicates (n=3) for BPA and BPS and four (n=4) for TCS and ACE. Each biological replicate consists of two technical replicate for BPA and BPS and one technical replicate for TCS and ACE per experimental group. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, p<0.05; ** p<0.01; *** p,0.001. Trends are illustrated by a dashed line, with p-value presented.

The panel of environmental contaminants under investigation did not affect the expression of the transporter SLC1A5 in BOECs.

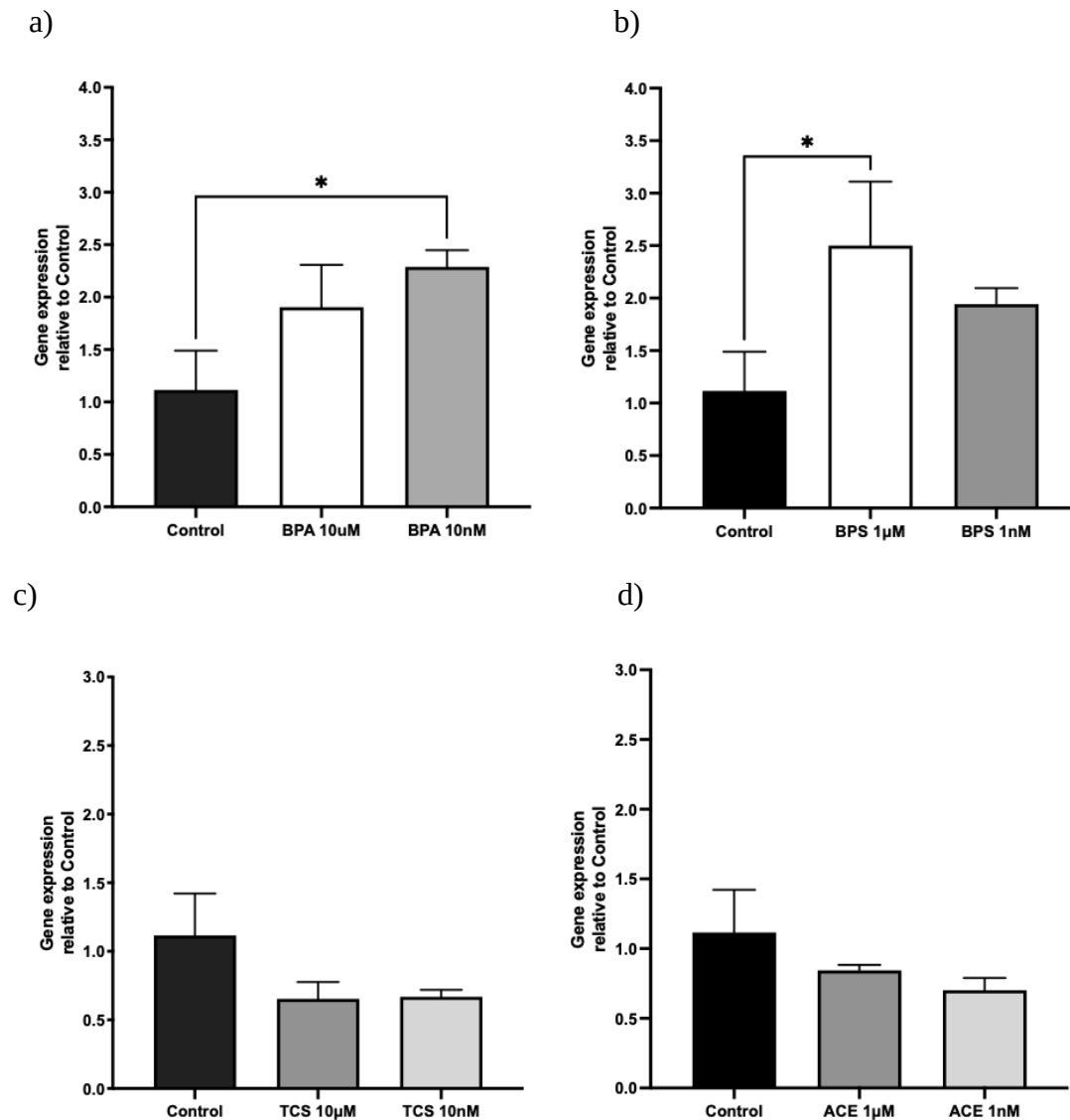


Figure 3.17: Relative gene expression of SLC7A1 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetamidiprid for 24 hours in air-liquid phase. These graphs represent data of three biological replicates (n=3) for BPA and BPS and four (n=4) for TCS and ACE. Each biological replicate consists of two technical replicate for BPA and BPS and one technical replicate for TCS and ACE per experimental group. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

Next, the expression of SLC7A2 in BOECS after exposure to BPA, BPS, TCS and ACE are presented in figure 3.16 a, b, c, d, respectively. The expression levels of SLC7A2 after exposure to bisphenols (BPA and BPS) was not significantly influenced by any concentration. However, a significant decrease of the SLC7A2 was observed in cells treated with low-dose TCS levels compared to control group, with a strong trend of decrease in 10µM- TCS ($p = 0.06$) compared

to control group (fig: 3.16c). Finally, ACE reduced the expression of SLC7A2 in BOECS compared to control irrespective of the administrated concentration of ACE (fig: 3.16d). More precisely, the expression levels were reduced by 0.95 folds after 1 μ M -ACE (0.18 vs 1.13 p=0.02) and by 0.925 folds after 1nM-ACE exposure (0.206 vs 1.13 p=0.02) compared to control group.

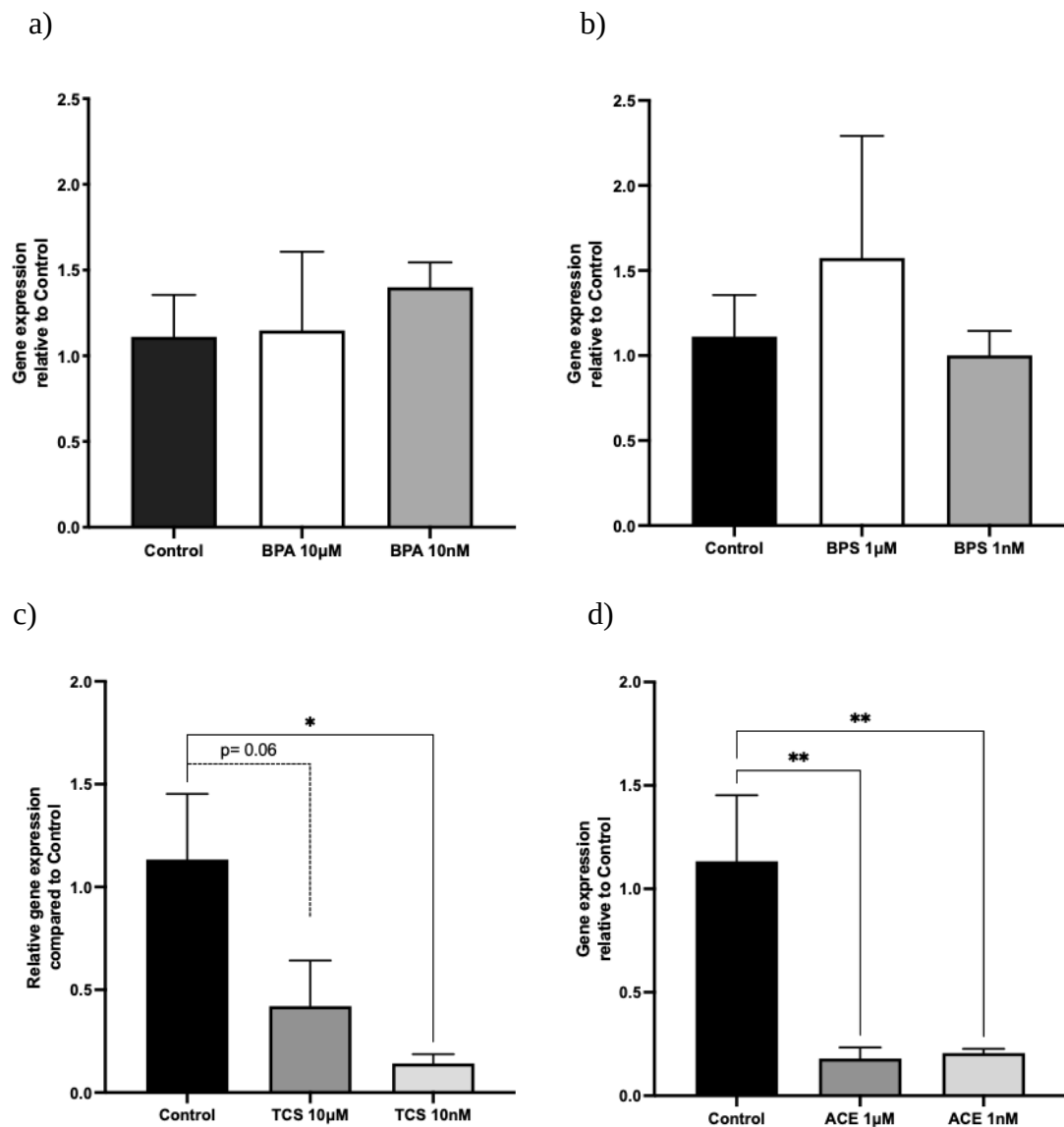


Figure 3.18: Relative gene expression of SLC7A2 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetaminophen for 24 hours in air-liquid phase. These graphs represent data of three biological replicates (n=3) for BPA and BPS and four (n=4) for TCS and ACE. Each biological replicate consists of two technical replicate for BPA and BPS and one technical replicate for TCS and ACE per experimental group. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, p<0.05; ** p<0.01; *** p,0.001. Trends are illustrated by a dashed line, with p-value presented.

The expression of SLC7A4 after exposure to environmental contaminants remained unchanged when BOECs were exposed to TCS or ACE (fig: 3.19 c, d respectively) However, 10 μ M and 10 nM of BPA significantly increased expression of SLC7A4 transporter compared to control group (2.14 vs 0.96 folds $p=0.004$) and (2.54 vs 0.96 folds $p=0.0004$) compared to control group (Fig3.19a). Furthermore, there was a significantly increase of SLC7A4 expression when BOEC were exposed to BPS irrespective of the administrated dose (Fig 3.19 b)

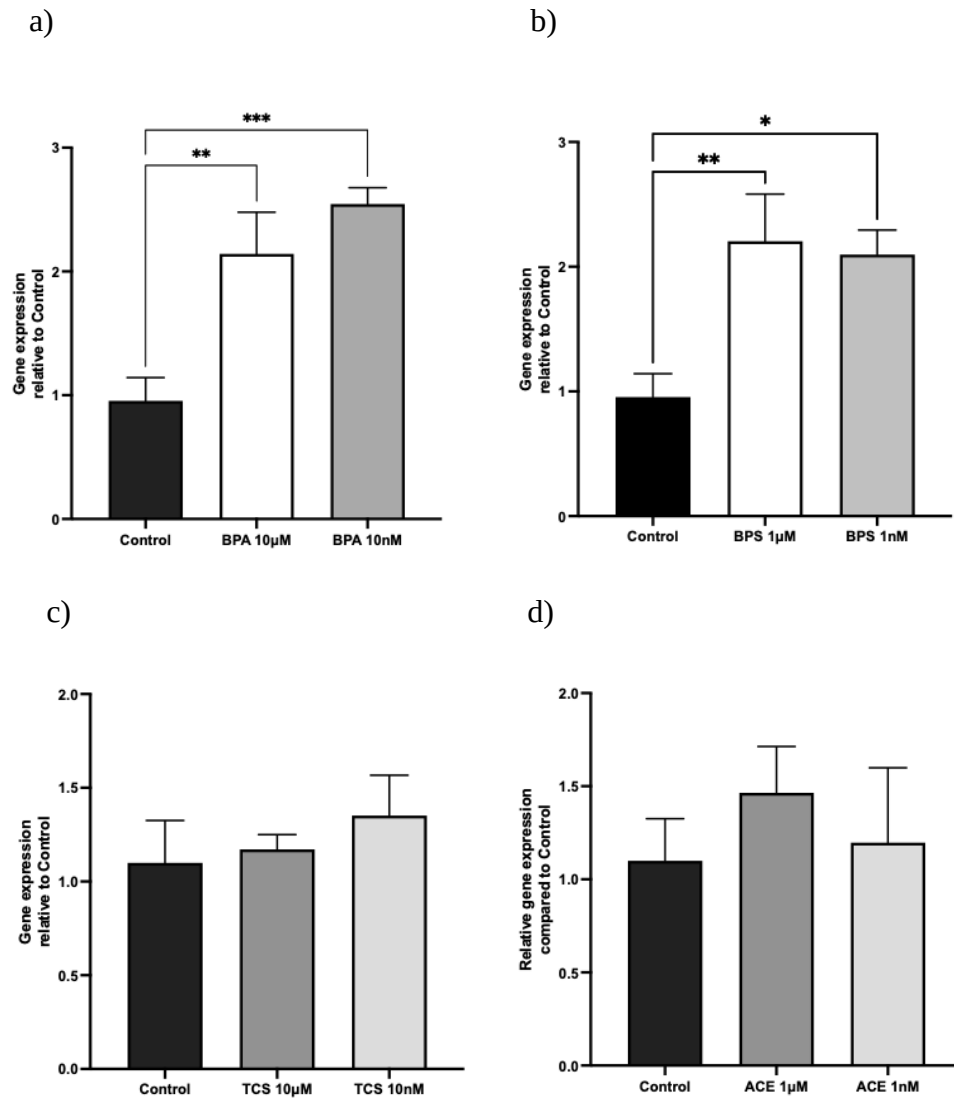


Figure 3.19: Relative gene expression of SLC7A4 amino acid transporter in BOEC exposed to a) Bisphenol A b) Bisphenol S, c) Triclosan and d) Acetaminophen for 24 hours in air-liquid phase. These graphs represent data of three biological replicates ($n=3$) for BPA and BPS and four ($n=4$) for TCS and ACE. Each biological replicate consists of two technical replicates for BPA and BPS and one technical replicate for TCS and ACE per experimental group. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, $p<0.05$; ** $p<0.01$; *** $p<0.001$. Trends are illustrated by a dashed line, with p -value presented.

Based on the results in figure 3.9c, where TCS prompted a change in glucose concentration transported/secreted by the BOECs into the ivDOF, expression the SLC2A1 (glucose transporter) was investigated (fig 3.20). These data resulting from 4 biological replicates with 1 technical replicate per experimental group. The expression of the SLC2A1 glucose transporter after TCS exposure was similar to those of the untreated group.

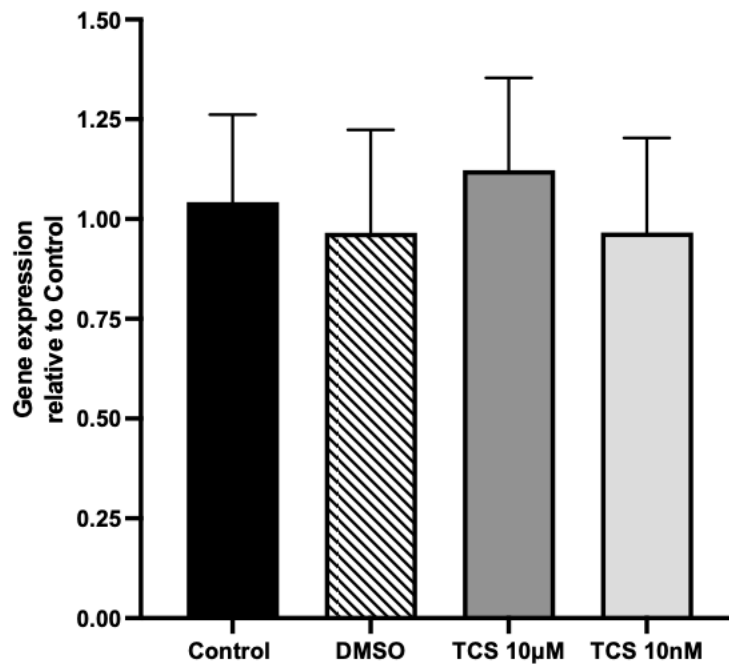


Figure 3.20: Gene expression of SLC2A1 glucose transporter in BOEC exposed to Triclosan for 24 hours in air-liquid phase. These graphs represent data of four biological replicates (n=4) with 1 technical replicate per experimental group per biological replicate. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

3.6 Discussion

Under physiological conditions, fertilisation, and pre-implantation development in mammals, occurs in the oviduct. Normally, this organ provides an optimised environment for the early reproductive events, such as final sperm maturation (capacitation), fertilisation and early stages of embryo development. However, the luminal environment of the oviduct is not an isolated system and exposure of environmental compounds is thought to induce changes on oviductal environment and subsequently in the reproductive processes mentioned above.

In this chapter the effects of environmental contaminants of interest (BPA, BPS, TCS and ACE) on bovine periconceptual environment was investigated. Using bovine as a model the metabolic function as well as the secretions of the oviduct epithelial cells, which are essential during the early reproductive events, were determined.

Initially the metabolic function of BOEC was investigated by determining essential energy substrates such as glucose, pyruvate, lactate. BOEC were treated with the chosen panel of environmental contaminants for 7 consecutive days, and glucose, pyruvate and lactate concentration were quantified in the spent media in 24 hours intervals. The results revealed that exposure to BPA decreased the pyruvate and increased lactate concentration in the spent media after long exposure while the glucose uptake remained unchanged during the whole period of exposure. One major fate of glucose is the conversion to pyruvate which subsequently either converted to Acetyl-CoA through pyruvate hydrogenase (PDH) enzyme and enters the mitochondria to participate to TCA cycle for energy production or is converted to Lactate through lactate hydrogenase enzyme (LDH). This final step is a reversible reaction that is dependent the pH of the environment (J. M. Berg et al., 2002). However, extracellular pyruvate can be taken up by cells and converted directly to lactate. The fact that the concentration of pyruvate was decreased and at the same time lactate was increased in the spent medium suggest that BPA presence in the cell culture might affect the flow of nutrients through the glycolysis pathway, possibly by altering the equilibrium of the last chemical reaction where pyruvate is converted to lactate. Impaired glycolysis after exposure to BPA has been identified previously in other systems such as in liver of mouse (Ji et al., 2020) and rat (Azevedo et al., 2019). Furthermore, impaired energy metabolism was identified in zebrafish testis where it was observed that BPA led to reduction of lactate production by reducing the LDH activity (Batista-Silva et al., 2022). Increased lactate and pyruvate production was identified in human ovarian

adenocarcinoma cell line OVCAR-3 which exposed to BPA in vitro (Shi et al., 2017). These differences among the studies could be due to different concentrations, period of exposure and models that have been used. However, common ground among all these studies was the impairment of the glycolysis metabolic pathway after BPA exposure.

Interestingly, when treated with BPS for 24h, BOECs seemed to *increase* the concentration of pyruvate in the spent medium. Moreover, treatment with TCS and ACE during the first 48h of culture also prompted an increase in pyruvate concentration in the spent medium. However, this effect was eased after 48 hours of culture in all cases, and no significant changes were noticed on glucose or lactate during the whole period of exposure. Pyruvate is an essential nutrient used as energy substrate during the preimplantation embryo development and has been identified in oviduct fluid (Gardner & Leese, 1990). The nutrients composition in the periconceptual environment has been linked with embryo development (Gegenfurtner et al., 2019). and alteration on concentration or composition of the oviduct fluid has been correlated with adverse effects on embryo development (Li et al., 2007). For this reason, alteration on pyruvate concentration in the oviductal environment could impact the preimplantation development.

However, during the first 72hours of BOEC culture the cells were not confluent. Therefore, the difference that was noticed on pyruvate concentration could possibly be due to difference on BOEC viability after the treatment. To test this assumption, the viability of the BOEC after exposure to the panel of environmental contaminants compared to untreated group was assessed. The results revealed that cell viability was impacted by BPA in the first 24hours, by BPS the first 48hours of exposure and in the case of TCS and ACE during the 72 hours of culture. After these points no statistically significant difference on viability of treated BOEC compared to untreated group was noticed. Based on this observation it is likely the difference in pyruvate concentration in the spent medium of cells treated with BPS, TCS, ACE and be due to the differences in cell number during the exposure. However, since the cell viability after 96hours of exposure was similar to the untreated group, the differences in pyruvate and lactate concentration after BPA treatment were unlikely to be influenced by the cell number suggesting that the BOEC metabolic status was impacted rather than their survival ability against BPA.

In summary, these data demonstrated that treating BOECs with our panel of environmental contaminants prompted subtle, but clear impacts on cellular physiology.

Next the impact of the panel of contaminants on secretions of BOECs was investigated. During these experiments, BOEC were cultured in an air-liquid interface which imitate more closely the *in vivo* status (Simintiras et al., 2016). First, Transepithelial Electrical resistance (TEER) of BOEC in the presence of the treatments was assessed. TEER increases when tight junctions between the cells are formed (Lo et al., 1999; Anderson, 2001). This method has previously been used to confirm the formation of an intact monolayer of bovine (Simintiras & Sturme, 2017) and porcine (Chen et al., 2015) oviduct epithelial cells. By measuring TEER on BOEC exposed to the treatments over the course of 7 days, we confirmed that TEER was increasing in the same pattern as the untreated group. These observations indicated that the formation of the tight junctions was not significantly influenced by the presence of the contaminants allowing the BOEC to form an intact monolayer.

When cultured in an ALI, oviduct epithelial cells create a thin film of fluid, representative of the fluid of the oviduct (Simintiras et al 2016). This fluid is termed ivDOF and it is possible to quantify the concentrations of energy substrates, glucose, pyruvate, lactate and 18 amino acids, secreted by BOEC. When cells were exposed to TCS, the concentration of glucose in ivDOF was significantly increased compared to that of the controls. The glucose concentration in ivDOF was decreased after 24 hours of exposure to BPA however this trend of decrease was eased after 48 hours, indicative of an acute response. BPS and ACE did not impact the glucose concentration in the oviductal secretions at any point of culture.

Despite some fluctuations, lactate was not statistically influenced by the presence of any of the contaminants. Finally, pyruvate concentration was significantly increased during the first 24hours of exposure to TCS and ACE, a difference which was eased after 48 hours of culture.

The oviductal environment is considered the optimal environment for the early reproductive events, supplying the necessary nutrients through the oviductal fluid secreted by the epithelial cells (Leese, 1988). Among other molecules, glucose, pyruvate and lactate are included in the oviductal fluid and they are used as main energy substrates during preimplantation embryo development (Leese, 2012). Exposure to certain environmental changes during vital periods of development and growth can significantly impact an individual's health both immediately and

over the long term (Barker, 2007). Indeed, alteration on the concentrations of molecules such glucose, insulin, non-esterified fatty acids have been associated with metabolic imbalance and linked with poor reproductive outcome in dairy cattle (Lonergan et al., 2016). For this reason, changes of the nutrients' composition in the oviductal fluid could have an impact on early stage of embryo development. This is important because concentrations of glucose and pyruvate within the fluid created by oviduct cells were modified when BOECS were treated with TCS and ACE. Both of these molecules are used as the key energy substrates during embryo development with the pyruvate being the preferred metabolite during cleavage stages and glucose consumption been increased during blastocyst stage (Martin & Leese, 1995; Thompson, 2000; Chi et al., 2020). High glucose during preimplantation development has previously been linked with altered gene expression related to glucose metabolism in equine (Sánchez-Calabuig et al., 2021) accumulation of reactive oxygen species (ROS) and mitochondrial dysfunction on zebrafish (Li et al., 2022), impaired metabolic pathways such as glycolysis, oxidative phosphorylation, TCA cycle and amino acid metabolism in mouse (Hong et al., 2023) and cardiovascular dysplasia on chicken (Jin et al., 2019). Moreover, changes in glucose concentration, accompanied with changes in lipids concentrations, have been linked previously with impaired oocyte metabolism and reduced embryos quality in bovine (De Bie et al., 2017).

Apart from glucose, pyruvate concentration was significantly increased in BOEC secretions after 24 hours of TCS and ACE exposure. As mentioned earlier, pyruvate plays an important role in early reproductive events. Pyruvate is a derivative of glycolysis and is essential for the ATP production in the mitochondria. However, glycolysis is low during the early stages of embryo development and for this reason, cleavage stage embryos need to receive the necessary amount of pyruvate through their environment (oviductal fluid in physiological conception or media in assisted reproduction) (Leese, 2003). Pyruvate importance on early stages of embryo development has been underlined by many studies over the years (Leese & Barton, 1984; Butcher et al., 1998; Orsi & Leese, 2001; Bradley & Swann, 2019; Hengye Zhang et al., 2021; da Fonseca Junior et al., 2023). Due to its importance, pyruvate has been suggested as a potential marker for the embryo quality (Conaghan et al., 1993; Mádr et al., 2015) and its consumption during preimplantation period has been linked with embryo developmental competence (Guerif, McKeegan, Henry J. Leese, et al., 2013a). Crucially pyruvate has recently been shown to promote zygotic genome activation process in mouse (Nagaraj et al., 2017) and porcine (Zhang, Zheng, et al., 2022). Low levels of pyruvate disrupt the M- and Z-decay

pathways (Hengye Zhang et al., 2021) both pathways are involved in maternal mRNA clearance, an essential process for the maternal to zygotic genome activation transition (Sha et al., 2020). Such findings indicate the importance of TCS and ACE-induced modifications of the secretions of the oviduct in ways that could have a tangible impact on the developing embryo.

These experiments are observational, and so mechanism by which ACE and TCS induce alterations in oviduct epithelial cells could not be identified. However, a possible explanation for these outcomes could be the TCS and ACE have an impact on metabolic pathways of the BOEC or possibly affect the transporters' function or expression of these molecules. To investigate this, Glucose transporter SLC2A1 (GLUT1) expression was investigated after BOEC exposure to TCS for 24 hours, however no statistically significant difference was identified, indicating that the differences observed were downstream of the nutrient transporter. Interestingly, an in vivo study on mice showed that treatment with TCS reduced expression of GLUT1 in the placenta (Cao et al., 2017), suggesting that GLUT may be sensitive to TCS. Further investigation into metabolic and molecular aspect needs to be done in the future to identify the mechanism of action of these contaminants on BOEC nutrients transport

Amino acids concentration in BOEC secretions was also significantly modified by the presence of environmental contaminants. BPA had the greatest impact on amino acid transport as statistical differences were identified on 15 (His, Trp, Val, Iso, Leu, Thr, Lys, Gln, Gly, Arg, Ala, Tyr, Asp, Asn, Ser) out of 18 amino acids that were measured. In all cases the concentration of the affected amino acids was significantly lower in the treated compared to control group. BPS also influenced the amino acid transport/secretion in the ivDOF as 6 (Trp, Lys, Asp, Gly, Arg, Ala) out of 18 amino acids where significantly different to untreated groups. Finally, TCS and ACE presence in the media affected the amino acid concentration transported/secreted into the ivDOF as 3 out of 18 amino acids, Gly, Arg, Lys, and Asp, Glu, Arg respectively, where significantly different to untreated groups. Overall, we can say that amino acid composition in the oviductal environment was impacted by the presence of these environmental compounds.

To the best of our knowledge the effects of the environmental contaminants on amino acid transport/secretion in the oviduct fluid has not been investigated before. However, the importance of amino acids during embryo development has been studied over the years using

different animal models (Gardner & Lane, 1993; Partridge & Leese, 1996; Pinyopummintr & Bavister, 1996; Humpherson et al., 2005) and presented in a recent review by Leese et al 2021 (Leese et al., 2021). Amino acids have multiple roles in embryo development, by participating in protein synthesis (Epstein & Smith, 1973), metabolic pathways as energy substrates (Partridge & Leese, 1996), or in oxidative stress regulation by producing antioxidants such as glutathione (Menezo et al., 2016) and pH regulation (Baltz, 2001). They are also involved in biosynthetic processes such as purines and pyrimidines synthesis (Alexiou & Leese, 1992). Amino acid turnover during preimplantation period has been suggested as a biomarker for embryo viability and developmental capacity in human (Houghton et al., 2002; Brison et al., 2004). In a recent study, where the association of follicular fluid and blastocyst formation in bovine was investigated, the levels of specific amino acids in the follicular fluid were linked with higher blastocyst formation rates (Sanei et al., 2023), suggesting that the level of amino acids in periconceptual environment play an important role in embryo developmental competence.

All the above information indicates that amino acids are essential for the embryos and any alterations on the levels, or the composition of the amino acids could impact their development. The fact that the presence of environmental contaminants changed the amino acids secretions allows us to speculate that embryo development would respond to this altered environment. However, the exact changed on the embryo development could not be discussed based the current observations. Future work could focus on the effects on embryos which develop in such altered environment.

The most commonly affected amino acids among the environmental contaminant exposed BOECs were arginine (Arg), alanine (Ala), aspartate (Asp), lysine (Lys) and tryptophan (Trp) with Arg being affected in all cases of exposure. The mechanisms behind the altered amino acid transport/secretion into the ivDOF are unclear, however it may point to alteration of amino acid transporters. SLC1A4 and SLC1A5 are amino acid transporters through which neutral such as Ala and acidic amino acids such as Asp can be transported through the ACS system (Gao et al., 2009). SLC7A1, SLC7A2, and SLC7A4 are cationic amino acid transporter and Arg and Lys are transported through γ^+ system (Gao et al., 2009; Forde et al., 2014).

Given the shared transporters, the expression of SLC1A4 and SLC1A5 were assessed. Interestingly, expression of SLC1A4 responded to BPA and ACE, whilst SLC1A5 remained

unchanged. This observation suggest that aspartate transport was possible impaired due to the alteration on the SLC1A4 and not on SLC1A5 expression in BOEC. Despite a slight increase on SLC1A4 expression after BPS exposure, no significant differences were identified in SLC1A4 and SLC1A5 expression suggesting that more investigation needs to be done in the future to identify why the aspartate concentration in ivDOF changed after BPS exposure. Furthermore, significant differences were identified on the expression of the cationic amino acid transporters (SLC7A1, SLC7A2, SLC7A4) after exposure to environmental contaminants. More specifically, BPA and BPS presence in the media significantly increased expression of SLC7A1 and SLC7A4, but not SLC7A2, in BOEC. This observation suggested that Arg and Lys transport in ivDOF was impaired due to the alteration on SLC7A1 and SLC7A4 expression after bisphenol exposure. Meanwhile, SLC7A2 expression on BOEC was significantly lower after TCS and ACE exposure suggesting that the altered Lys and Arg concentration in ivDOF was due to the effect on SLC7A2 expression. Impairment on placental nutrient transport has been previously reported in pregnant mice after TCS exposure (Cao et al., 2017). Altered amino acid metabolism after ACE exposure has also been investigated in an in vivo study on mice (Cartereau et al., 2018).

Overall, in this chapter, we have seen that exposure of periconceptual environment in environmental contaminants of interest changed the physiological function of the cells which subsequently cause an impairment on the oviductal epithelial secretion by altering the energy substrates' composition (glucose, pyruvate, and amino acids). The altered amino acid composition after exposure is resulting from changes on the expression of the amino acid transporters. It is very likely that the observed changes on oviductal fluid composition could possibly affect early reproductive processes such as oocyte maturation and preimplantation embryo development. The effects of environmental compounds on oocytes and embryo's function and metabolism will be investigated in the next chapters.

Chapter 4:
The impact of environmental contaminants on bovine oocytes

4.1 Introduction

4.1.1 Oocyte metabolic function during maturation

Mammalian follicular environment and oocyte metabolism has been widely studied (reviewed by Warzych and Lipinska, 2020) and has been linked with the developmental competence of oocyte and the subsequent embryo. Understanding metabolic processes during the early reproductive events, such as oocyte maturation, fertilisation and early stages of development might pave the way to improve in vitro culture conditions by recognising the essential molecules and energy substrates for each process. This topic has been reviewed from many scientific groups over the years with the recent and most well-rounded one being written by (Richani et al., 2021).

Research on oocyte metabolic function has tended to focus on the latter stages of development and maturation. However, the metabolic activity during oogenesis and folliculogenesis was been reviewed by (Collado-Fernandez et al., 2013). After leaving the follicle at ovulation, the oocyte remains enclosed in a compact layer of somatic cells, known as the cumulus cells. The oocyte and its accompanying layer of cumulus cells are intimately connected through transzonal projections creating the cumulus-oocyte-complex (COC) (Li & Albertini, 2013). Cumulus cells provide numerous functions; they serve as a physical barrier for sperm, as well as providing support the oocyte final stages of maturation through the provision of essential nutrients. Oocytes have a low capacity to take up glucose from the external environment, due to low phosphofructokinase (Dumesic et al., 2015) and lack of specific transporters expression (Sutton-McDowall et al., 2010; Richani et al., 2021). In addition some specific amino acids (Pelland et al., 2009) such as alanine, lysine, histidine, and glycine are also poorly transported. By contrast, cumulus cells have high glycolytic activity, meaning that glucose is metabolised and converted to pyruvate by the cumulus cells. This pyruvate is subsequently channelled directly to the oocyte as energy substrate. As expanded on chapter 1 section 1.6.2, pyruvate is converted to acetyl-CoA and, through the synergy of TCA cycle and electron transport chain, ATP is produced through oxidative phosphorylation into the mitochondria. Beyond the provision of ATP, pyruvate metabolism has been also associated with maturation status. It seems that pyruvate facilitates the mammalian oocyte to continue the maturation process and reach metaphase II (MII) (Downs et al., 2002; Johnson et al., 2007). Differences on metabolic status of pyruvate has been identified between immature and mature mouse COCs cultured in

media supplemented with gonadotrophins. More specifically, COCs failed to reach MII phase consumed pyruvate whilst those which reach the final maturation status produced pyruvate (Roberts et al., 2004).

Even though glycolysis appears to be the main pathway that glucose is metabolised by cumulus cells glucose is metabolised by other routes. For example, the pentose phosphate pathway (PPP) has been correlated with meiotic progression to MII stage in mouse (Downs et al., 1998), porcine (Sato et al., 2007) and bovine (Cetica et al., 2002) COCs. This pathway also influences the antioxidant mechanism of COCS by replenishing glutathione, an antioxidant of paramount importance during maturation, which is reduced using NADPH a by-product of PPP.

Interestingly, ATP in oocytes is predominantly produced through oxidative metabolism in mitochondria using pyruvate as energy substrate which is provided after glucose metabolised by cumulus cells or by using fatty acids. The ATP consumption seems to fluctuate during maturation process, as in metaphase I (MI) or MII stage is higher than GV stage (Dalton et al., 2014). While glucose metabolism does produce ATP, lipid metabolism -notably fatty acids - through β -oxidation pathway has been confirmed to be used for energy production in different species of COCs (Sturmey & Leese, 2003; Van Hoeck et al., 2011; Hoeck et al., 2013). Fatty acid B-oxidation produces high amounts of ATP and it has also correlated with the resumption of COC maturation (Downs et al., 2009). Indeed, there are evidence supporting that oocyte maturation is supported by b-oxidation in several species (Sturmey et al., 2009; Dunning et al., 2010; McKeegan et al., 2021)

Furthermore, other local factors such as growth factors, cytokines and steroids hormones are secreted by the somatic cells which surrounds the oocyte. It has been found that this dialogue between the oocyte and the somatic cells facilitated by these local factors during the maturation and ovulation process also play an important role on the oocyte nourishment (Dumollard et al., 2007; Sreerangaraja et al., 2020).

The steroid hormones produced by the somatic cells of the follicle aim to assist the oocyte development and survival (Wang et al., 2006; Sreerangaraja et al., 2020). The first step of steroidogenesis is the transfer of cholesterol into the mitochondria which are the main sites of the steroid production. This process is facilitated by the steroidogenic acute regulatory protein (STAR protein). The biosynthesis of the progesterone (P4) and oestradiol (E2) is based on the

two-cell-two-gonadotrophin mechanism, where the theca cells resulting on P4 and androgen production which subsequently are transferred to granulosa cells and act as precursors of the E2 production, a process which has been described in detail in Chapter 1, section 1.2.2.

The importance of the steroid hormone secreted by COCs during in vitro maturation, was previously investigated. In a series of the in vitro experiments bovine COCs were used as models. The results have shown that the inhibition of steroidogenesis by using aminoglutethimide (AGT) prevent nuclear maturation and the cumulus expansion of the COCs. The AGT blocks the cytochrome P450 action resulting in the inhibition of several steroid hydroxylation steps. However, when the COCs were cultured in a conditioned media from COCs in the presence of AGT, the maturation rate and the cumulus expansion rate was similar to control group suggesting that the endogenous steroids secretion of the COCs are essential for the oocyte maturation (Wang et al., 2006)

As described above, the ATP production as well as the endogenous steroids secretion is crucial for the proper function and completion of the oocyte maturation. The common factor of steroidogenesis and ATP production during the maturation process is that both take place in mitochondria. Mitochondria morphology, function or number has been reviewed and related with the oocyte quality and developmental competence (Van Blerkom, 2011) A review published recently, including in vitro experiments conducted in both human and animal models, concluded that various types of environmental chemicals have the potential to cause mitochondrial stress and interfere with mitochondrial function (Reddam et al., 2022) Environmental chemical exposure could possibly affect the oocyte developmental competence by interrupting the proper function of the processes have been described above.

4.2 Hypothesis and Aims

Bisphenol A, Bisphenol S, Triclosan and Acetamiprid, have been identified in human bodily fluids and circulate into the human body through the blood stream. As Bisphenol A has been previously identified in the follicular fluid (Van Blerkom, 2011; Sreerangaraja Urs *et al.*, 2020), we hypothesised that the environmental contaminants of interests could reach the ovarian tissue through the blood stream and enter the follicular fluid and impact the oocyte maturation. To test this hypothesis bovine COCs were used as a model. The aims of this project are to determine the impact of these chemicals on COCs function by investigating the following:

- ⇒ The impact on COCs energy substrates uptake and release.
- ⇒ The impact on COCs steroids release during IVM.
- ⇒ The ability of COCs to reach the completion of the maturation process.
- ⇒ The effects of the exposure on COCs mitochondrial activity and finally
- ⇒ The effects on the nuclear status of the generated zygotes.

4.3 Methods

4.3.1 Environmental contaminants stock preparation and storage

As previously explained (Chapter 3, fig:3.1), DMSO was chosen as the solvent of the environmental contaminants BPA, BPS, TCS, and ACE, the effects of which were investigated during these set of experiments. Two different concentrations were picked to be investigated for each environmental contaminant (Table 4.1). Each environmental contaminant was diluted into 100% DMSO (CAS: 67-68-5) creating a stock of 5000x of each final concentration and stored in aliquots at -20°C in glass vials for maximum of 3 months to prevent degradation. The day before the experiment the 5000x stock was further diluted in embryo tested H₂O to a 1000x stock and stored in 4°C for 7 days. Finally, the 1000x stocks were further diluted into Bovine Maturation Media (BMM) to the desired concentration, the morning before the isolation of the COCs and incubated at 39°C, 5% CO₂ at least two hours before the addition of the COCs into the treated media. The reason for the extra step of dilution into the embryo tested water was to reduce the amount of DMSO in the final exposure.

Table 4.1: The final concentration of environmental contaminants that COCs exposed

<u>Environmental contaminant</u>	<u>Concentration</u>	<u>Concentration</u>
<i>Bisphenol A (BPA)</i>	10nM	1nM
<i>Bisphenol S (BPS)</i>	10nM	1nM
<i>Triclosan (TCS)</i>	10nM	1nM
<i>Acetamiprid (ACE)</i>	10nM	1nM

4.3.2 Experimental design

In this chapter the effects of the environmental contaminants on Cumulus Oocyte Complexes (COCs) function were investigated. For all the experiments the COCs were isolated from bovine ovaries as described in chapter 2 sections 2.3.3 and 2.3.4

Initially, the effects of the environmental contaminants on the nuclear status of the exposed COCs was investigated (fig 4.1). Subsequently, the steroid hormones and energy metabolites productions during In Vitro Maturation (IVM) were accessed. The results are presented from

Figure 4.2 to 4.4 and from Figure 4.5 to 4.7, respectively. In addition, the OCR of the oocytes after acute exposure to the compounds was examined and the data are illustrated from Figure 4.8 to 4.11. Finally, the effects on pronucleus formation on zygotes after exposure during the in vitro fertilisation was also accessed (fig 4.12). The possible effects of control vehicle (DMSO) against the control group for each endpoint are presented in Figure 4.13_Appendix to 4.17_Appendix

The nuclear status of 928 bovine COCs was assessed after exposure to the environmental contaminants, BPA, BPS, TCS or ACE during IVM. After IVM the COCs were collected and incubated in HM media supplemented with hyaluronidase for 5 minutes to remove the cumulus cells subsequently the denuded oocytes were washed twice in 0.02% PBS-PVP and placed in 0.1% Hoechst in ethanol overnight at 4°C for fixation and staining. The next day the fixed-stained oocytes were washed twice in 0.02% PBS-PVP and place in glycerol on μ -Slide 15 Well 3D (abidi). A Zeiss inverted fluorescent microscope (Axiovert A1) was used and the oocyte imaging was performed at 361 excitation and 497 emission (nm). The oocytes were categorised in the following groups a) GV, oocytes with germinal vesicles nucleus after the completion of the IVM b) MI, oocytes reach the metaphase I, c) MII, oocyte complete the metaphase II d) degenerated, oocytes with abnormal nucleus morphology. Oocytes from 3 individual biological replicates per experimental group was investigated and the data are presented in Figure 4.1.

The COCs were exposed to two different concentration 1 nM and 10 nM of BPA, BPS, TCS or ACE during IVM. The preparation and the storage of the stocks has been described in section 4.3.1. The conditioned media was collected the next day (22 to 23 hours later) and stored in -80oC until further analysis. These samples were used to determine the quantity of steroid hormones, 17 β -estradiol and progesterone (fig 4.2 – 4.4) as well as the energy substrates, pyruvate and lactate (fig: 4.5 -4.7), released into the media by using ELISA (Chapter 2, section 2.6.2) and microfluorometric assays (Chapter 2, section 2.4.2) respectively. Each graph is result of multiple biological replicates per group and the exact number is mentioned in each figure legend. Each assay is described in detail is Chapter 2.

Subsequently, the mitochondrial activity of COCs acutely exposed to the compounds of interest was investigated by using Oxygen Consumption Rate (OCR) as marker. The protocol that was followed is described in detail in Chapter 2, section 2.4.5. Each graph is result of at least 3

biological replicates per group and the exact number is mentioned in each figure legend. The results are presented from Figure 4.8 to Figure 4.11.

Finally, the impact of the environmental contaminants on zygotes was examined by investigating the pronucleus formation 18 to 21 hours after fertilisation. For this experiment untreated mature COCs were fertilised by sperm as described in Chapter 2, section 2.3.5. The FERT-TALP media was supplemented with the treatments (table 4.1) and the exposure lasted for the whole duration of the fertilisation process. The next day the remaining cumulus cells were removed by vortexing for 1min, and the zygotes fixed and stained as described in the previous paragraph. The same microscope was used for the imaging and the zygotes were categorised into 4 groups a) unfertilised, zygotes without any sign of pronucleus or with the appearance of one polar body b) 2PN, zygotes with 2 pronucleus, c) >2PN, zygotes with more than 2 pronucleus and d) 2-cell embryos, zygotes which complete the first cell division. Results are presented in Figure 4.12

4.3.1 Statistical Analysis

GraphPad Prism 9 was used for all statistical analysis. Shapiro-Wilk test was used in order to define whether a parametric or non-parametric analysis is required. When the data was normally distributed, One-Way ANOVA was used followed by Tukey's post-hoc test. However, when normal distribution was not followed, a Kruskal-Wallis test was performed followed by Dunn's post-hoc test. In the case of percentages arcsine correction was performed initially and the results were analysed with One-way ANOVA or with two-way ANOVA followed by Tukey's post-hoc test. The exact method of analysis is mentioned in the legend of each graph.

4.4 Results

4.4.1 Oocyte nuclear maturation and exposure to environmental contaminants

Initially, the effects of environmental contaminants on nuclear maturation during IVM was investigated. COCs were exposed to the treatments (table) for 21h-23h during IVM and the next day, nuclear staining was performed on denuded oocytes as described above in order to identify the nuclear status of the treated oocytes. The nuclear status was divided into 4 categories: oocytes that enter metaphase II (MII) stage, oocytes in metaphase I (MI) stage, oocytes that did not enter the final stage of maturation and remained in germinal vesicles (GV) stage and finally oocytes with abnormal nuclear status defined as degenerated. As illustrated in the graphs in Figure 4.1 the treatments did not significantly influence the maturation of COCs in terms of the nuclear status. The percentage of COCs reaching the metaphase II (MII) was similar between the untreated group and the treated groups, irrespective of the environmental contaminants or the dose that was used. The number of COCs per experimental group, that tested for the nuclear status after the maturation, is illustrated in the Table 4.2 and each number correspond to 3 biological replicates per group. Control-vehicle (DMSO) did not have an effect on COCs nuclear status compared to control group (fig 4.15_Appendix)

Table 4.2: Number of COCs tested for nuclear status. Each group correspond to 3 biological replicates (n=3)

COCs per experimental group

<i>Total number of COCs</i>	Control	DMSO	BPA (10nM)	BPA (1nM)	BPS (10nM)	BPS (1nM)	TCS (10nM)	TCS (1nM)	ACE (10nM)	ACE (1nM)
928	112	100	86	100	85	93	92	87	85	88

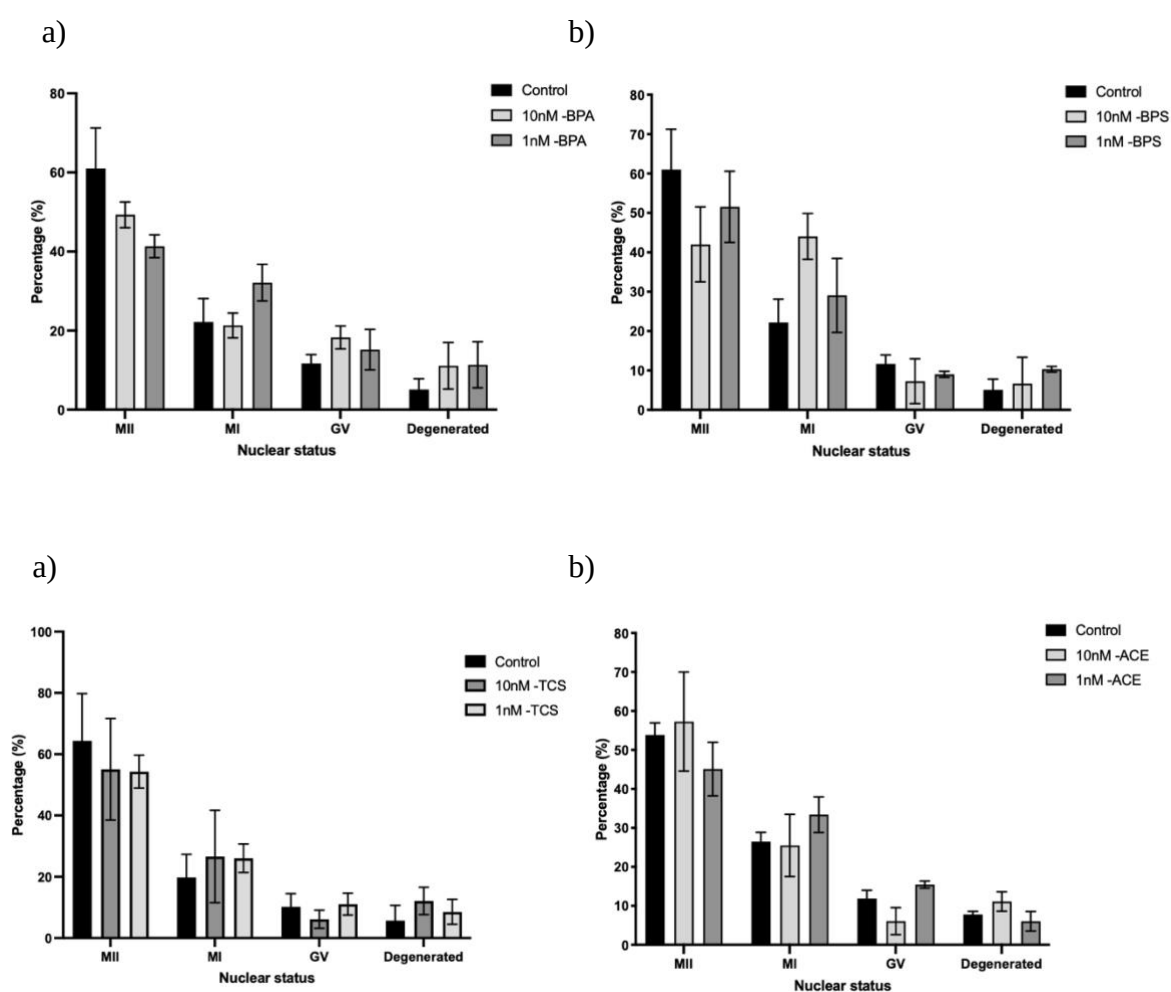


Figure 4.1: Nuclear status of bovine COCs exposed to two different concentrations (10 nM and 1 nM) of a) BPA, b) BPS, c) TCS and d) ACE during IVM. MII represent oocytes in metaphase II, MI represent oocytes metaphase I, GV represent oocytes in germinal vesicles stage and degenerated group includes oocytes with degenerated nucleus. As the data are presented in percentages, initially they underwent arcsine transformation and subsequently, Two-way ANOVA followed by Tuckey test was performed, * $p < 0.05$, data are presented in mean $\pm$ SD. Three biological replicates were used for the investigation of each environmental compounds ($n=3$).

4.4.2 Effects of environmental contaminants on steroid hormones production during IVM

Next the 17 β -oestradiol and progesterone production, in the presence of the environmental contaminants, during IVM, was investigated and the results are illustrated from figure 4.2 to 4.4. The presence of bisphenols in IVM media significantly impacted the production of 17 β -oestradiol (Fig:4.2a). COCs exposed to high dose of BPA (10nM) released significantly lower amount of 17 β -estradiol compared to control group (2.81 vs 3.61 pmol/hour/oocyte $p=0.0041$) as did COCs treated with 1nM of BPS (3.61 vs 2.91pmol/hour/oocyte $p=0.026$). However, 17 β -

oestradiol levels were not influenced by the high dose of BPS suggesting that BPS interferes in a non-monotonic way on the production of this hormone. Finally, the progesterone production during IVM was not significantly influenced by BPA nor BPS at any of the administrated concentrations (Fig:4.2b).

Regarding the impact of TCS on steroid hormone production, 17 β -oestradiol and progesterone, the results are presented in Figure 4.3. As we can notice from the graphs, 17b-estradiol was not significantly influenced by the presence of TCS. However, when the COCs were exposed to low dose of TCS (1nM), the levels of progesterone production were significantly higher compared to control group (2.85 vs 4.78 pg/hour/oocyte p=0.028), whilst the high dose of TCS was not suggesting a non-monotonic effect of action.

When the COCs were exposed to 10 nM or 1 nM of ACE, the production of 17 β -oestradiol or progesterone was not significantly impacted, suggesting that ACE does not affecting the steroids production during in vitro maturation (fig 4.4) as the other three contaminants. Importantly, the control vehicle (DMSO) had no impact on hormone release (fig 4.13_Appendix).

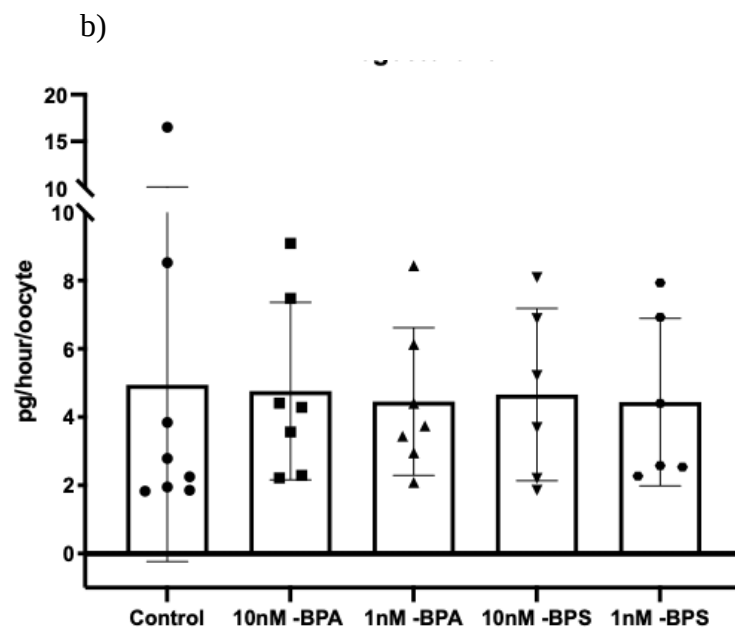
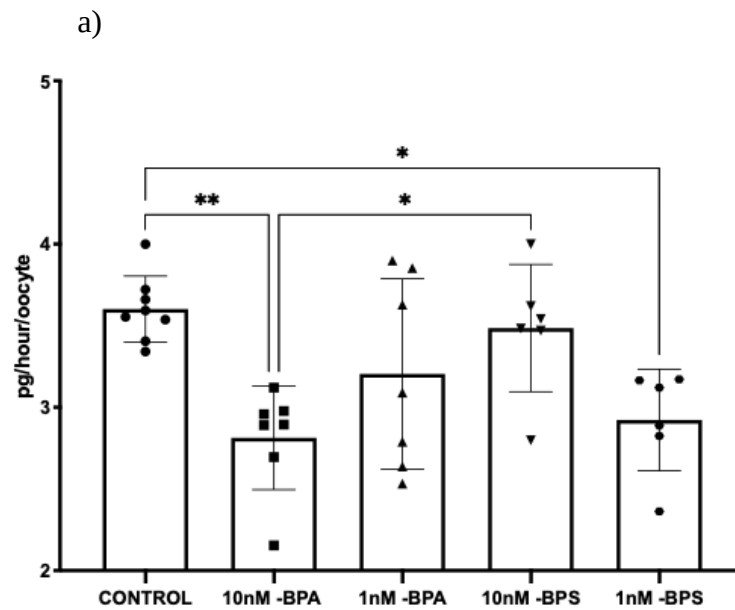


Figure 4.2: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to two different concentrations of Bisphenol A and Bisphenol S at 10 nM or 1 nM during in vitro maturation (n=7 for BPA and n=6 for BPS, each dot represents an individual replicate). Differences were assessed using one-way ANOVA followed by Tukey test. * p<0.05. Data are presented in mean $\pm$ SD

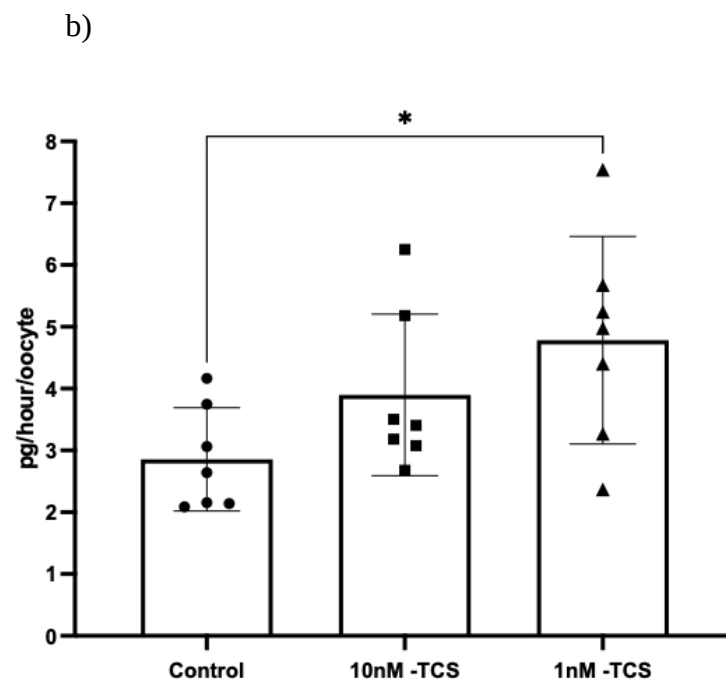
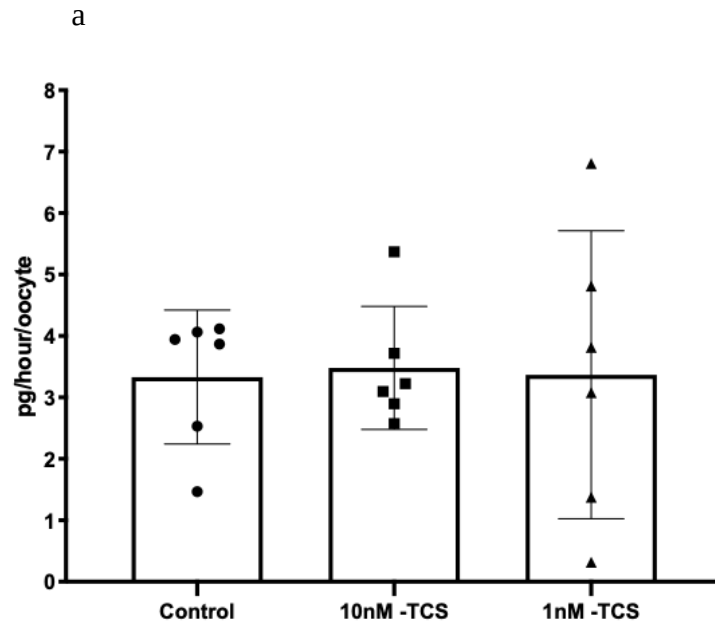


Figure 4.3: Release of a) 17b-estradiol and b) progesterone from bovine COCs exposed to two different concentrations of Triclosan at 10 nM or 1 nM during in vitro maturation (n=7 each dot represents an individual replicate). Differences were assessed using one-way ANOVA followed by Tukey test. * p<0.05 Data are presented in mean $\pm$ SD

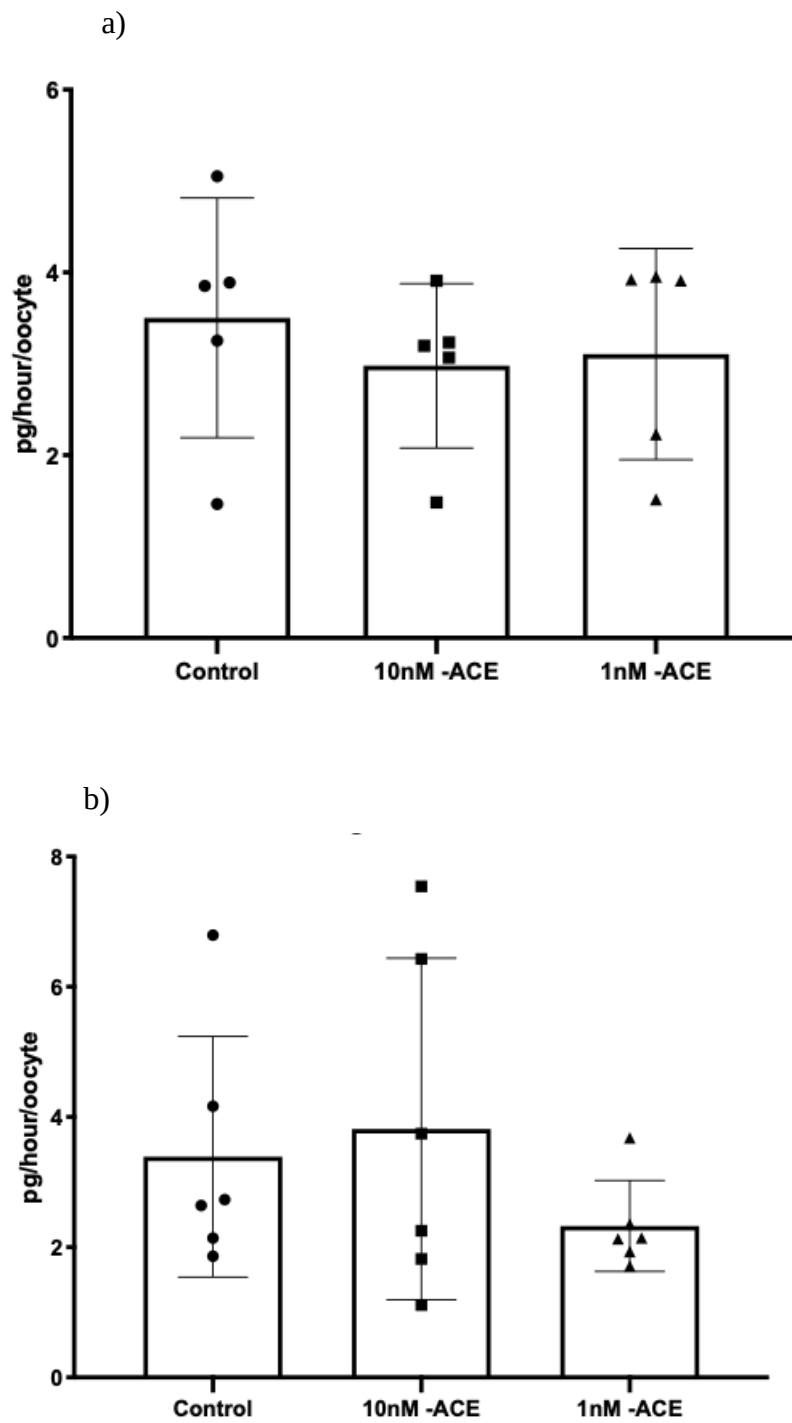


Figure 4.4: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to two different concentrations of Acetamiprid at 10 nM or 1 nM during in vitro maturation (n=5 each dot represents an individual replicate). Differences were assessed using one-way ANOVA followed by Tukey test. * $p < 0.05$ Data are presented in mean $\pm$ SD

4.4.3 The impact of environmental contaminants on oocytes' energy substrates

Next, the spent IVM media was used to quantify pyruvate and lactate released or depleted by the COCs during maturation (Fig:4.5- 4.7). Pyruvate and lactate production was not significantly influenced when the COCs were exposed to BPA or BPS during IVM.

When bovine COCs were treated with TCS, pyruvate metabolism was affected, irrespective of the administrated concentration. Untreated COCs released 82.84 pmol/hour/COCs pyruvate during IVM whilst COCs exposed to 1 nM or 10 nM TCS consumed pyruvate (106.4 pmol/hour/COCs and 112.8 pmol/hour/COCs, respectively). However, this metabolic change of pyruvate was not reflected on lactate production as it remained unchanged (fig: 4.6) among the experimental groups.

The same pattern of response in terms of the energy substrates, pyruvate and lactate was observed when COCs were exposed to 1 nM and 10 nM of ACE. ACE-exposed COCs consumed pyruvate whereas the untreated COCs produce pyruvate during IVM. Meanwhile, similarly to TCS, the lactate production remains in relative same levels among the experimental groups. Finally, DMSO did not significantly affect pyruvate and lactate production compared to untreated group (fig 4.14_Appendix)

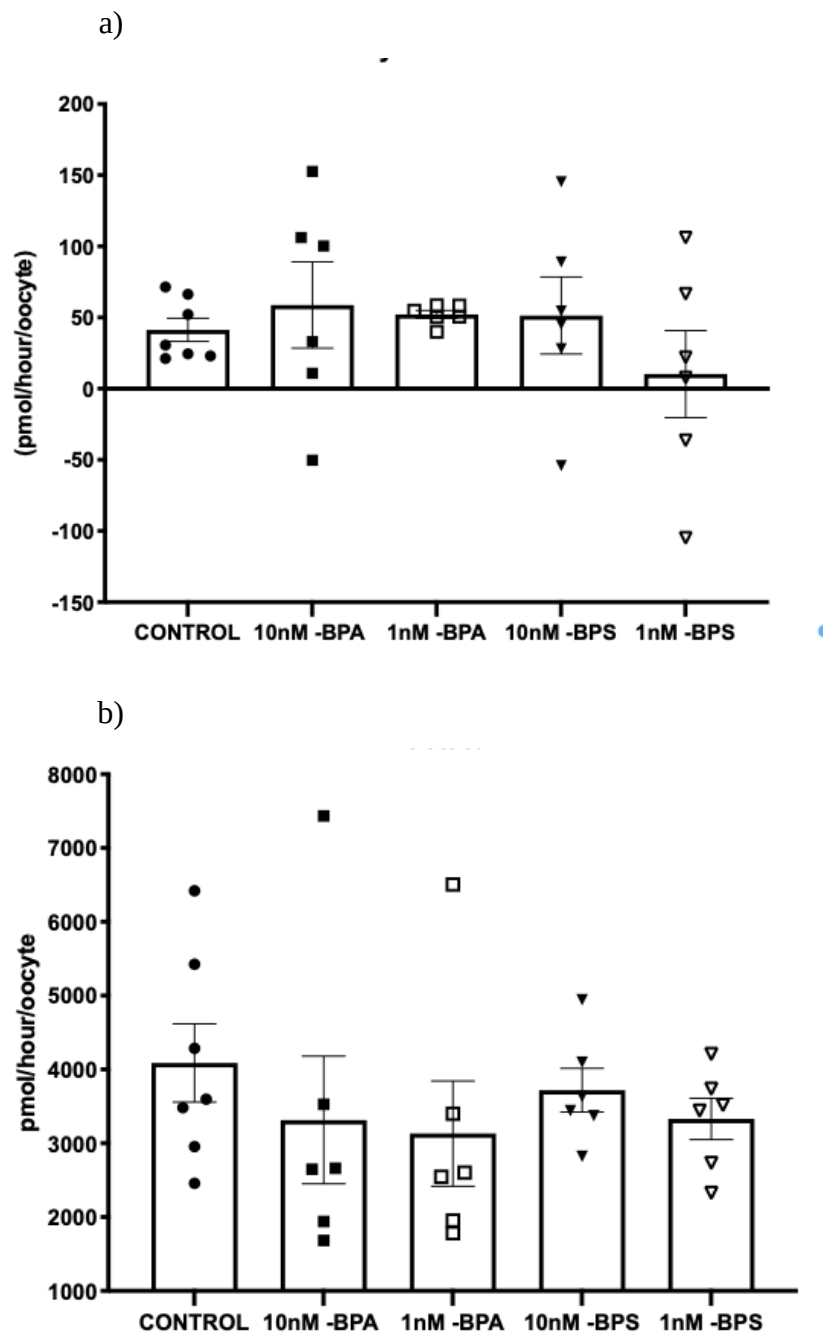


Figure 4.5: Release or uptake of a) pyruvate and b) lactate from bovine COCs exposed to two different concentrations of Bisphenols (BPA and BPS) at 10 nM or 1 nM during in vitro maturation (n=6 each dot represents an individual replicate). Differences were assessed using one-way ANOVA followed by Tukey test. * p<0.05 Data are presented in mean $\pm$ SD

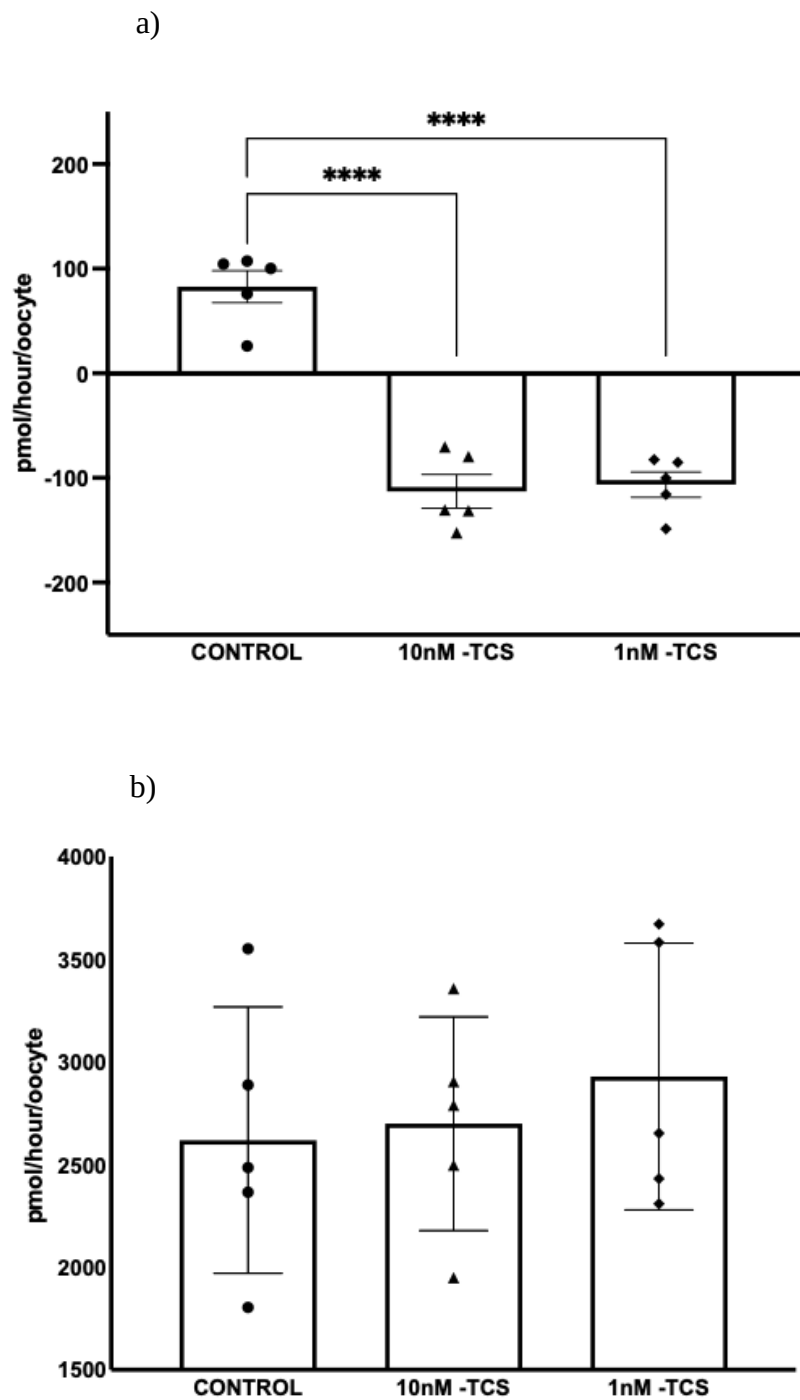


Figure 4.6: Release or uptake of a) pyruvate and b) lactate from bovine COCs exposed to two different concentrations of Triclosan at 10 nM or 1 nM during in vitro maturation (n=5, each dot represents an individual replicate). Differences were assessed using one-way ANOVA followed by Tukey test. * p<0.05 Data are presented in mean $\pm$ SD.

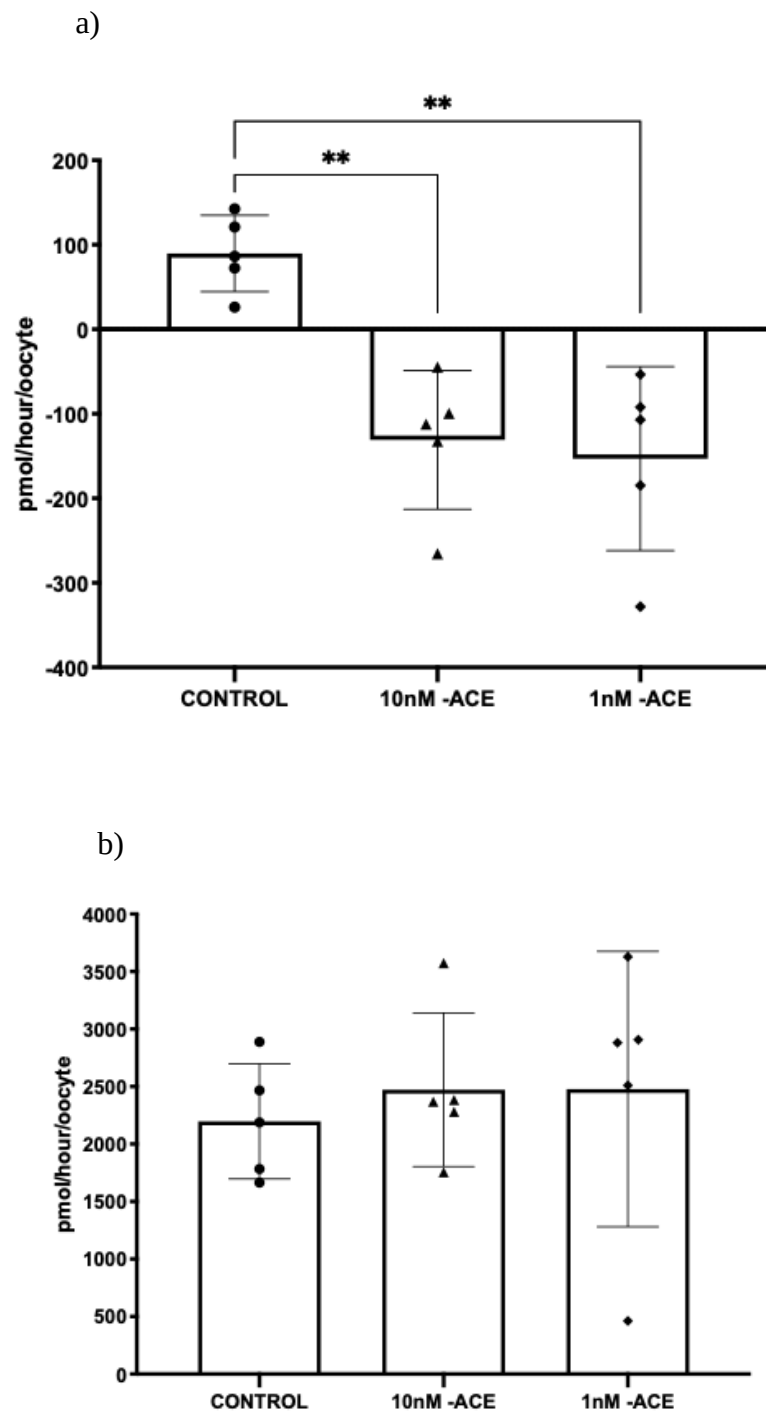


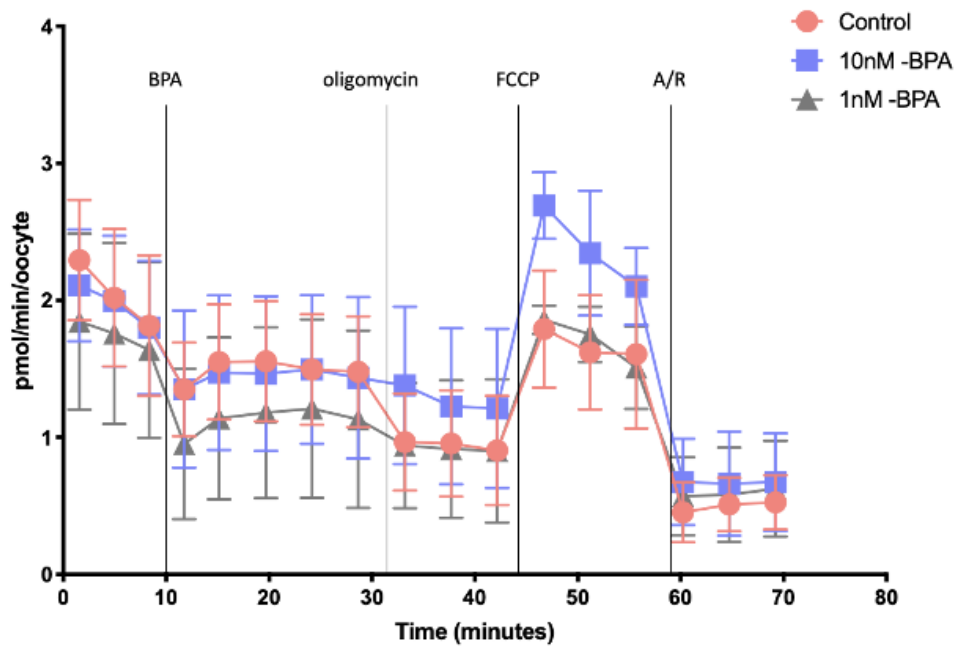
Figure 4.7: Release or uptake of a) pyruvate and b) lactate from bovine COCs exposed to two different concentrations of Acetamiprid at 10 nM or 1 nM during in vitro maturation (n=5, each dot represents an individual replicate). Differences were assessed using one-way ANOVA followed by Tukey test. * p<0.05 Data are presented in mean $\pm$ SD.

4.4.4 The impact on mitochondrial activity using Oxygen Consumption Rate (OCR) as a marker

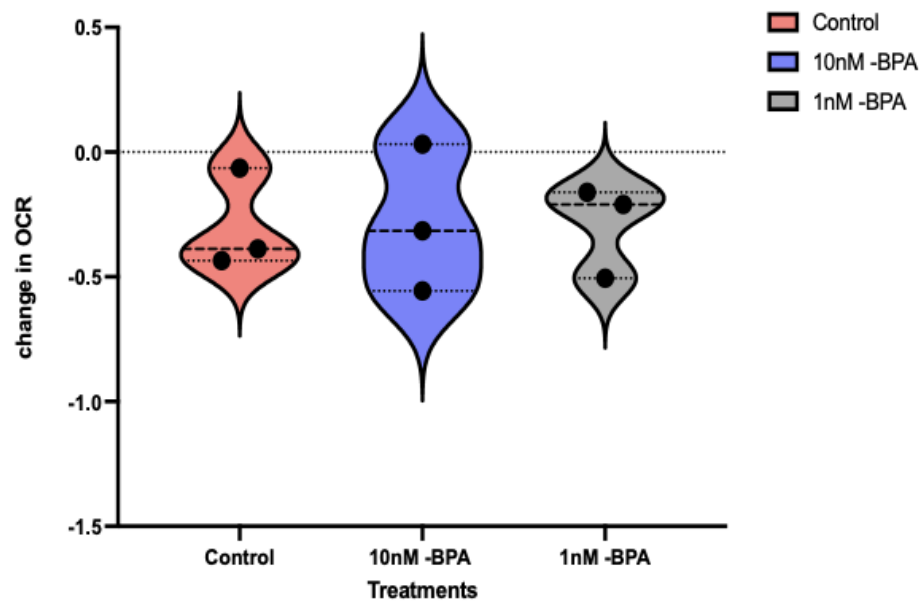
In the next set of experiments, by using oxygen consumption rate (OCR) as marker, the mitochondrial activity of the bovine oocytes after acute exposure to the BPA (Fig:4.8), BPS (Fig:4.9), TCS (Fig:4.10), and ACE (Fig: 4.11) was assessed. Immature oocytes were used for these experiments immediately after the oocyte's retrieval from the ovary. In the case of BPA, BPS and ACE exposure, bovine corona enclosed oocytes (CEOs) were used by treating the isolated COCs with 80IU/ml hyaluronidase for 3 to 5min. However, during the TCS exposure COCs were used.

The OCR data after CEO were acutely exposed to 0nM, 1 nM or 10 nM BPA are illustrated in figure 4.8 and they are resulting out of three biological replicates. Figure 4.8a represents the OCR line graph per experimental group and the time point where BPA and subsequently each mitochondrial inhibitor was added (described in detail in Chapter 2, section 2.4.5). The OCR after the addition of BPA was not significantly different from the untreated group (0nM) regardless of the administrated concentration (Fig: 4.8b). However, when the components of the OCR (ATP coupled, proton leak, spare capacity and non-mitochondrial) were calculated as percentage of the basal respiration after the BPA addition, the proton leak of 10 nM BPA was significantly increased compared to 1 nM BPA group, ($39.14 \pm 11.78\%$ vs $25.84 \pm 10.27\%$ $p=0.021$ respectively).

a)



b)



c)

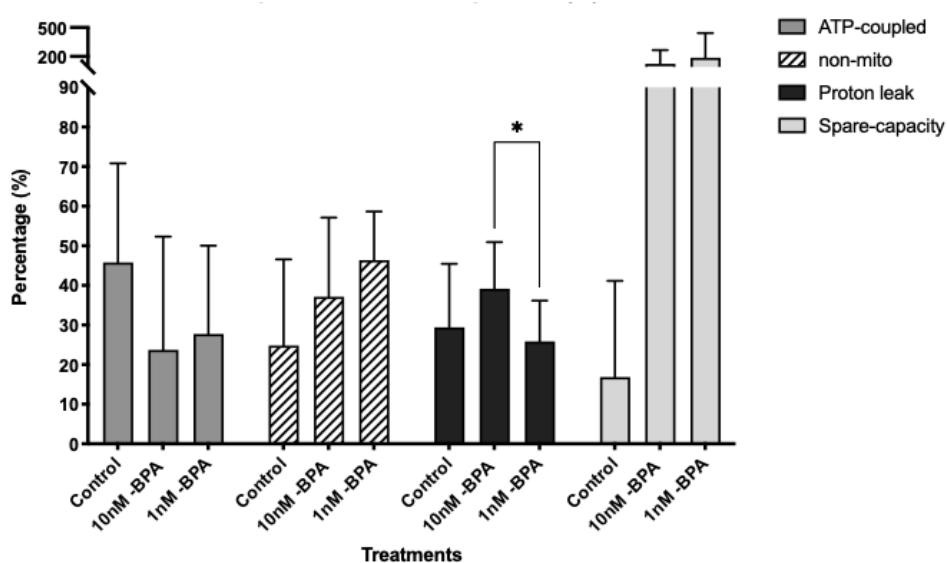
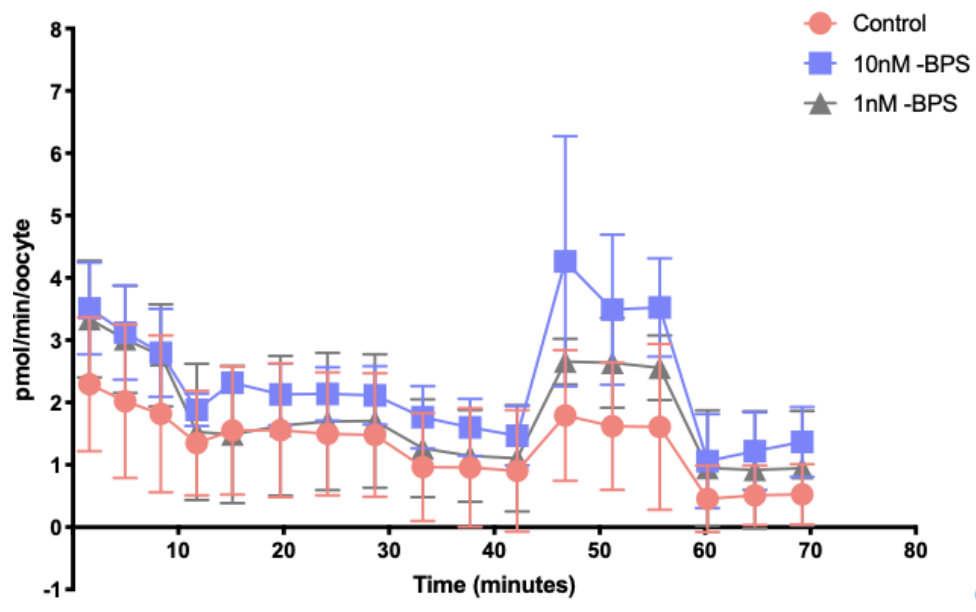


Figure 4.8: The impact on OCR immature CEOs acutely exposed to Bisphenol A (BPA) immediately after isolation from the ovary. a) OCR in COCs in response to BPA, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPA treatment, c) mitochondrial parameters as a percentage of basal OCR after BPA injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test, * $p < 0.05$ Data are presented in mean $\pm$ SD. These graphs include data of 3 biological replicates (OCR, $n=3$).

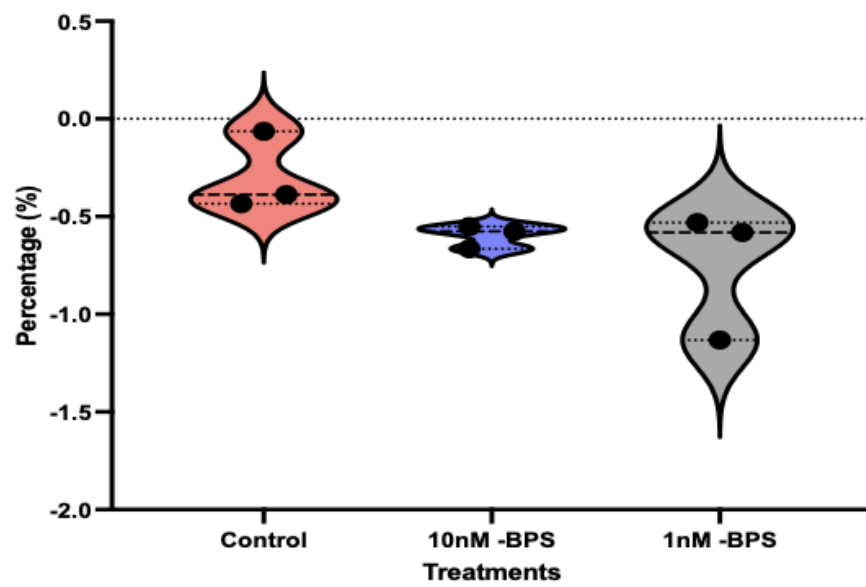
Similar, to BPA, when the CEOs were treated with BPS during the OCR assay, the response of OCR was not significantly different to control group (Fig: 4.9b). Nevertheless, strong trend of differences, compared to control group, was observed on non-mitochondrial OCR when CEO exposed to 10 nM and 1 nM BPS, with p-value $p=0.057$ and 0.072 respectively (Fig:4.9c).

The data of figures 4.8 and 4.9 are results of three biological replicates per experimental group, however, as it can be seen, there is notable variation among the biological replicates and some strong trends of differences are illustrated however due to low number of replicates further investigation needs to be conducted in the future.

a)



b)



c)

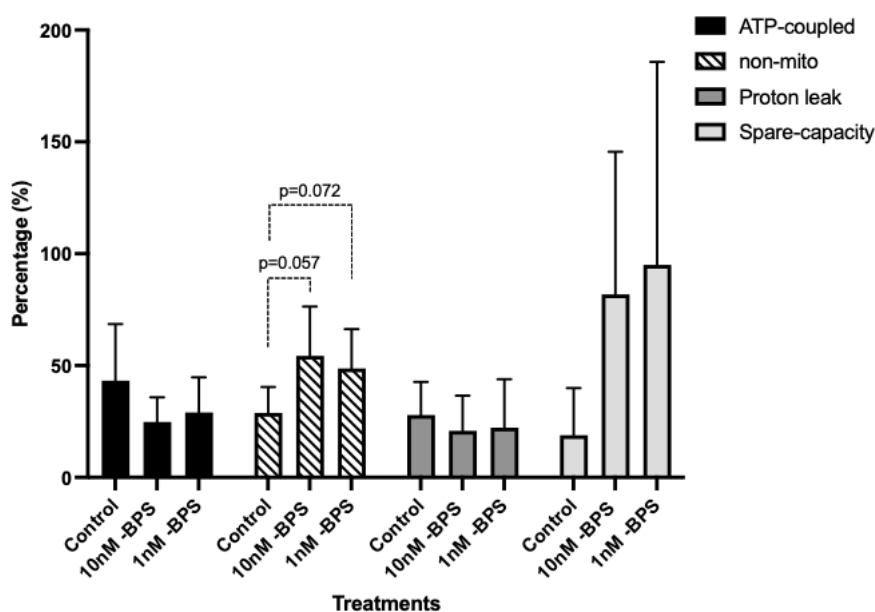
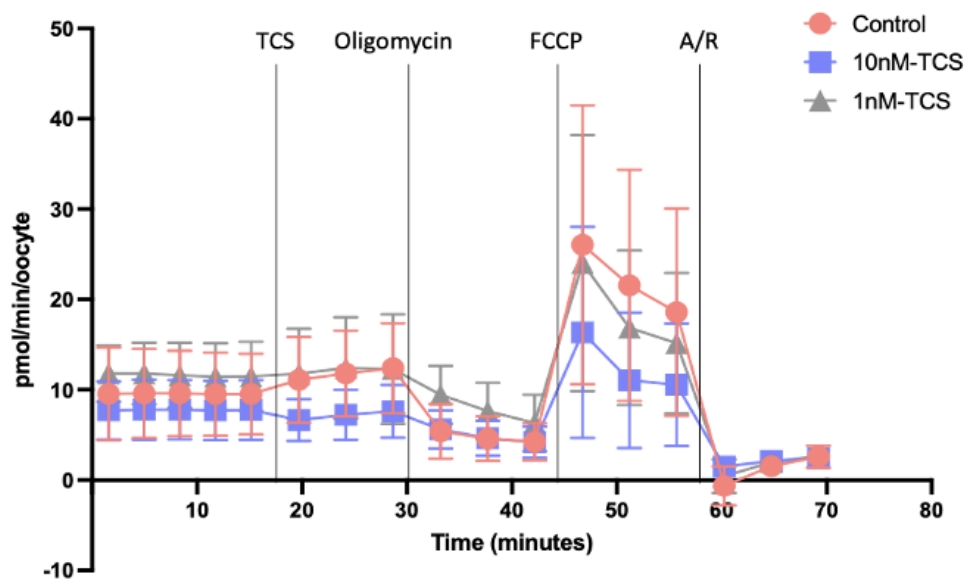


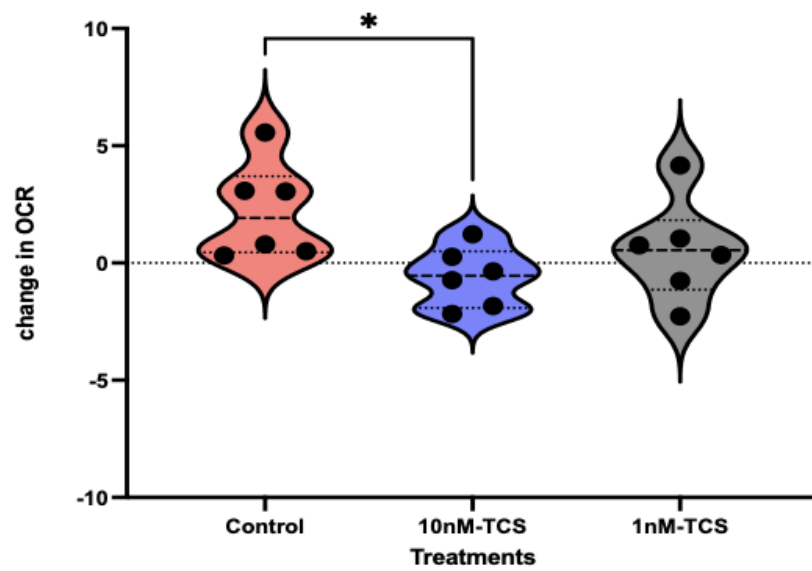
Figure 4.9: The impact on OCR immature COCs acutely exposed to BPS immediately after isolation from the ovary. a) OCR in COCs in response to Bisphenol S, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPS treatment, c) mitochondrial parameters as a percentage of basal OCR after BPS injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test, * $p < 0.05$ Data are presented in mean $\pm$ SD. Trends are indicated with dash line with the p-value presented. These graphs include data of 3 biological replicates (OCR, $n=3$).

The mitochondrial activity of the COCs exposed to TCS was assessed next. COCs were exposed to 0, 1 and 10 nM TCS immediately after the isolation from the ovary during the OCR assay. The analysis of basal respiration before and after the TCS exposure revealed that the addition of fresh BMM media in the untreated group due to nutrients and energy substrates addition resulted in a positive change in OCR however, that was not observed in the TCS-treated groups. Additionally, it seems that the 10nM-TCS exposure provokes a reduction in OCR which was statistically significantly different to control group (Fig:4.10b). ATP-coupled oxygen consumption was decreased about 25% in both TCS-treated groups as the ATP-coupled OCR in untreated group is $58.7\% \pm 5.2\%$ whereas for 10nM-TCS group is $33.1\% \pm 2.5\%$ and for 1nM-TCS $33.3 \pm 5.6\%$ (fig.4.10c). In addition, the non-mitochondrial activity was increased by almost 18% in 10nM-TCS treated COCs compared to untreated group ($28.4\% \pm 3\%$ vs $10.5\% \pm 3.2\%$ $p=0.023$). Finally, a trend of increase in OCR related to proton leak was noticed when the COCS where exposed to the 1 nM of TCS compared to untreated group ($p=0.065$).

a)



b)



c)

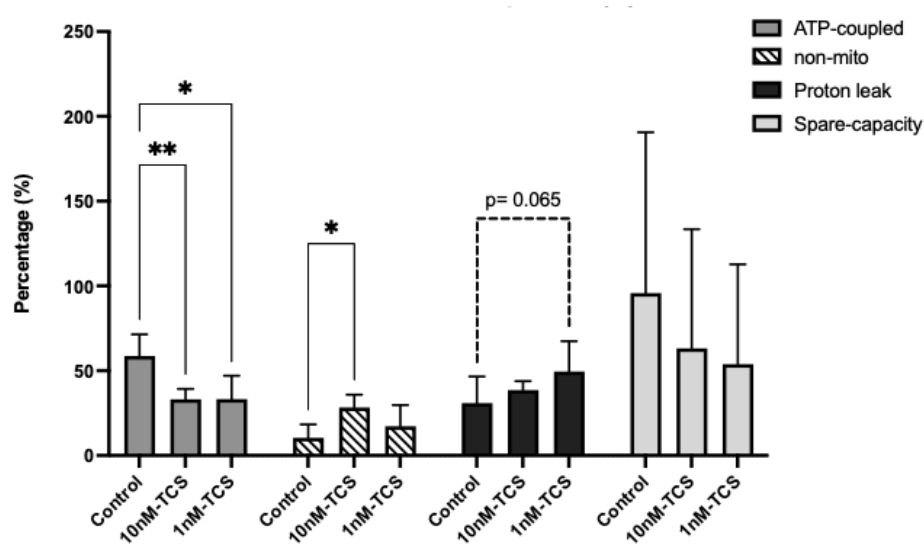
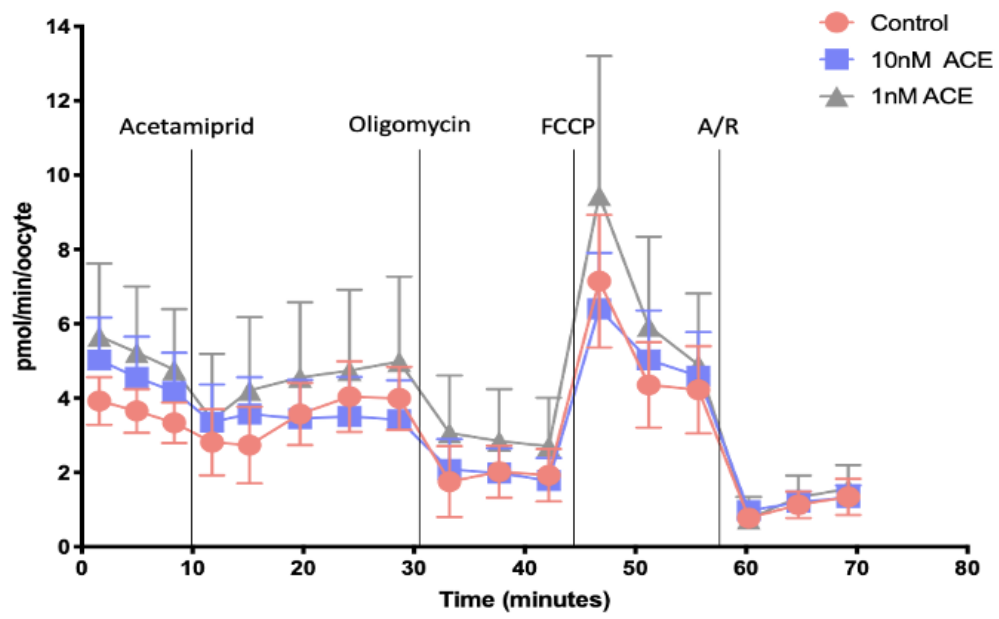


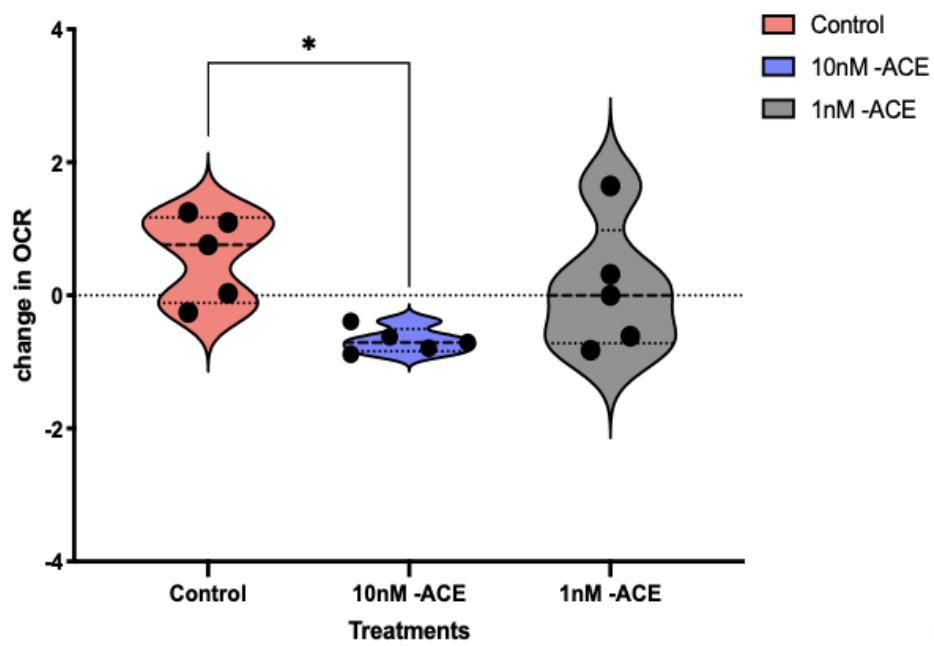
Figure 4.10: The impact on OCR immature COCs acutely exposed to TCS immediately after isolation from the ovary. a) OCR in COCs in response to Triclosan and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the TCS treatment, c) mitochondrial parameters as a percentage of basal OCR after TCS injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test. * $p < 0.05$ Data are presented in mean $\pm$ SD. Trends are indicated with dash line with the p-value presented. These graphs include data of 6 biological replicates (OCR, $n=6$).

Next, the effects of ACE on the OCR of bovine CEOs were investigated. Similarly, to the TCS basal respiration of the CEOs exposed to the high concentration of ACE (10nM) was reduced, compared to control group (Fig:4.11b). However, when the OCR components were further analysed as percentage of the basal respiration after the treatment no statistically significant difference was identified among the groups (Fig: 4.11c). Finally, the effect of control-vehicle against the untreated group was assessed and no statistically significant difference was found among the groups (fig 4.16_Appendix)

a)



b)



c)

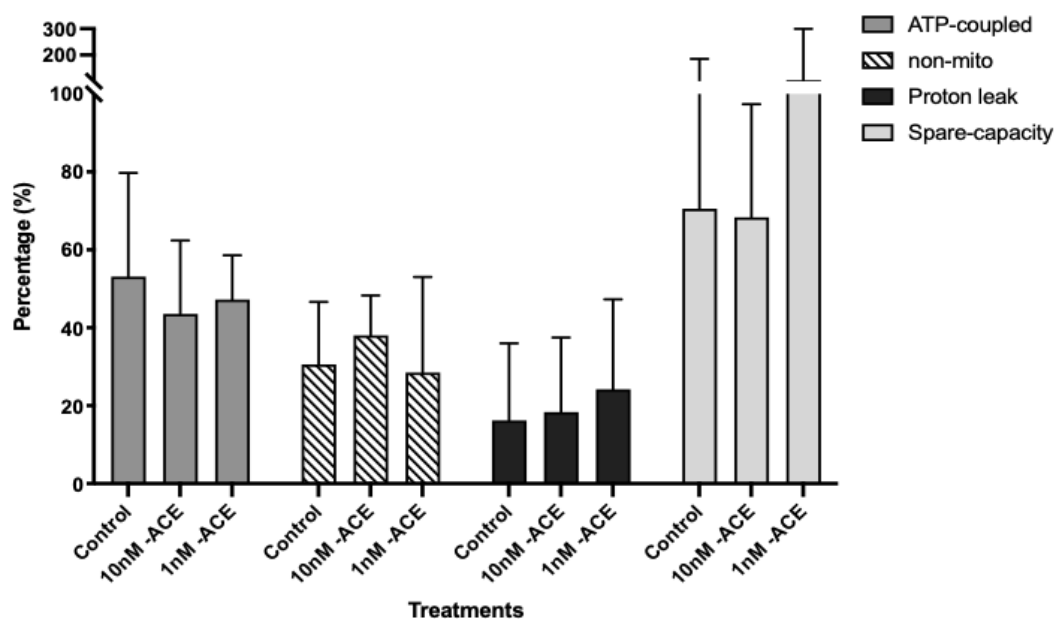


Figure 4.11: The impact on OCR immature CEOs acutely exposed to ACE immediately after isolation from the ovary. a) OCR in COCs in response to Acetamiprid and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the ACE treatment, c) mitochondrial parameters as a percentage of basal OCR after ACE injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test. * $p < 0.05$ Data are presented in mean $\pm$ SD. Trends are indicated with dash line with the p-value presented. These graphs include data of 5 biological replicates (OCR, $n=5$).

4.4.5 The zygotes pronuclear status after exposure to environmental contaminants of interest

Finally, the potential effects of the environmental contaminants of interest (BPA, BPS, TCS, and ACE) on the fertilisation process investigated by assessing the zygotes pronuclear status. In total, 750 zygotes were assessed (see table 4.3). The percentage of zygotes with normal number of pronuclei (2PN group) were significantly reduced when they were exposed to 10 nM of BPA ($4.6\% \pm 3.2\%$ vs $43.15\% \pm 12.5\%$, $p = 0.044$) compared to control and a strong trend of increase was noticed on the rate of unfertilised oocytes after exposure to the same concentration of BPA ($p=0.058$, fig: 4.12a). Meanwhile, a strong trend of decrease on normal zygotes (2PN group) and concurrently a statistically significant increase of zygotes with abnormal number of pronucleus ($>2PN$) was also observed after BPS exposure irrespective of

the administrated concentration (fig: 4.12b). Furthermore, TCS exposure resulted in a trend of increase on the zygotes with more than 2 pronuclei was found after exposure to 1 nM and 10 nM TCS compared to the untreated group ($p= 0.067$ and $p=0.06$ respectively, fig: 4.12c). Finally, a significant rise on the zygotes rate with abnormal number of pronucleus after exposure to 1 nM of ACE was identified compared to control group ($23.3\% \pm 6.8\%$ vs $9.2\% \pm 9\%$, $p=0.005$). DMSO did not affect the pro-nucleus formation (fig 4.17_Appendix)

Table 4.3: Number of zygotes tested for pro-nucleus formation. Each group represent the number of 3 biological replicates

<i>Zygotes total number</i>	<i>Zygotes per treatment group</i>									
	Control	DMSO	BPA (10nM)	BPA (1nM)	BPS (10nM)	BPS (1nM)	TCS (10nM)	TCS (1nM)	ACE (10nM)	ACE (1nM)
750	101	72	69	73	72	67	73	68	85	70

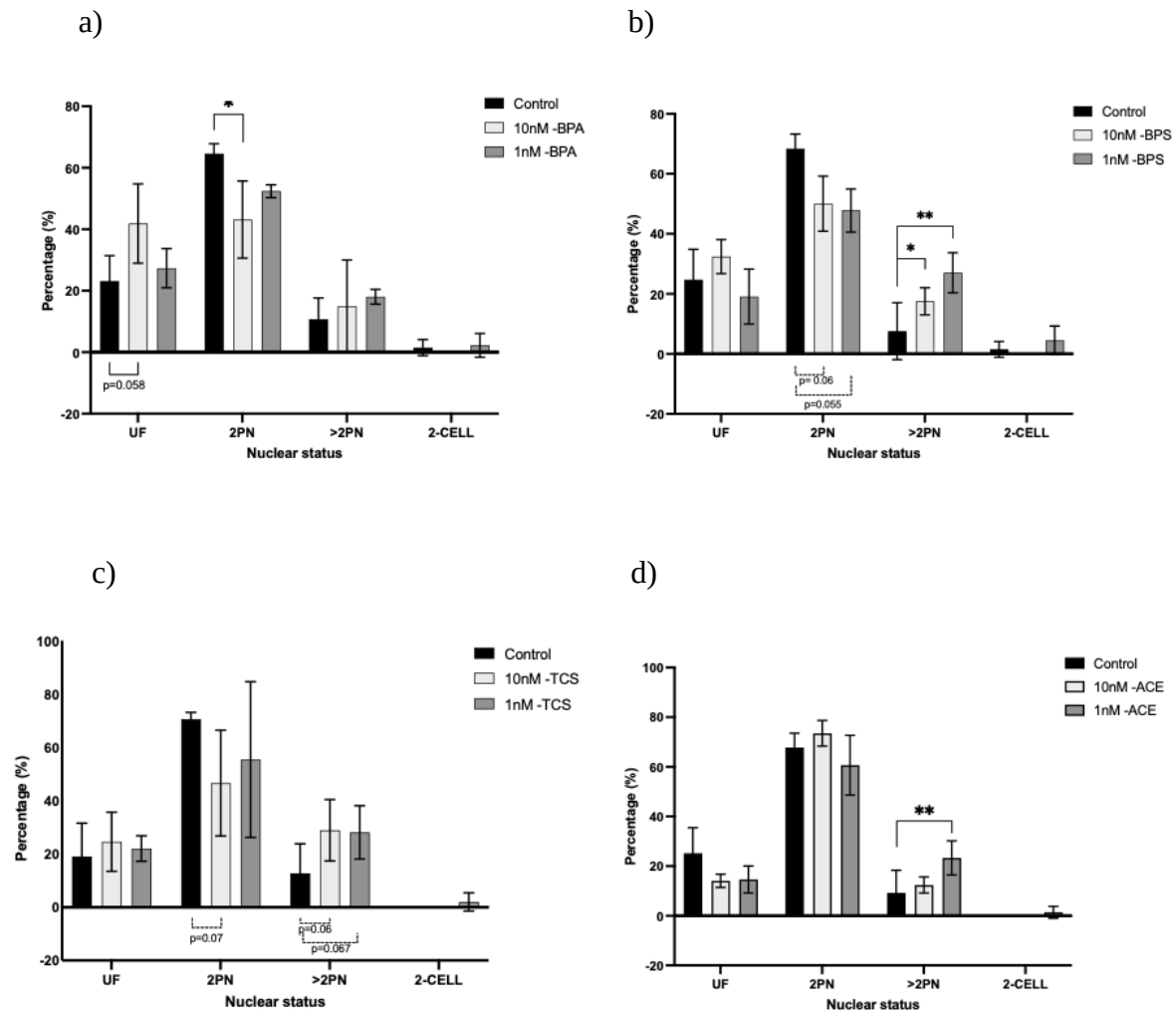


Figure 4.12: Pro-nucleus formation of zygotes exposed to a) BPA b) BPS, c) TCS and d) ACE during in-vitro fertilisation. **Uf group**, represent the unfertilised oocytes, **2PN group** represent zygotes with 2 pronucleus, **>2PN group** represent the zygotes with more than 2 PN, and finally, **2-cell group** represent the zygotes that have complete the first cell division. As the data are presented in percentages, initially they underwent arcsine transformation and subsequently, Two-way ANOVA followed by Tuckey test was performed. The results are presented in mean and $\pm$ SD. Three biological replicates were used for the investigation of each environmental compounds (n=3).

4.5 Discussion

In this chapter the impact on environmental contaminants of interest (BPA, BPS, TCS, ACE) on Cumulus Oocyte Complexes (COCs) function was investigated. Using bovine as a model, oocytes maturation, steroid production and metabolic activity was assessed. The administrated concentrations of 1 nM and 10 nM are representative of levels previously identified in human bodily fluids (Adolfsson-Erici et al., 2002; Allmyr et al., 2006, 2008; Arbuckle *et al.*, 2015; Azzouz *et al.*, 2016; Calafat et al., 2008; Fu et al., 2024; Han et al., 2018; Jin et al., 2020; Lee et al., 2018; Liao et al., 2012; Liu et al., 2017; Tuzimski et al., 2019; Zhang & Lu, 2022) which, in the majority of the cases, occurs in the nanomolar range.

The results revealed that exposure to environmental contaminants altered steroidogenesis by affecting the 17 β -oestradiol and progesterone production. In addition, oocyte metabolism was affected as indicated by modified pyruvate metabolism and mitochondrial function.

However, not all the environmental contaminants had the same effects on bovine oocyte function. It seems that 17b-estadiol production was significantly influenced and the progesterone production remained unchanged in the presence of bisphenols (BPA, BPS, fig 4.2) whilst the opposite happened in the case of TCS where 1nM dose significantly increased the progesterone levels compared to untreated group (fig 4.3). COCs' 17b-estradiol and progesterone production during IVM remained unchanged in the presence of ACE.

Steroid hormone production after chemical exposure to environmental contaminants of interest has been previously reported. BPA is the most well-studied chemical among the contaminants that were studied during this thesis. BPA is a reproductive toxicant and its effects on reproductive system has been previously suggested in animal models and human (Machtinger & Orvieto, 2014; Peretz et al., 2014). Regarding steroidogenesis, it has been reported that bisphenols alter oestradiol and progesterone secretions in ovine (Téteau et al., 2020), swine, human (Mansur et al., 2016) granulosa cells. BPS impact on granulosa and theca cells steroidogenesis was studied on bovine as well (Campen et al., 2018) Many differences on the results regarding the oestradiol and progesterone secretion were identified among the studies however these differences could result due to different concentrations, time of exposure, and species that have been used. Another reason of these differences could be a possible different mechanism of action used by these contaminants. In a study published recently (Téteau et al.,

2023) using ovine as a model it was suggested that both BPA and BPS impair steroidogenesis in granulosa cells however, they act via different molecular pathways. However, in almost all cases BPA and BPS concentrations used in these studies was in micromolar scale which is higher than what was used during our study. Only in ovine (Téteau et al., 2020) and bovine (Campen et al., 2018) model a wide range from 1 nM to 200 μ M and 1 fM to 100 μ M were used respectively. In both cases 1 nM and 10 nM of BPA and/or BPS did not alter the progesterone secretion which align with our results. However, in contrast with our results, the same concentrations in these studies did not cause any alteration in oestradiol production.

The results of the present study revealed that bovine COCs treated with 1 nM TCS released significantly more progesterone than controls. Similar observations have been reported previously for human (Du et al., 2021) and rat (Chen et al., 2019) granulosa cells in a range of different TCS concentration (1nM -10 μ M)). However, when rats were supplemented with TCS in food at 50 mg/kg/day for 28 consecutive days, progesterone levels in serum fell significantly (Arismendi et al., 2022). Decreases in progesterone levels in response to TCS have been reported in mice *in vivo* exposed to 10 and 100 mg/kg/day (Cao et al., 2018). In the same studies the E2 levels in serum were quantified. TCS (1 nM -10 μ M) has been reported to significantly increase production of E2 in human and rat granulosa cells (Chen et al., 2019; Du et al., 2021); contrasting our observations which indicate that 17 β -oestradiol production during *in vitro* maturation of bovine COCs remained unaffected by TCS. However, our results agree with findings from whole animal dose studies in rat (Arismendi et al., 2022) and mouse (Cao et al., 2018), using TCS levels of 50 mg/kg/day and 10, 100 mg/kg/day, respectively, where no alteration of E2 levels in serum was also demonstrated.

The observed differences in our study that evidence an influence of BPA, BPS, and TCS on P4 and E2 levels may be explained by the duration of the exposure or the animal model that have been used. Additionally, this may result from different administered concentrations, indicating a nonmonotonic action of the environmental contaminants. The nonmonotonic action of BPS and TCS can further be supported from our results, since the 1nM dose impacted the E2 and P4 production respectively, however E2 and P4 production remained unchanged when the COCs were exposed to 10 nM of BPS or TCS. Non-monotonic action of EDCs has been previously reported (Vandenberg et al., 2012), notably BPA, (Vandenberg, 2014). BPS and TCS both have a similar chemical structure to BPA, both of which exhibit estrogenic action and structural similarity to 17 β -oestradiol. Importantly, our findings align with the conclusion

of studies suggesting the endocrine disrupting properties of BPS and TCS. However, further investigation on the non-monotonic action needs to be done.

Finally, the results showed that ACE exposure during in vitro maturation did not affect the steroid hormone production irrespective of the administrated concentrations. To the best of our knowledge, there is no other in vitro study to investigate the impact of ACE on steroid production during in vitro maturation. However, it has previously been reported that ACE can affect testosterone synthesis in Leydig cells in rat (Kong et al., 2017). Adverse effects on reproductive system on male rat and mouse after exposure to ACE have been reported in other studies as well (Arıcan et al., 2020; Toghan et al., 2022). By contrast, there are few studies on the impact of ACE on female reproductive system. However, in a recently published study in which the protective effects of melatonin against the ACE exposure were investigated in female mice in vivo, it was suggested that exposure to 10 mg/kg and 20 mg/kg impacted the histology of the ovaries and the oocyte quality.

ACE belongs in a pesticide category known as neonicotinoids, which also contains the pesticide imidacloprid (IMI). IMI has confirmed negative impact on follicle development, and hormone synthesis. Oestradiol and progesterone levels were not influenced the first 24h of exposure, in agreement with our results when the COCs were exposed to ACE during in vitro maturation. However, increase in progesterone production was noticed at 72 hours of exposure (Mourikes et al., 2023). Despite the fact that IMI and ACE belong to the same pesticide category any comparison between those two studies should be done with caution as ACE and IMI are different contaminants. The ACE effects on steroid production during IVM should be further investigated in the future to be able to make conclusions.

Beyond steroidogenesis, the metabolism of COCs exposed to environmental contaminants was investigated by measuring essential energy substrates molecules such as pyruvate and lactate. No statistically significant difference on pyruvate or lactate was identified when the COCs were exposed to bisphenols (BPA, BPS) at any administrated concentration. However, a big variation among the biological replicates within the experimental groups was noticed (10nM-BPA, 10nM-BPS, 1nM-BPS). For this reason, further investigation needs to be done in the future to make conclusion. When the COCs were treated with TCS or ACE during IVM pyruvate was depleted, irrespective of the administrated concentration, whereas controls released pyruvate.

Pyruvate is an essential energy substrate during early embryo development (Nagaraj et al., 2017). Furthermore, pyruvate is required for the completion of meiotic maturation in mouse (Downs et al., 2002; Johnson et al., 2007). In a study conducted on mouse COCs using maturation media supplemented with FSH during IVM, pyruvate was produced by COCs that had reached the MII stage however pyruvate was consumed by nuclear immature COCs (Roberts, Stark, Iatropoulou, David L. Becker, et al., 2004). Pyruvate is also produced as the product of glycolysis in the cytoplasm from where it is either converted to lactate or enters the mitochondria, converted to acetyl-CoA which enters the TCA cycle. Based on our observations of altered pyruvate handling by COCs treated with TCS or ACE, we proposed that these COCs were either entering meiotic arrest or displaying mitochondrial dysfunction.

To explore the cause for disrupted pyruvate metabolism, the nuclear status, and the mitochondrial function by determining Oxygen Consumption Rate (OCR) on COCs exposed to environmental contaminants was subsequently investigated. The results confirmed that maturation status was not influenced by any treatment. Importantly, a number of studies have proposed that maturation of COCs from a range of species may be affected by BPA (Machtinger et al., 2013), BPS (Žalmanová et al., 2017; Desmarchais et al., 2020), TCS (Park et al., 2020) and ACE (Ishikawa et al., 2015). However, in all cases the maturation process was disrupted when the oocytes were exposed to higher micromolar range of concentration – far in excess of any concentration ever measured within the body. Only one other study has assessed the impact of BPS in the nanomolar range (Desmarchais et al., 2020). Similar to our findings that work determined that 1 nM dose did not influence the levels of oocytes reaching MII stage.

Given that the observed disruptions in pyruvate metabolism were unlikely to be as a result of maturation, mitochondrial function by using Oxygen Consumption Rate (OCR) as marker was measured in COCs exposed to compounds of interest immediately after aspiration. Mitochondrial function was determined by using mitochondrial inhibitors oligomycin and Antimycin/Rotenone mix which inhibit Complex V, and Complex III/Complex I respectively, as well as FCCP which acts as a mitochondrial uncoupler (Muller et al., 2019). OCR rate was not significantly influenced by the presences of bisphenols (BPA, BPS). However, when the individual components of OCR were further analyzed (ATP-coupled, non-mitochondrial, proton leak and spare capacity) a strong trend of increase on the OCR generated by non-

mitochondrial activity was noticed when the COCs were exposed to 1 nM and 10 nM BPS compared to untreated group. To the best of our knowledge there is not published data regarding the effects of BPS on oocytes' oxygen consumption rate. However, impaired mitochondrial function after BPS exposure has been previously reported in in vitro study on neuronal cells (Zhang et al., 2024) and in testes of rats in vivo exposed to BPS for 10 days (Darghouthi et al., 2022). Even though there was no statistically significant difference on the OCR components, increases in the non-mitochondrial OCR was noticed when the oocytes were exposed to BPA (Fig 4.8). The increase of the non-mitochondrial OCR could arise via an increase of anaerobic glycolysis suggesting a metabolic adaptation in the presence of bisphenols. However, this single observation is not enough to lead as to this conclusion and for this reason further investigation towards this way needs to be done.

The results also revealed that the total OCR was significantly reduced in COCs exposed to 10 nM dose of TCS. Further analysis of this result showed that the ATP-coupled OCR when expressed as percentage of the total OCR was significantly reduced in TCS-treated COCs compared to this of the untreated group. Moreover, the rate of non-mitochondrial OCR was significantly increased after 10 nM TCS exposure and a strong trend of increase was observed in proton leak proportion after COCs exposure to 1 nM TCS compared to control group. These results confirm mitochondrial dysfunction after TCS exposure of bovine COCs during IVM. TCS-induced mitochondrial toxicity has previously been confirmed in different mammalian cells including porcine sperm (using exposure levels of 1-10 µg/ml; (Ajao et al., 2015)), human granulosa cells (Du et al., 2021) and porcine oocytes and embryos (Kim et al., 2020; Park et al., 2020) after direct exposure in micromolar range (from 1 to 100 µM).

Finally, treatment with ACE also caused a significant reduction in the OCR of the bovine oocytes. However, when the OCR was expressed as percentage of its components there was not statistically significant difference among the groups. For this reason, no firm conclusion regarding the effects of ACE on oocyte mitochondrial function can be made at this point and further investigation needs to be done in the future.

So far using bovine oocytes, we have demonstrated that exposure to environmental contaminants of our interest in concentrations related to those that have been identified leads to altered steroidogenesis and disrupted metabolic and mitochondrial function.

Next, we investigated the potential effects of these chemicals on the fertilisation process by assessing the zygotes nuclear status. The results revealed that the high dose of BPA reduced the normal zygotes (2PN group) and at the same time a strong trend of increase was noticed on the oocytes remained unfertilised. Moreover, there was a statistically significant increase of zygotes with abnormal number of pronucleus ($>2PN$) in the BPS and TCS-treated groups. Finally, ACE prompted a significant increase in zygotes with abnormal number of pronucleus after exposure to 1 nM concentration. To the best of our knowledge the pronuclei formation after exposure to environmental contaminants of interest during IVF has not been investigated before. However, it is well known that the formation, the time of appearance, the position and breakdown of the pronuclei have been considered as markers associated with embryo viability and developmental competence (Li et al., 2013; Coticchio et al., 2018, 2022; Ezoe et al., 2022). The fact that an abnormal number of pronucleus ($>2PN$) was observed after the exposure to the chemicals suggest that the fertilisation process was impaired which could subsequently lead to disrupted preimplantation development. The cause of the abnormal number of pronucleus could be either to disrupted mechanism which block the polyspermy or due to polyploid. However, further investigation needs to be performed in the future in order to identify the cause of this observation.

In this chapter we have demonstrated that oocyte quality was negatively impacted by the presence of environmental contaminants of interest as essential processes such as steroidogenesis, metabolic and mitochondrial function as well as pronuclei formation were disrupted. Oocyte quality and the environment in which development occurs are major determinants of ongoing embryo viability and developmental competence. The subsequent preimplantation stages of development are of critical importance since impairment during this period of development can have detrimental effects on pregnancy outcomes and, more alarmingly, on lifelong health of the offspring, captured cogently by the Developmental Origins of Health and Disease hypothesis (Barker, 2007). According to this hypothesis chemical exposure of gametes and preimplantation embryos could alter the developmental fate of an organism. For this reason, the effects of the panel of environmental contaminants on embryo viability and developmental competence will be demonstrated in the following chapter.

Chapter 5:

The impact of environmental contaminants on early stages of embryo development

5.1 Introduction

5.1.1 Embryo metabolism

During the early stages of development, the embryo undergoes a range of biochemical and physiological alterations such as cell division, embryonic genome activation, compaction, formation of the blastocoel cavity, and first cell differentiation. All these modifications require energy which is acquired by metabolising energy substrates through specific metabolic pathways.

One of the most important molecules for energy production during the whole preimplantation period is pyruvate. Pyruvate is preferably consumed over glucose during the cleavage stages from zygote until compaction (Butcher et al., 1998; Guerif, McKeegan, Henry J. Leese, et al., 2013b) and oxidised through the TCA pathway and finally produce ATP into the mitochondria (Alberts et al., 2002). Apart from the role as an energy source it seems that pyruvate has a regulatory role in embryonic genome activation. In an experiment conducted in mouse and human embryos, when pyruvate was absent during a specific developmental period the embryos were not able to enter the cellular division necessary prior to genome activation (Nagaraj et al., 2017).

Cleavage stages of embryo development are characterised as a period with low metabolic activity. It has been suggested that this occurs in order to decrease the probability of DNA damage stimulated by ROS activity (Leese, 2002). Following the cleavage stage, there is a trend of increase in glucose, pyruvate and lactate consumption at morula stage which reaches the peak during the blastocyst formation. At this stage, a shift in energy substrate preference has been noticed (glycolytic shift), with glucose becoming the main source for energy production. As suggested by Martin and Leese it is possible that embryos undergo this metabolic shift in order to survive hypoxia during implantation by utilise glucose to convert to lactate (Martin & Leese, 1995). During this stage, the oxygen consumption also increases, leaving the oxidative metabolism as the predominant metabolic pathway for ATP production. In a study conducted in porcine embryos it seems that 97% of the ATP production is result of oxidative phosphorylation and only 3% result from the glycolysis pathway (Sturmey & Leese, 2003). Furthermore, other molecules such as amino acids and fatty acids are used as energy metabolites through the embryo development. Interestingly, this broad pattern of embryo

energy metabolism appears broadly conserved across mammalian species (Lewis & Sturmey, 2015; Malkowska et al., 2022).

Embryo metabolic activity has been correlated with its developmental competence (Gardner et al., 2001; Houghton et al., 2002; Brison et al., 2004; Sturmey et al., 2008b; Hemmings et al., 2013; Mádr et al., 2015). For example, Brison et al showed more than 20 years ago that clinical pregnancy in human embryos was related to amino acid metabolism (Brison et al., 2004). Since then, multiple studies have shown correlative patterns between embryo viability and the depletion or release of a number of substrates from the media (Gardner et al., 2011; Gardner & Wale, 2013; Guerif et al., 2013; Motiei et al., 2020).

Leese and colleagues (Leese et al., 2016) proposed that the metabolism of a healthy embryo falls into what they described as the “Goldilocks zone”. This notion proposes that embryos with higher developmental probabilities are those which their metabolic activity in terms of energy substrate and ATP consumption/production fluctuate within a “lagom” range (not too little not too much). This concept has been supported by a study conducted in bovine embryos showed that the embryos with maximum developmental potential were those which consumed an intermediate amount of pyruvate during the cleavage stages (Guerif et al., 2013).

Taking into consideration the Developmental Origins of Health and Disease (DOHaD) theory (Barker, 2007), any alteration on the energy substrates intake and metabolism of the embryo during early developmental stage could have a great impact on the health of the individual in long term. For instance, high levels of lipids during early stages of development and during different stages of pregnancy have been correlated with the development of diseases such as diabetes, cardiovascular diseases, later in life of the offspring (Baardman et al., 2013; Zhao et al., 2024). Apart from the insufficient nutrient's intake, other factors such as chemical exposure, can negatively impact the embryo metabolism. In this chapter, we investigate how the panel of the environmental contaminants of our interest could affect the metabolism and the developmental competence of the bovine preimplantation embryo.

The panel of the environmental contaminants under investigation (Bisphenol A, Bisphenol S, Triclosan, and Acetamiprid) have all been detected in human bodily secretions and transferred through the bloodstream (Adolfsson-Erici et al., 2002; Allmyr et al., 2006, 2008; Calafat et al., 2008; Liao, Liu, Alomirah, et al., 2012; Liu et al., 2017; Han et al., 2018; Lee et al., 2018;

Tuzimski et al., 2019; Jin et al., 2020; Zhang & Lu, 2022; Fu et al., 2024). Moreover, Bisphenol A (Ikezuki et al., 2002; Paoli et al., 2020) and recently Bisphenol S (Žalmanová et al., 2023) have been identified in human follicular fluid. Given the chemical similarities between Bisphenol A, Bisphenol S, Triclosan and Acetamiprid, we propose that each of the contaminants could cross the barrier of reproductive organs such as ovarian and fallopian tube tissue and enter the follicular or/and the oviduct fluid. It is therefore plausible that oocytes and embryos may be directly exposed to these compounds during the early stages of development.

5.2 Hypothesis and Aims

In the previous chapter we identified effects on COCs metabolic activity after exposure to environmental contaminants, Next, we hypothesise that this effect could have a subsequent impact on embryo development and metabolism. Furthermore, since the panel of environmental contaminants have been previously identified in bodily fluids, we hypothesis that direct exposure during early embryo development is possible. To test this, the metabolic activity of embryos derived from chemically exposed COCs as well as directly chemically-exposed embryos was investigated. These will be assessed by using bovine as model by isolating cumulus-oocyte complexes (COCs) to produce embryo and expose them to the chemical of our interest in different stages of development. The objectives of this chapter are to evaluate the impact of these chemicals on embryo developmental competence and metabolic function by investigating the following:

- ⇒ The effects on developmental competence on the embryos derived from chemically exposed COCs
- ⇒ The impact on energy substrate intake and release of the blastocyst derived from chemically exposed COCs
- ⇒ The effects on embryo metabolism after exposure to the environmental contaminants during different stages of embryo development (cleavage stage and blastocyst stage).
- ⇒ The impact on oxygen consumption rate during the blastocyst stage.

5.3 Experimental design

5.3.1 Environmental contaminants stock preparation and storage

Environmental contaminants were prepared as described in Chapter 4, section 4.3.1. Briefly, 5000x stock of each contaminant were diluted in 100% DMSO and then subsequently further diluted preparing 1000x stock in embryo tested H₂O on the day of the experiment. Two different concentrations of each environmental contaminant were investigated (Table 5.1).

Table 5.1: The final concentration of environmental contaminants that COCs exposed

<u>Environmental contaminant</u>	<u>Concentration</u>	<u>Concentration</u>
<i>Bisphenol A (BPA)</i>	10nM	1nM
<i>Bisphenol S (BPS)</i>	10nM	1nM
<i>Triclosan (TCS)</i>	10nM	1nM
<i>Acetamiprid (ACE)</i>	10nM	1nM

5.3.2 Experiment 1: How does embryo development and metabolism respond when oocytes are exposed to environmental contaminants?

In this experiment, COCs during the IVM were exposed to environmental contaminants and the impact on developmental competence and metabolic function of subsequent embryos was measured. Bovine COCs were isolated as described in Chapter 2 section 2.3.3 and 3.3.4 and exposed to BPA, BPS, TCS and ACE in two different concentrations (Table 5.1) during IVM. Subsequently, the COCs were fertilised using IVF (Chapter 2 section 2.3.5) and subsequent embryos cultured in droplets under oil in groups of 20 to 25 embryos per drop. Developmental progression was assessed under the stereoscope on the second day (D2) to determine the cleavage rate and on day seven (D7) and eight (D8) to measure the blastocyst rate.

On D7 and D8, all blastocysts were transferred into 5µl drops (Chapter 2, section 2.3.7) and cultured individually for approximately 24 hours. After this, the embryos were removed and the plates containing the drops with the spent media were stored in -80°C until further analysis. The spent media was used to determine the glucose and pyruvate and lactate concentration (Chapter 2 section 2.4.2) as well as the amino acid concentration (Chapter 2, section 2.4.4).

The analysis was conducted within the period of 3 months. The exact time of the embryo entry and exit from the individual drop was recorded and used to determine each energy substrate intake or release in pmols per hour per embryo (pmol/hour/embryo). The embryos used were exposed to the environmental contaminants of interest only during IVM, for the rest of the process (IVF, IVC, individual IVC) no additional chemical exposure was performed. Data are representative of least 3 biological replicates, with the exact number is illustrated on the legend of each figure. The results are presented from figure 5.1 to figure 5.7. Finally, the effects of control vehicle (DMSO) compared to untreated group were examined and the result are included in Appendices section from Figure 5.22_Appendix and 5.23_Appendix.

5.3.3 Experiment 2: How does exposure to environmental contaminants during different stages of embryo development affect their metabolic activity?

During this experiment, the impact on metabolic function of embryos after direct exposure during different stages of embryo development was investigated. COCs were isolated and used for IVM, IVF and IVC as previously has been described in Chapter 2 section 2.3.4, 2.3.5 and 2.3.6, respectively. Initially, the fertilised eggs (zygotes) were transferred in grouped embryo cultured without the addition of any chemical in the media. Subsequently when the embryos had reached the desired developmental stage, they were transferred into individual culture (as described in chapter 2 section 2.3.7) during which they were exposed to the environmental contaminants of interest (Table 5.1). To determine the impact of the chemicals on the cleavage stage embryos, embryos which were at least 2-cell embryos on D2 or 4-cells on D3 were transferred into individual drops and cultured in the presence of the chemicals for 24 hours.

To assess the impact of environmental contaminants on blastocysts, the embryos were cultured for 7 days in groups of 20 to 25 embryos. On D7 and D8, all embryos that had reached the blastocyst stage were transferred and exposed to chemicals during individual culture for approximately 24 hours. The next day the embryos were removed.

The exact time and the developmental stage of each embryo was recorded on the entry and exit of each individual culture, and the plates were stored in -80°C until further analysis. The analysis was conducted within 3 months from the collection day. The spent media was used for the determination of glucose, pyruvate, lactate and amino acids consumption and release

(Chapter 2, sections 2.4.2 and 2.4.4). The results are illustrated in pmol/hour /embryo. Data are representative of at least 3 biological replicates, with the exact number presented in the legend of each figure. The results are presented from figure 5.8 to figure 5.18. Finally, DMSO effects on cleavage and blastocyst stage embryos' metabolites consumption/production was investigated as well and the results are presented in Figure 5.24_Appendix and Figure 5.25_Appendix, respectively.

5.3.4 Experiment 3: Do environmental contaminants affect oxygen consumption rate during blastocyst stage?

In this set of experiments, the mitochondrial function of embryos in the blastocyst stage was determined by using Oxygen Consumption Rate as a marker after acute exposure to TCS, BPA, and BPS. The protocol for the determination of the Oxygen Consumption Rate has been described in detail in Chapter 2 section 2.4.5 and is based on (Muller et al., 2019). Briefly, embryos at blastocyst stage were transferred into an assay plate and then exposed initially to the environmental contaminant under investigation, and subsequently to treated with 3 different mitochondrial inhibitors (oligomycin, FCCP, Antimycin/Rotenone mix). The oxygen consumption rate was measured during this assay. Each mitochondrial inhibitor inhibits different component of the electron transport chain reaction in the mitochondria; therefore, we are able to determine if ATP generation into the mitochondria can be affected by the presence of the chemical. The data are presented from figure 5.19 to figure 5.21. Data are representative of at least 3 biological replicates with the exact number is written on the legend of each figure. The OCR of embryos treated with the control vehicle was finally assessed and compared against the untreated group and the results are included in Figure 5.26_Appendix of the Appendices section.

5.3.5 Statistical Analysis

GraphPad Prism 9 was used for all statistical analysis. Shapiro-Wilk test was used in order to define whether a parametric or non-parametric analysis is required. When the data was normally distributed, One-Way ANOVA was used followed by Tukey's post-hoc test. However, when normal distribution was not followed, a Kruskal-Wallis test was performed followed by Dunn's post-hoc test. In the case of percentages, arcsine correction was performed initially, and the results were analysed with One-way ANOVA or with two-way ANOVA followed by Tukey's post-hoc test. The exact method of analysis is mentioned in the legend of each data figure.

5.4 Results

5.4.1 The impact of environmental contaminants during oocyte maturation on subsequent embryo developmental competence and metabolic activity

Figure 5.1 shows that exposing oocytes to any of the environmental contaminants during IVM had no impact on subsequent cleavage rate of the embryos. However, the blastocyst rate of embryos derived from chemically exposed COCs was impacted in some cases. As figure 5.2a depicts, the percentage of blastocysts that formed from COCs exposed to 10 nM BPA was significantly lower compared to the untreated group ($13.3 \pm 5.3\%$ vs $28.6 \pm 2.7\%$, $p = 0.0064$). Furthermore, there was a strong trend of decrease in the 1 nM BPA compared to control group ($p = 0.056$).

When presented at the lowest concentration (1nM), TCS exposure during IVM led to a significantly lower blastocyst rate compared to control group (Fig 5.2c, $12.22 \pm 7.7\%$ vs $29.1 \pm 10.1\%$ $p = 0.03$), however TCS 10 nM had no effect on the blastocyst rate. Similar to TCS, 1 nM of ACE (fig 5.2d) during IVM significantly decreased the blastocyst rate whereas, 10 nM dose had no effect. BPS-exposure during IVM did not affect blastocyst rate at any administrated dose (fig 5.2b).

Finally, cleavage or blastocyst rate of embryos derived from COCs treated with DMSO was not significantly different compared to control group (fig 5.22_Appendix).

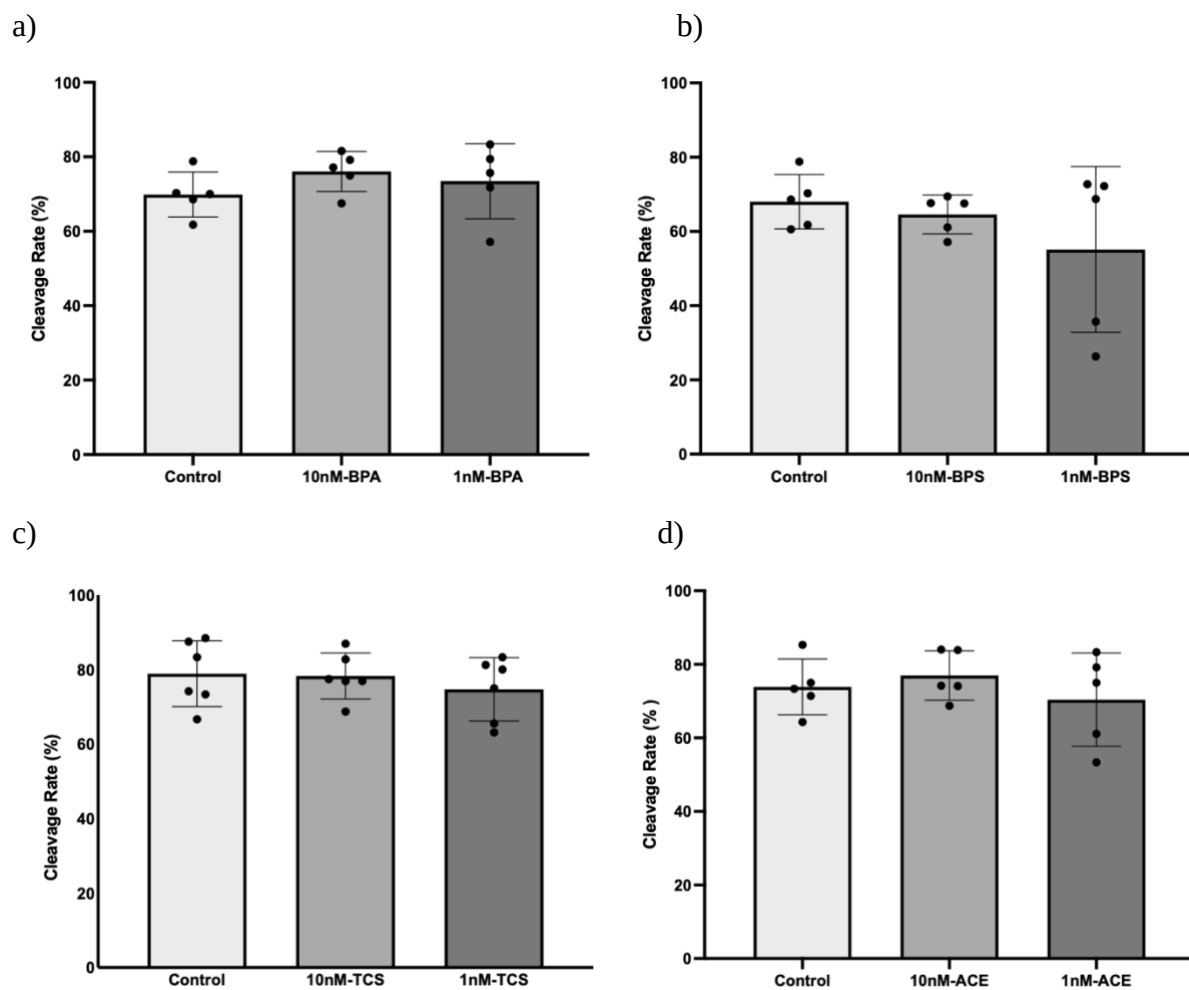


Figure 5.1: Cleavage rate of embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation. Each graph represent data of at least 5 biological replicates with each dot presenting an individual biological replicate (n=5 for BPA and n=5 for BPS, n=6 for TCS and n=5 for ACE). The data represents results of, 30 to 40 fertilised COCs per experimental group per biological replicate. Data are expressed as Mean $\pm$ SD. Differences were determined using One-Way ANOVA with Tukey's post-hoc test.

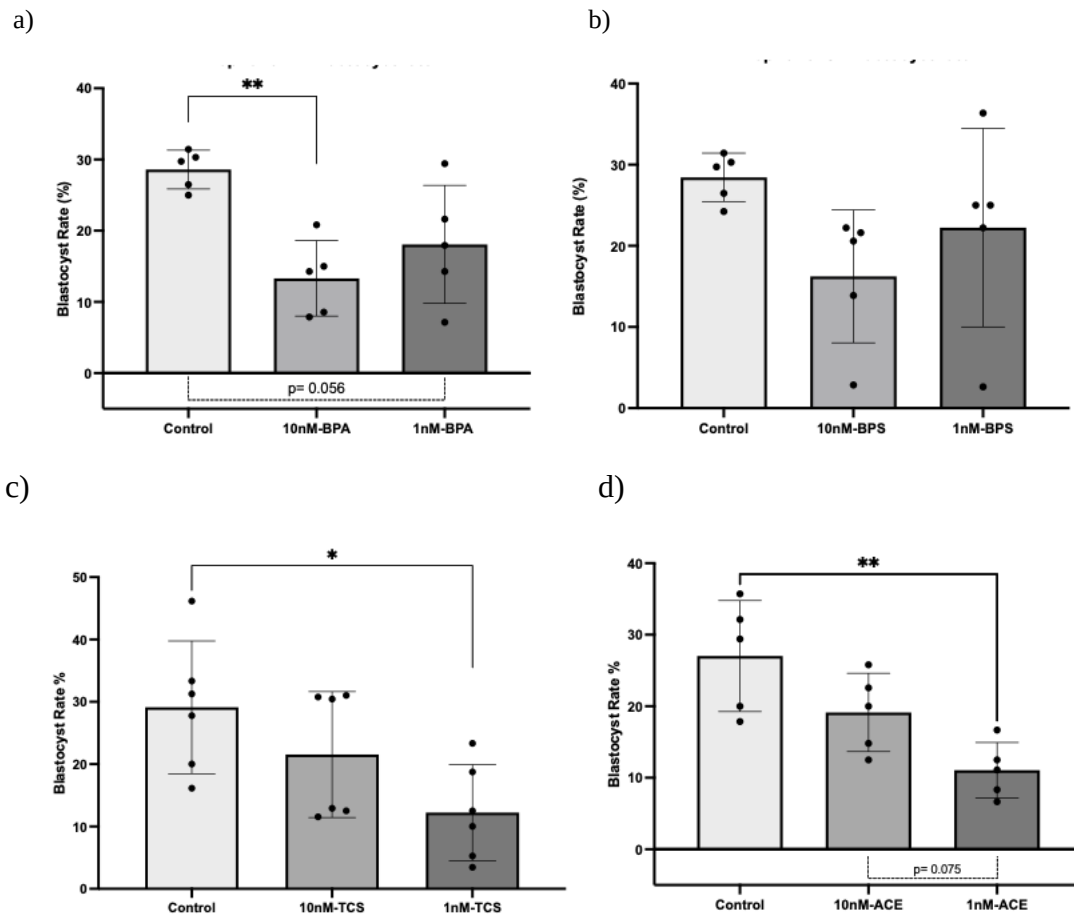


Figure 5.2: Blastocyst rate of embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during *in vitro* maturation. Each graph represent data of at least 5 biological replicates with each dot presenting an individual biological replicate (n=5 for BPA and n=5 for BPS, n=6 for TCS and n=5 for ACE). Data are expressed as Mean $\pm$ SD. Differences were determined using One-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

Next, metabolic function of embryos was assessed. Initially, glucose and pyruvate consumption, and lactate production were determined at the blastocyst stage. Glucose consumption was not impacted by any of the chemicals (fig 5.3). However, the blastocysts derived from 1 nM BPA-exposed COCs consumed significantly higher more pyruvate compared to control group (31.1 ± 14.8 pmol/hour/embryo vs 20.6 ± 17.8 pmol/hour/embryo, $p= 0.048$). Interestingly, exposing oocytes to the higher dose (10nM) did not have any effect on pyruvate consumption by resulting blastocysts. Similarly, to BPA, the low dose of ACE significantly increased the pyruvate consumption on blastocyst stage (fig:5.4d) 21.7 ± 12.01 pmol/hour/embryo vs 12.8 ± 8.85 pmol/hour/embryo, $p= 0.014$). Finally, blastocysts arising from oocytes treated with TCS had a significantly higher pyruvate consumption compared to control group irrespective of the administrated dose (fig:5.4c).

Oocyte exposure to BPS had no legacy effect on pyruvate consumption by blastocysts. Moreover, lactate production (fig:5.5) was not affected by BPA, BPS and ACE, however, blastocysts derived from oocytes treated with 10 nM of TCS released less lactate into the medium compared to control group and also compared to low dose (1 nM) of TCS. Finally, it seems that DMSO did not had any great impact on embryos' glucose, pyruvate consumption and lactate production (fig 5.23_Appendix).

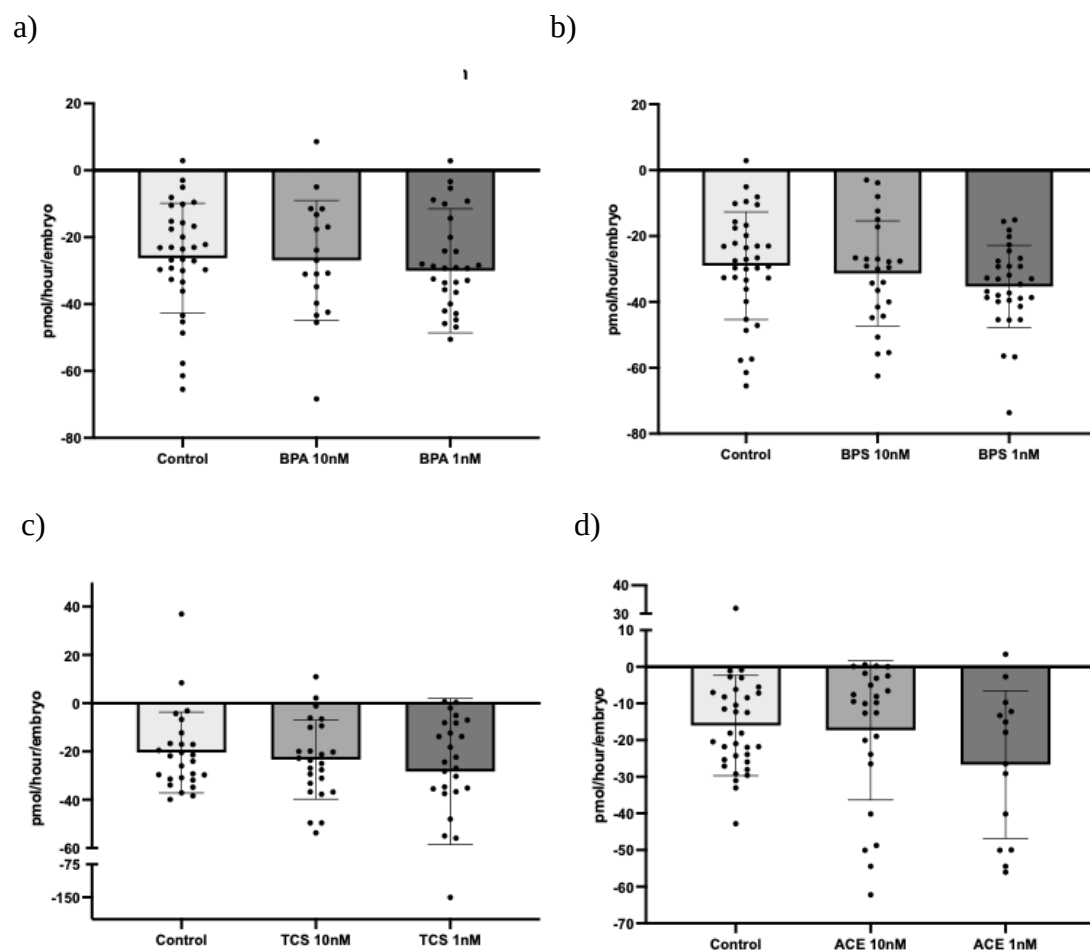


Figure 5.3: Glucose consumption of blastocyst stage embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during *in vitro* maturation. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set and differences were determined using Kruskal-Wallis followed by Dunn's post-hoc test.

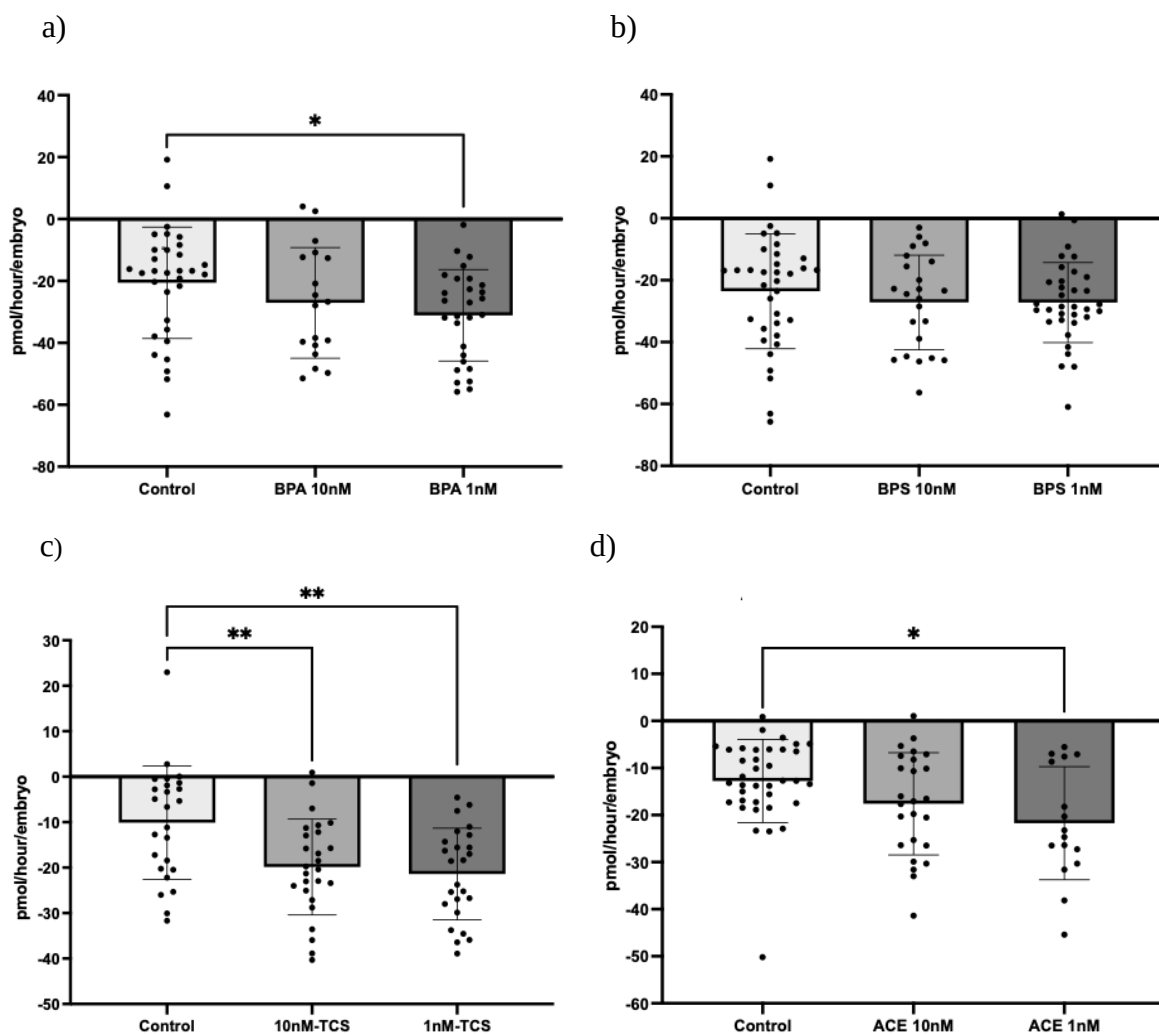


Figure 5.4: Pyruvate consumption of blastocyst stage embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

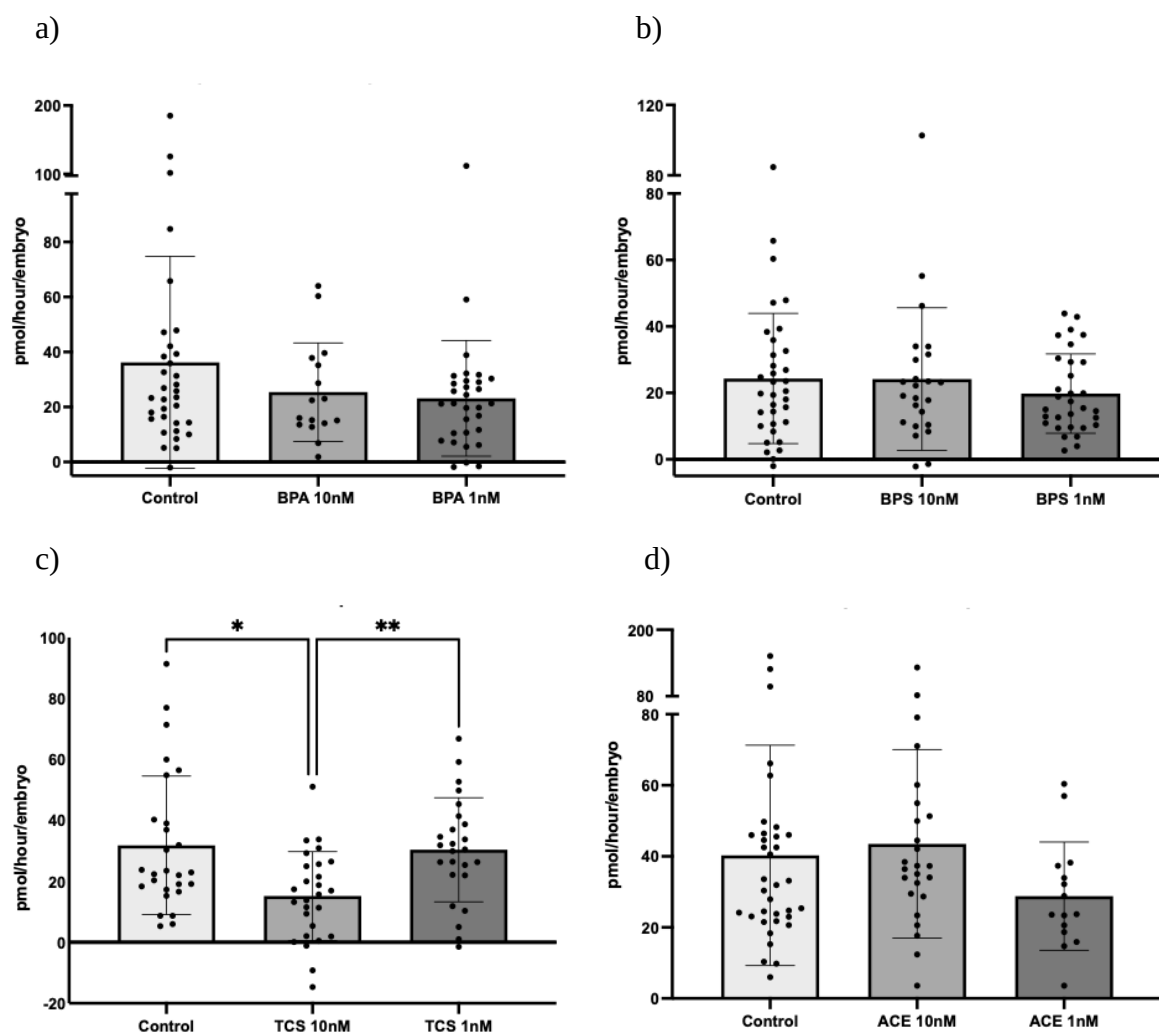


Figure 5.5: Lactate production of blastocyst stage embryos derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

The data presented in figures 5.3 to 5.5 included all blastocysts collected from group culture. However, during the 24h culture period, some blastocysts arrested. Figures 5.3 to 5.5 includes all data from these embryos. To further scrutinise the findings, the data were reinterpreted by dividing blastocysts into two groups, those which were remained intact and those which arrested during the 24hours of individual culture. These data are presented in figure 5.6.

Figure 5.6a shows that blastocysts from oocytes treated with BPA and that remained viable for the 24h individual culture had significantly higher pyruvate depletion than control equivalents. By contrast, embryos derived from oocytes exposed to TCS but subsequently arrested during the culture depleted significantly more pyruvate prior to their arrest (fig: 5.6b); these same embryos demonstrated reduced lactate release (dead, fig: 5.6c).

Interestingly, despite the fact that initially the ACE-exposure did not seem to affect the glucose consumption (Fig 5.3d) during the blastocyst stage, further analysis focussing on embryos that survived for the entire culture period revealed that glucose consumption was elevated in those embryos derived from oocytes exposed to 10 nM ACE compared to control and to 1 nM ACE group. Finally, the significant difference on pyruvate consumption on ACE group compared to control group, (illustrated in fig: 5.4d) seems that have been caused by the blastocysts that survived the whole period of the assay (alive, fig: 5.6f).

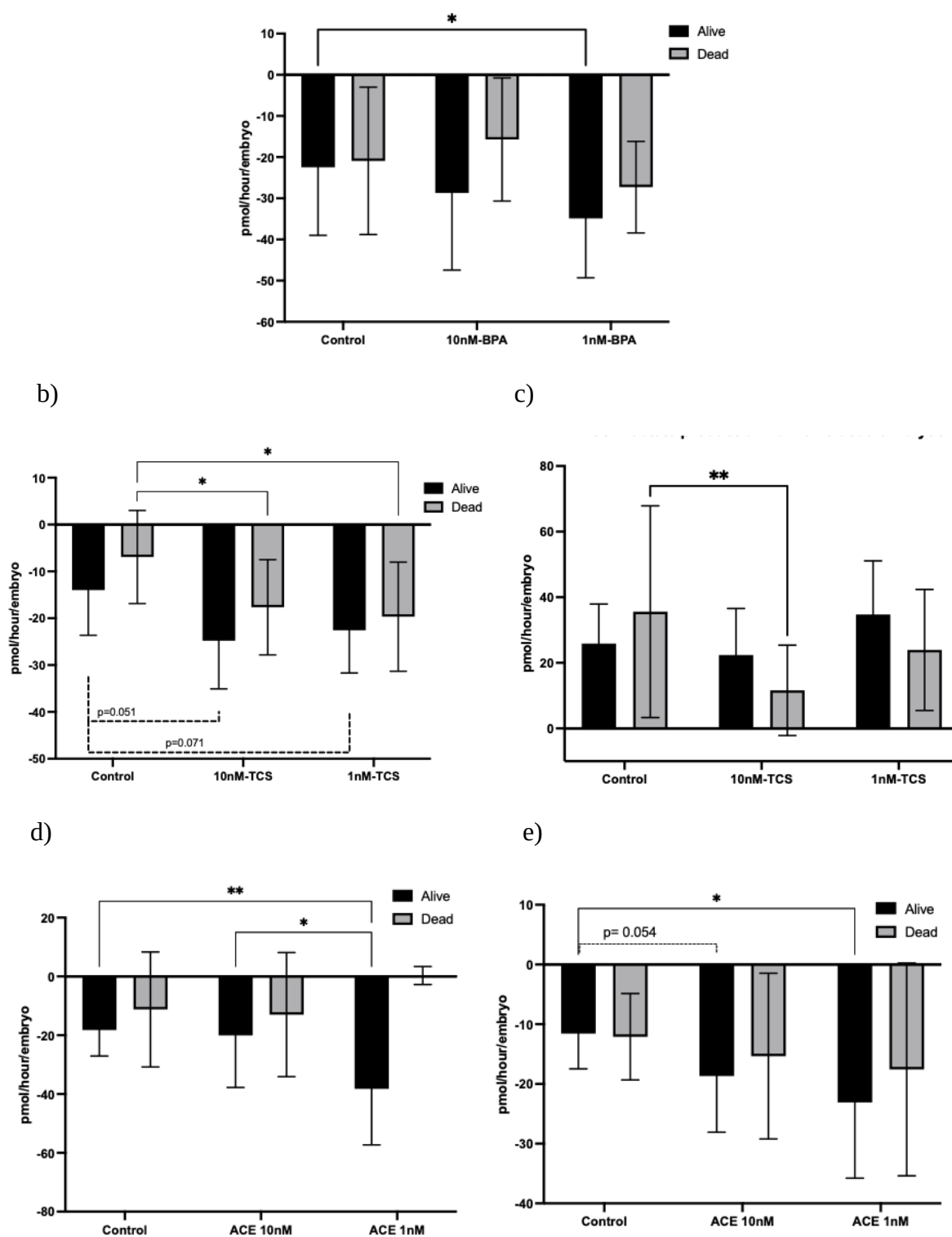


Figure 5.6: Comparison of glucose, pyruvate consumption and lactate production between alive and dead on blastocysts, derived from chemically exposed COCs to a) BPA, b) & c) TCS and d) & e) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; ** $p < 0.01$; * $p, 0.001$. Trends are illustrated by a dashed line, with p-value presented.**

Finally, the turnover of 18 amino acids was determined on blastocysts which derived from chemically exposed COCs during IVM (fig: 5.7). BPA-treatment influenced the turnover of three amino acids: glutamine (Gln), arginine (Arg) and tryptophan (Trp). The glutamine production was significantly increased by blastocysts derived from BPA-exposed COCs irrespective of the administrated dose. Interestingly, while untreated blastocysts consume arginine, blastocysts of the BPA groups released arginine during the individual culture (Fig: 5.7a). Tryptophan consumption was also significantly increased on 1 nM BPA compared to control group (Fig: 5.7a). This significant increase on tryptophan consumption was observed in blastocyst derived from BPS-exposed COCs irrespective of the administrated dose. BPS also had an impact on metabolic status of lysine as the blastocysts derived from the 1 nM BPS group consumed lysine whilst lysine was produced by control, control-vehicle and 10 nM BPS groups. TCS did not have a notable impact on amino acids turnover, and finally, 1 nM ACE had a significant impact on metabolic status of tyrosine compared to control group (Fig: 5.7d).

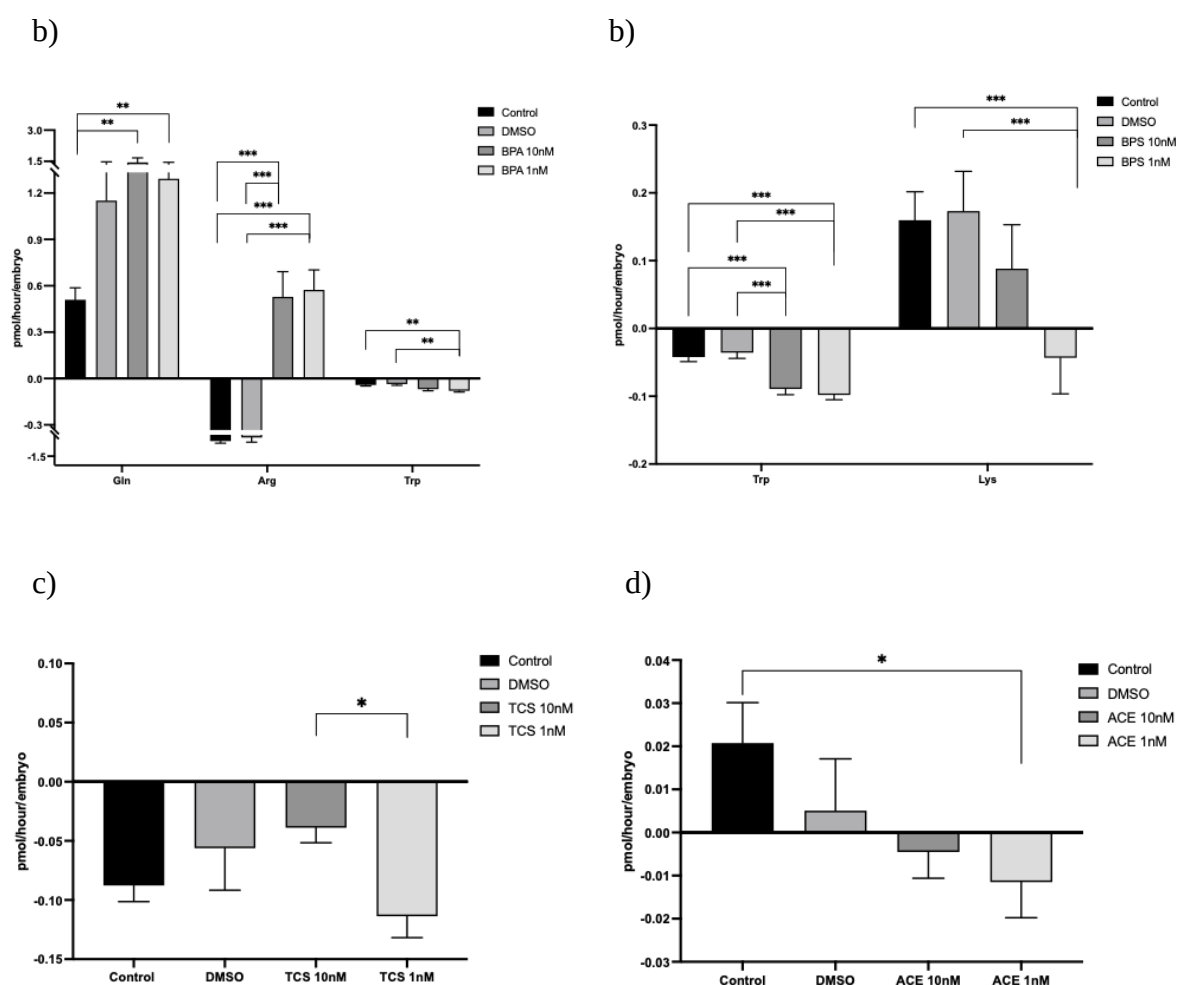


Figure 5.7: The turnover of amino acids with statistically significant difference on blastocysts derived from chemically exposed COCs to a) BPA and b) BPS, c) TCS and d) ACE. The COCs exposed at 10 nM or 1 nM of each chemical during in vitro maturation. Each graph represent data of at least 3 biological replicates. Data are expressed as Mean $\pm$ SEM. Shapiro-Walk test was conducted for each data set. Differences were determined using Multiple Mann-Whitney test using Holm-Sidak post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

5.4.2 The impact on embryo metabolism after direct exposure to environmental contaminants on different stages of embryo development.

In the second set of experiments the impact on metabolic status of embryos exposed directly to environmental contaminants of interest during the preimplantation phase was investigated. Glucose, pyruvate, lactate and amino acid turnover was quantified after direct exposure on different embryo developmental stages.

The amount of glucose consumed by embryos exposed during cleavage stage of development (day 2 and day 3) is presented in figure 5.18. Treatment with BPA and TCS prompted an increase in glucose consumption irrespective of the administrated dose compared to control group (fig 5.8a and fig 5.8c respectively). Glucose consumption was also significantly increased when the embryos were exposed to 1nM BPS (fig 5.8b). However, the 10 nM BPS did not cause a significant change on glucose consumption suggesting a non-monotonic effect of BPS.

Pyruvate consumption by cleavage embryos exposed to BPA, BPS and ACE was elevated (fig 5.9). Treatment with 10 nM BPA significantly increased pyruvate consumption compared to control group (22.03 ± 12 pmol/hour/embryo vs 12.2 ± 9.5 pmol/hour/embryo $p < 0.0001$), however this was also significantly different than that of embryos exposed to 1 nM BPA (fig 5.9a). Pyruvate consumption was also significantly increased when the cleavage embryos were exposed to BPS, compared to control group, irrespective of the administrated dose. However, the fact that the 1 nM BPS was significantly higher compared to 10nM BPS shows the non-monotonic effect of BPS (fig 5.9b). Finally, the low dose of ACE (1nM) significantly increased the pyruvate consumption of the cleavage embryos compared to control group (18.6 ± 6.4 pmol/hour/embryo vs 12.2 ± 11.2 pmol/hour/embryo $p = 0.0016$, fig 5.9d) Lactate production was not significantly influenced by the presence of any of the environmental chemicals (fig 5.10).

The data presented on figures 5.8 to 5.10 included embryos on day 2 and day 3 of development. For this reason, next the embryos were divided into two groups, those that were exposed on day 2 and those that were exposed on day 3 of development to identify if there is any different on the timing of exposure. These data are presented in figure 5.11 to 5.13.

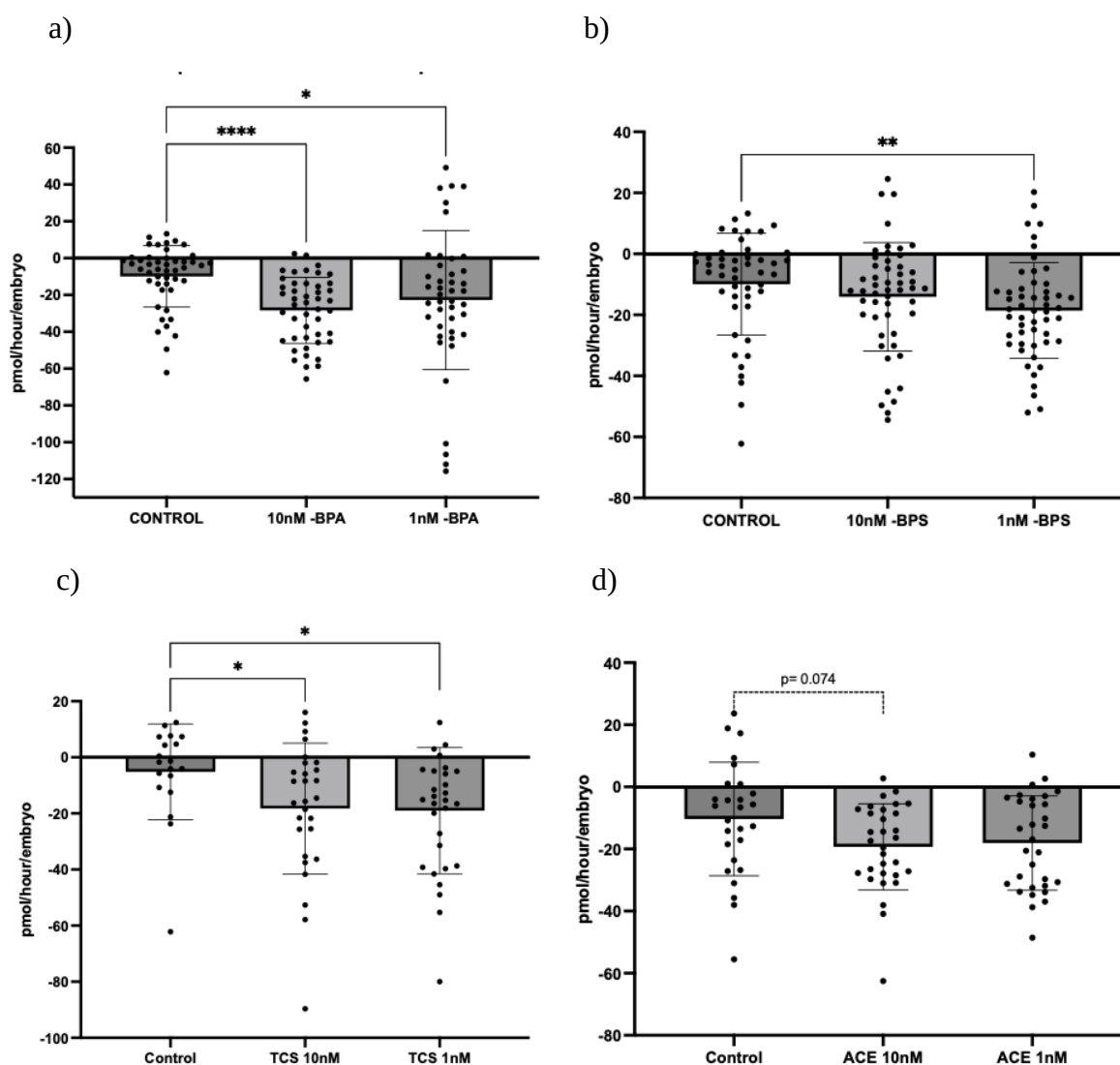


Figure 5.8: Glucose consumption of cleavage embryos (day 2 and day 3) exposed to a) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

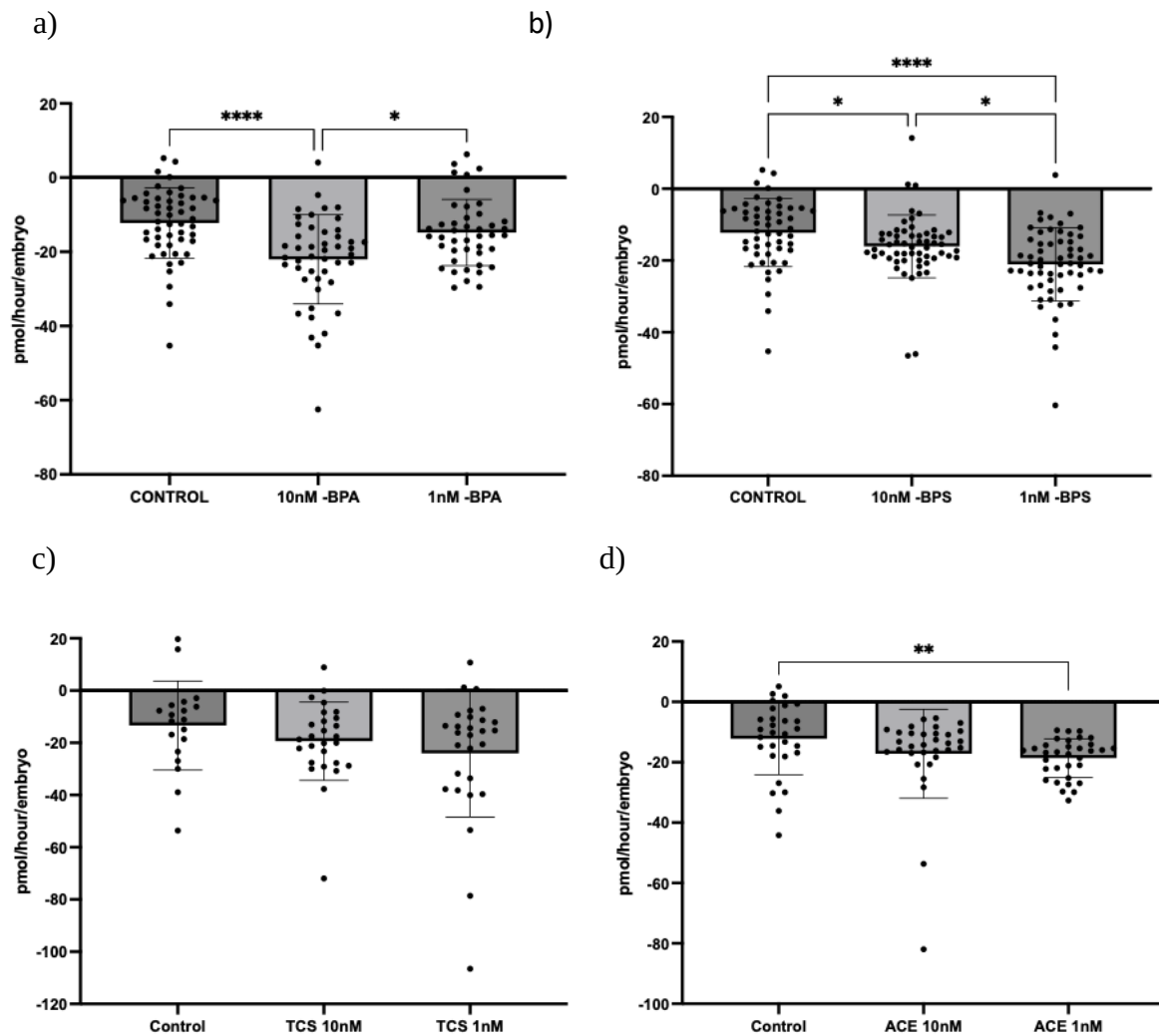


Figure 5.9: Pyruvate consumption of cleavage embryos (day 2 and day 3) exposed to a) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture. Each graph represents data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Wilk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

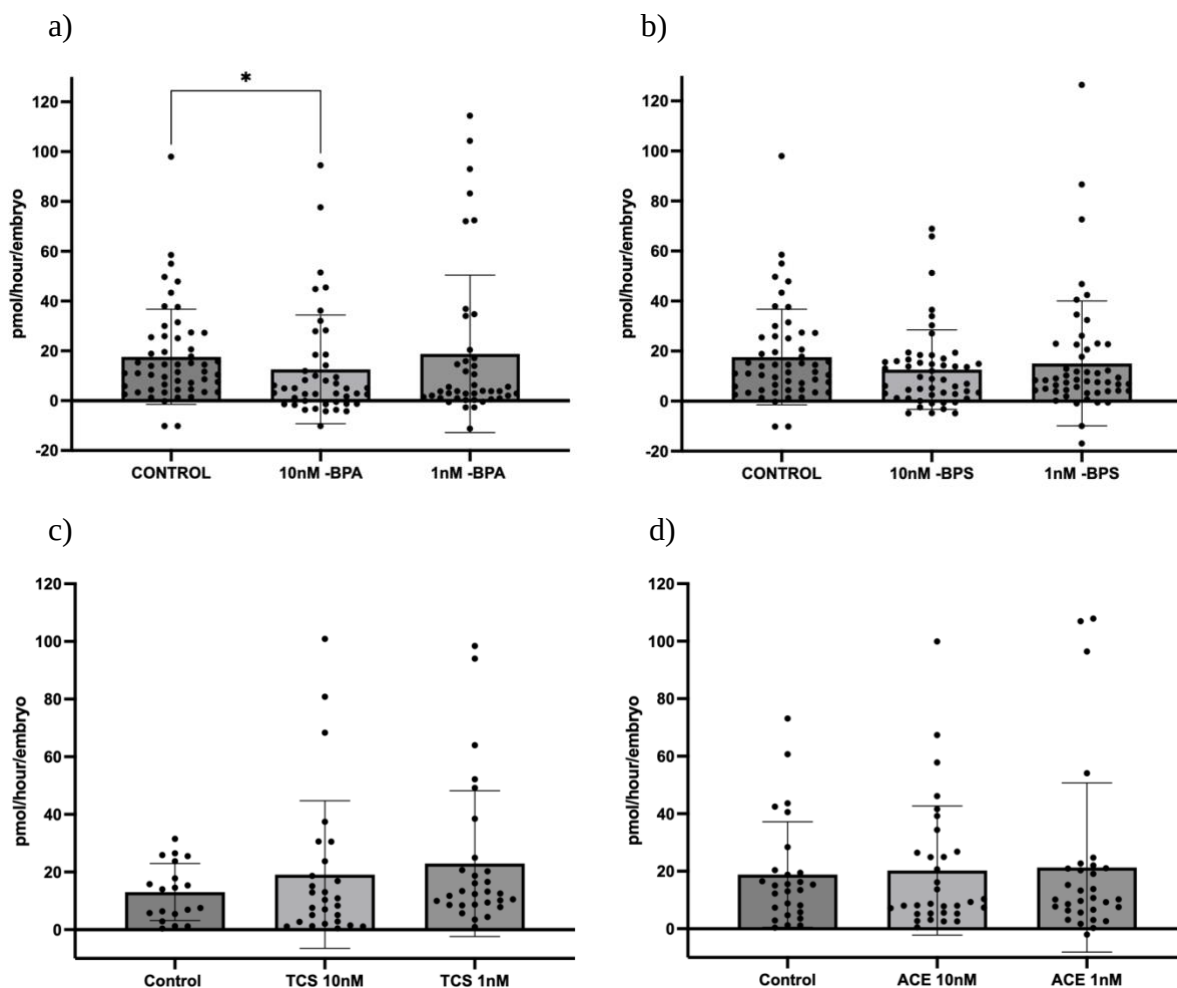


Figure 5.10: Lactate production of cleavage stage embryos (day 2 and day 3) exposed to α) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual *in vitro* culture. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

Glucose consumption of day 2 embryos was significantly increased after BPA exposure irrespective of the administrated dose compared to control group. This pattern remained on day 3, notably by embryos exposed to high concentration of BPA (fig 5.11a).

Treatment with 1 nM BPS it seemed that cleavage stage embryos (d2 and d3) significantly increased glucose consumption (figure 5.8b) however when the embryos divided by day of development (d2, d3) this difference disappeared (fig 5.11b). TCS-exposure had an impact on

glucose consumption of day 2 and day 3 embryos; the 10 nM TCS significantly influenced the glucose consumption of day 2 embryos and meanwhile TCS increased the glucose consumption of day 3 embryos irrespective of the TCS concentration the embryos were exposed (fig 5.11c).

Pyruvate consumption was significantly increased when the embryos were exposed to 10 nM of BPA on day 2 and day 3 of development (fig:5.12a). Meanwhile, both concentrations of BPS drove increased pyruvate consumption compared to control group on day 2 embryos. This significant increase of pyruvate depletion remained in day 3 embryos exposed to 1 nM BPS group (fig 5.12b). Finally, the statistically significant increase of pyruvate consumption of cleavage embryos exposed to 1 nM ACE (fig: 5.9d) is result of the exposure to 1 nM ACE on day 3 of development (fig: 5.12d)

The quantification of lactate production after exposure to environmental contaminants of interest on day 2 and day 3 embryos did not reveal any statistically significant difference among the experimental groups.

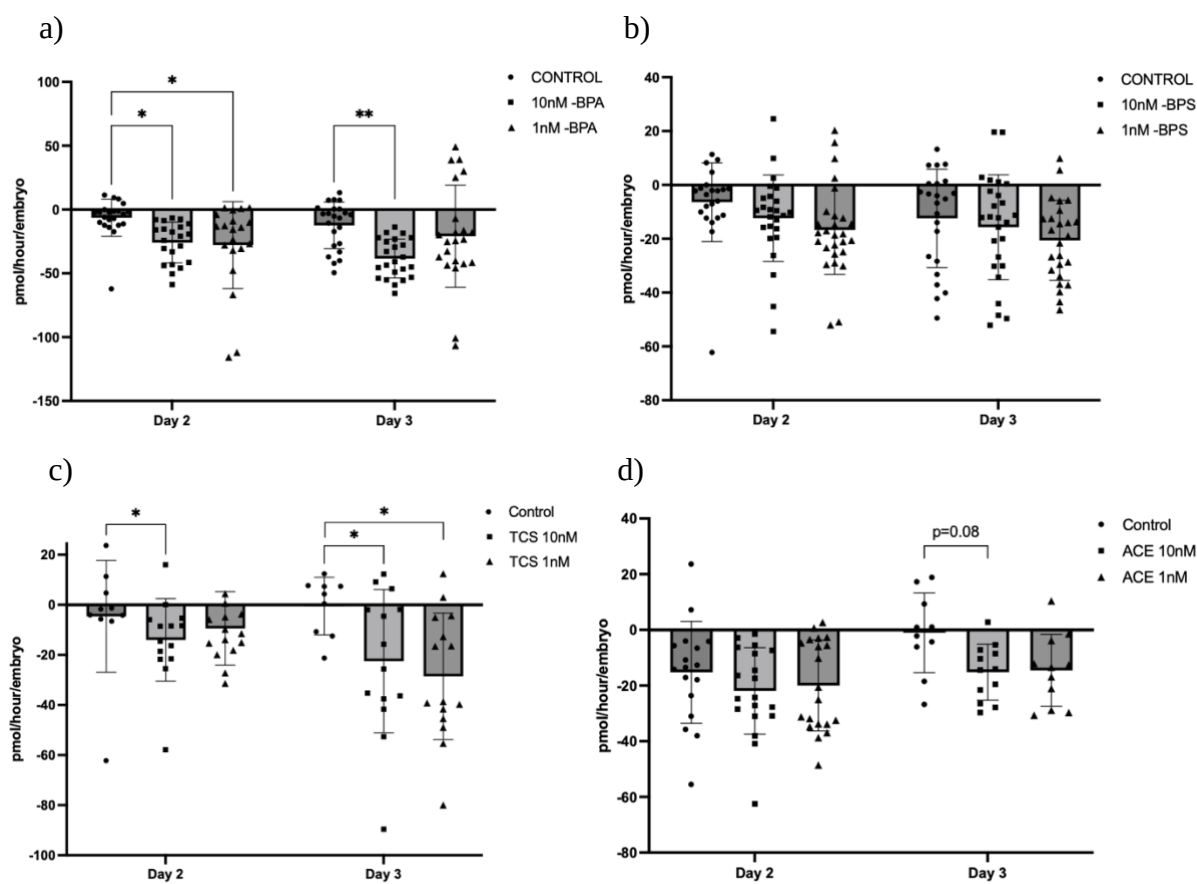


Figure 5.11: Glucose consumption of Day 2 and Day 3 embryos exposed to a) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

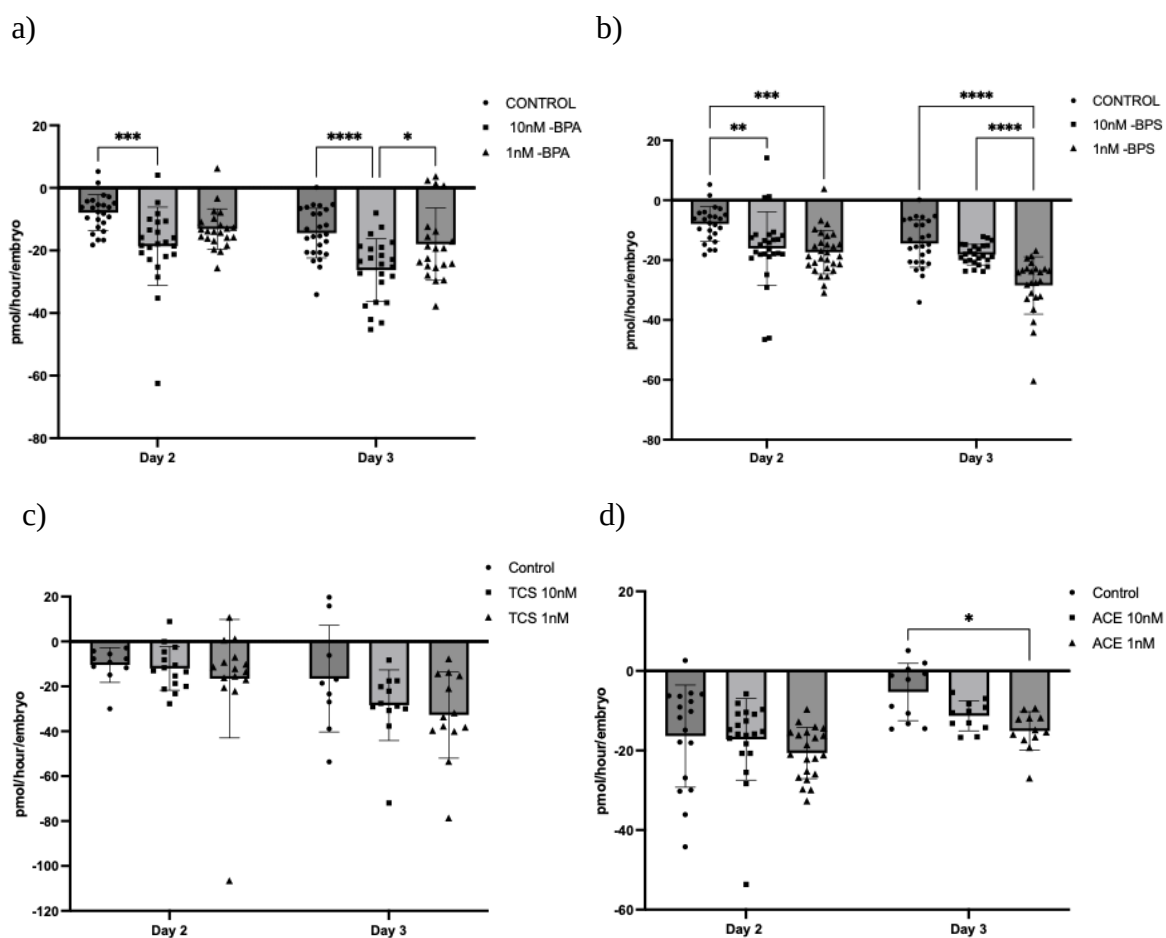


Figure 5.12: Pyruvate consumption of Day 2 and Day 3 embryos exposed to a) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

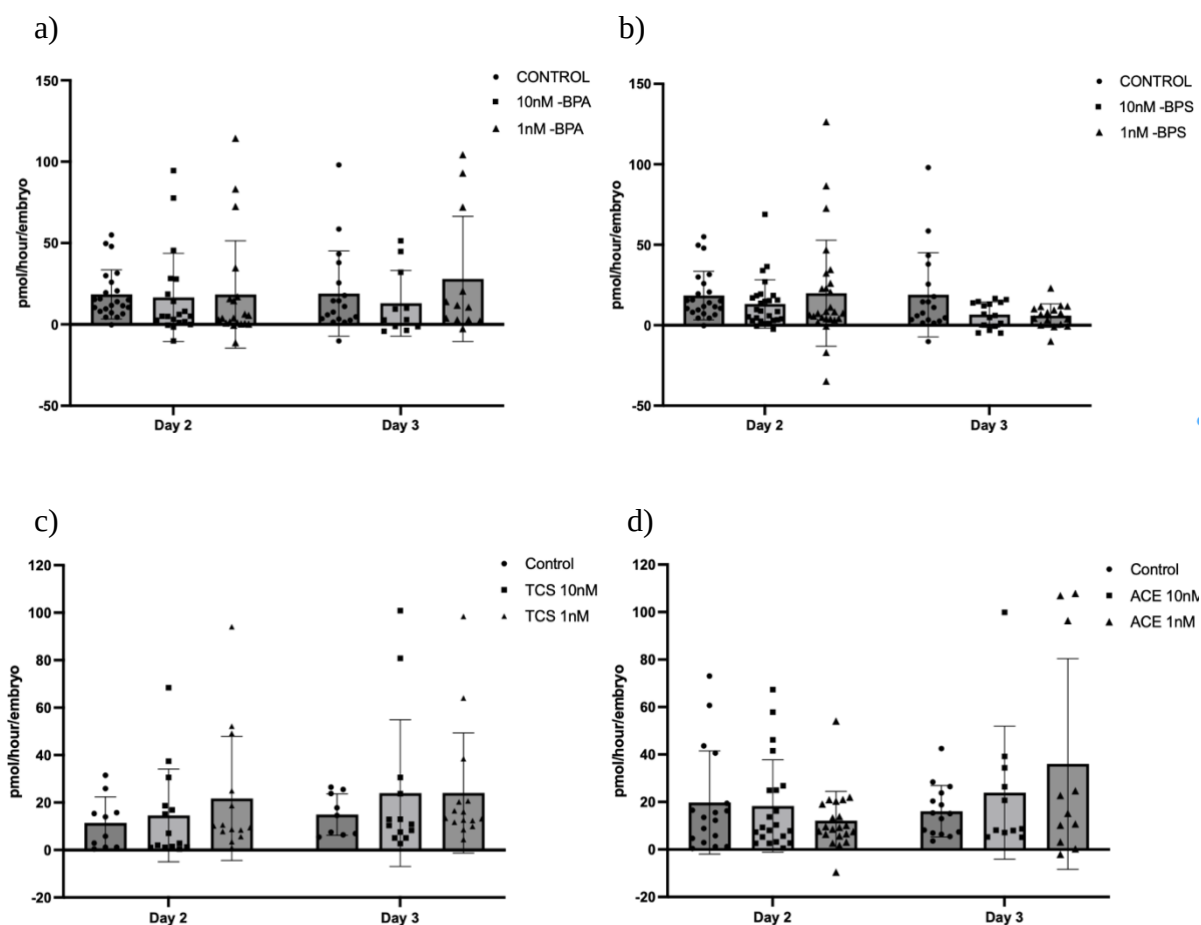
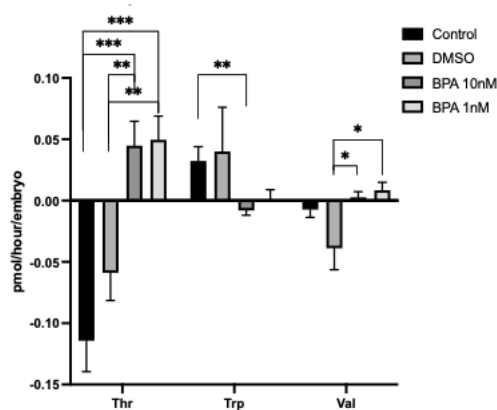


Figure 5.13: Lactate production of Day 2 and Day 3 embryos exposed to a) BPA and b) BPS, c) TCS and d) ACE. The embryos were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal- Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

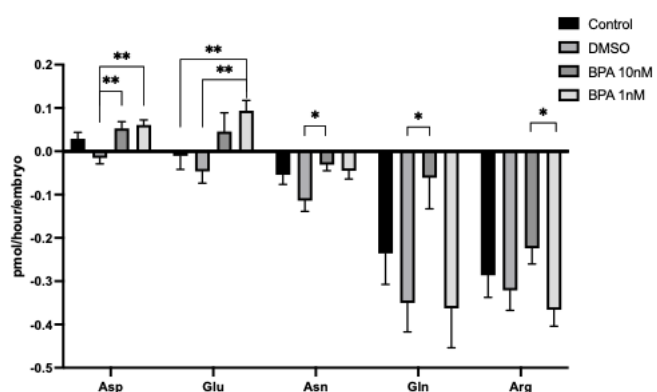
Next, the turnover of 18 amino acid (Asp, Glu, Asn, Ser, His, Gln, Gly, Thr, Arg, Ala, Tyr, Trp, Met, Val, Phe, Ile, Leu, Lys) was determined after the chemical exposure of embryos during the cleavage stage of development. The analysis revealed that there were significant changes in many amino acids in every case of chemical exposure (figure 5.14). When cleavage stage embryos were exposed to BPA statistical differences were identified in 8 amino acids (Thr, Trp, Val, Asp, Glu, Asn, Gln, Arg) which are presented in figure 5.14a & b. Interestingly, Thr, Trp, Val, Glu reverse their metabolic status after embryos were exposed to BPA irrespective of the administrated dose. Those 4 amino acids were consumed in the control

groups, whilst they were produced when the embryos exposed to BPA and vice-versa. (Fig5.14a & b). A similar pattern was observed for Thr and Trp when the cleavage embryos were exposed to BPS. Furthermore, a statistically significant increase of Gln and Asn consumption was observed when the embryos exposed to BPS compared to control group (fig: 514c). Regarding the amino acid turn over after TCS exposure, statistically significant differences were identified in 9 out of 18 amino acids (His, Leu, Trp, Thr, Arg, Glu, Gln, Gly, Ser). Among those, His, Leu, Thr, Gln, Gly and Ser metabolic status altered after exposure to TCS, irrespective of the administrated dose, compared to control group (fig: 5.14c & d). Finally, ACE exposure on cleavage stage embryos caused differences to 10 out of 18 amino acids (Iso, Phe, Leu, Met, Thr, Arg, Asp, Asn, Glu, Gly). Ile, and Met metabolic status shifted, compared to control group, after the exposure to ACE irrespective of the administrated dose. Changes in metabolic status have been shown also in the case of Leu and Asn when the embryos were exposed to high dose of ACE (10nM). Gly production was significantly decreased when the embryos were exposed to ACE (10 nM and 1 nM) compared to control group. By contrast, only the 10 nM ACE increased the ASN and Glu production compared to control group (fig: 5.14f & g).

a)



b)



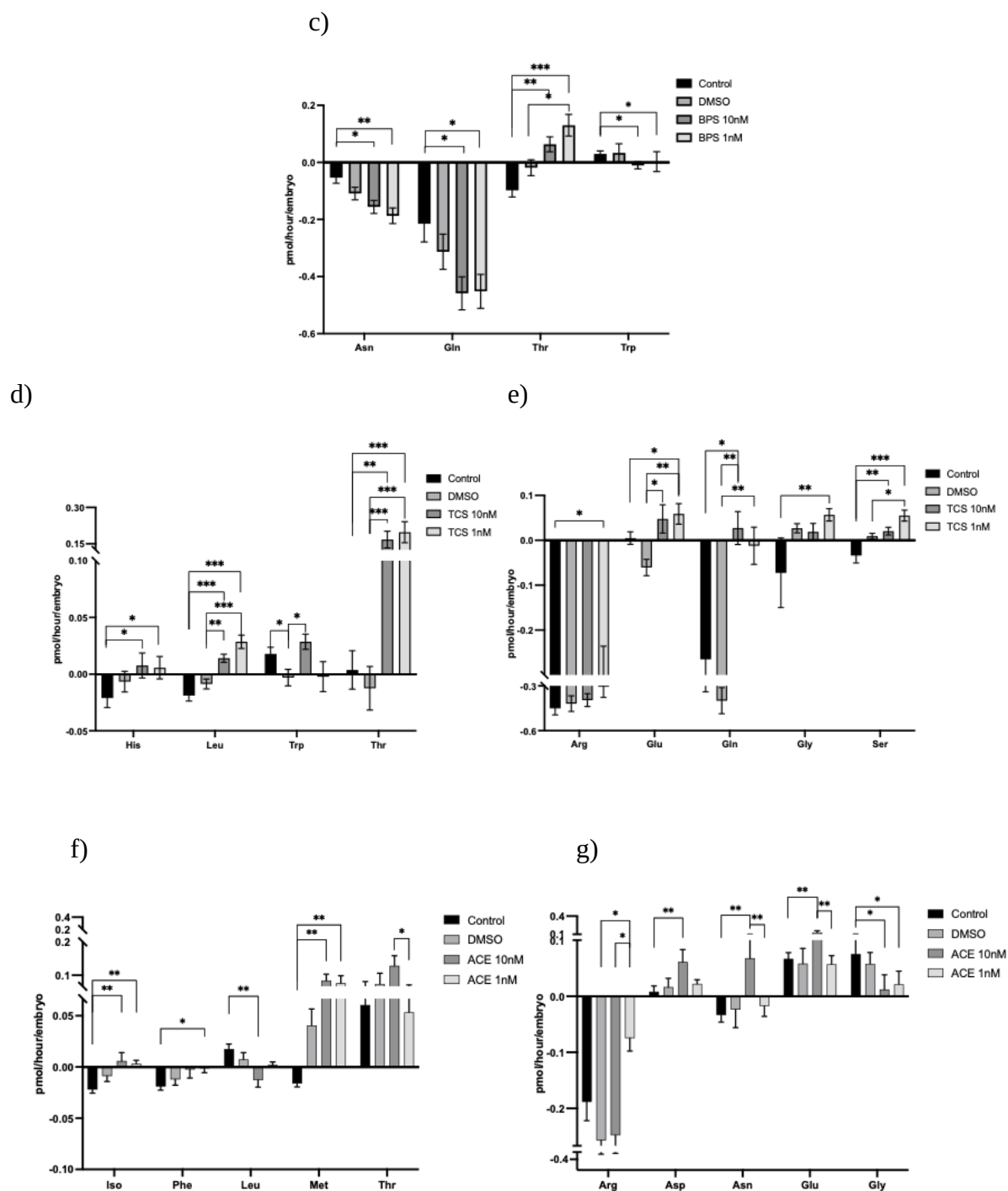


Figure 5.14: The effects of a) & b) BPA and c) BPS, d) & e) TCS and f) & g) ACE on amino acids turnover during cleavage stage of embryos development. Embryos were exposed at 10 nM or 1 nM of each chemical during in vitro culture. Each graph represent data of at least 3 biological replicates. Data are expressed as Mean $\pm$ SEM. Shapiro-Walk test was conducted for each data set. Differences were determined using Multiple Mann-Whitney test using Holm-Sidak post-hoc test *, $p<0.05$; **, $p<0.01$; *, $p<0.001$. Trends are illustrated by a dashed line, with p-value presented.**

Finally, the metabolic status of the embryos exposed to the environmental contaminants of interest during the blastocyst stage was investigated. Untreated controls consumed significantly less glucose than blastocysts exposed to 1 nM of BPS, with a strong trend observed in blastocysts exposed to high dose of TCS (10 nM, $p=0.065$). BPA and ACE did not significantly impact the glucose consumption of the blastocysts (Fig 5.15). However, ACE altered pyruvate consumption irrespective of the concentration that the blastocysts were exposed to (fig: 5.16d). Moreover, BPS treatment induced significantly elevated glucose depletion at the blastocyst stage (fig: 5.16b); a pattern also seen in response to TCS (fig: 5.16c) exposure. BPA did not impact the pyruvate depletion significantly. Finally, lactate production of the blastocyst remained unchanged after the environmental contaminant's exposure (fig: 5.17).

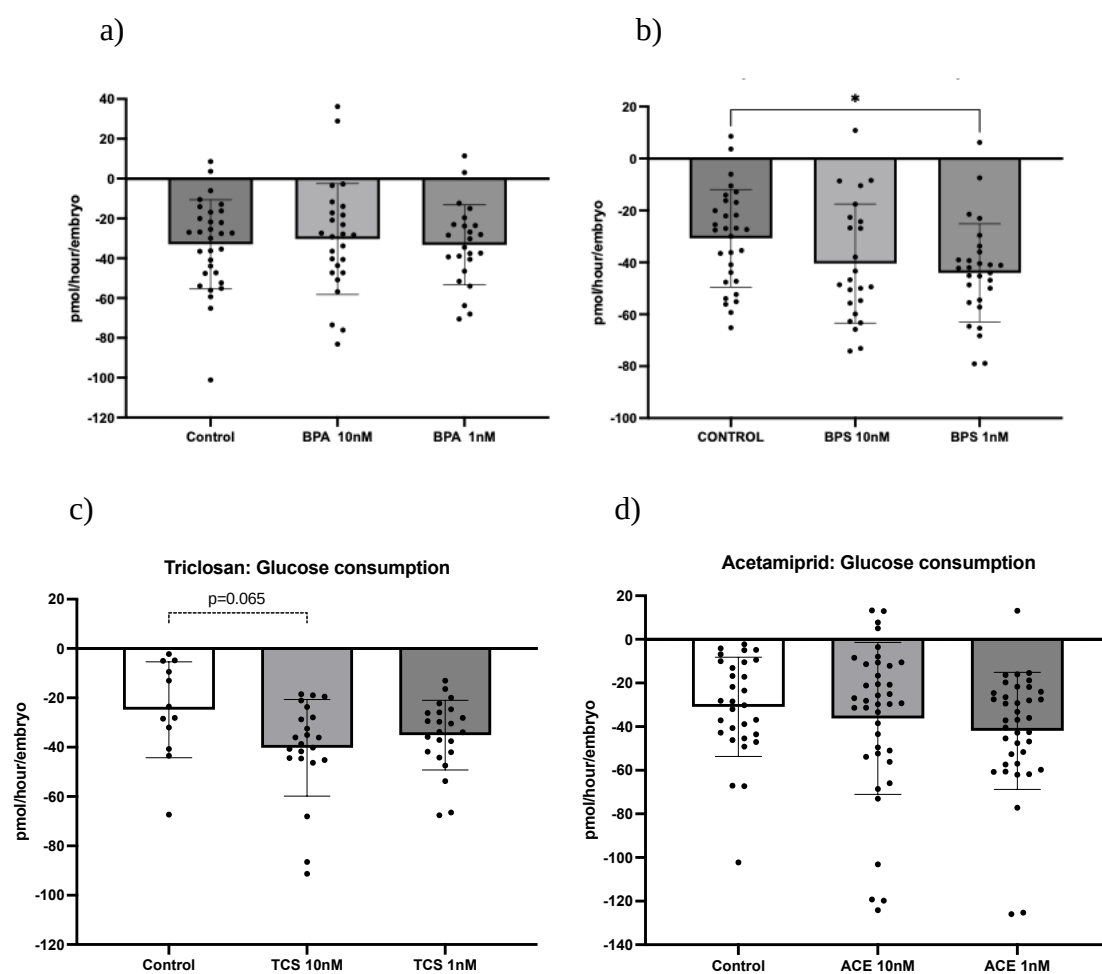


Figure 5.15: The effects of a) BPA and b) BPS, c) TCS and d) ACE on glucose consumption of during blastocyst stage exposure. 10 nM or 1 nM of each chemical was used during individual in vitro culture for 24 hours exposure during blastocyst stage. Each graph represents data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal-Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; * $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.**

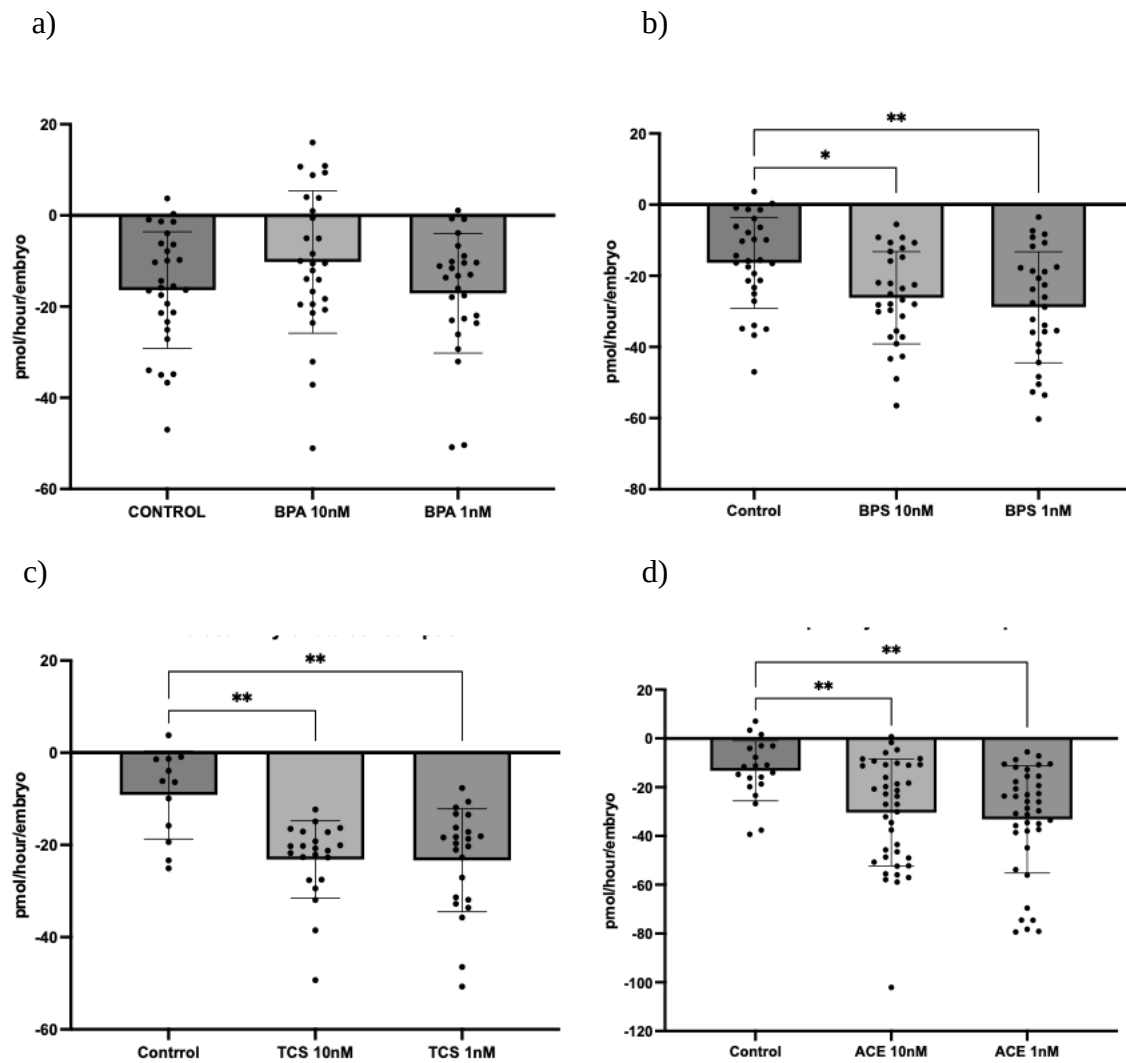


Figure 5.16: The effects of a) BPA and b) BPS, c) TCS and d) ACE on pyruvate consumption of during blastocyst stage exposure. 10 nM or 1 nM of each chemical was used during individual in vitro culture for 24 hours exposure during blastocyst stage. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal-Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

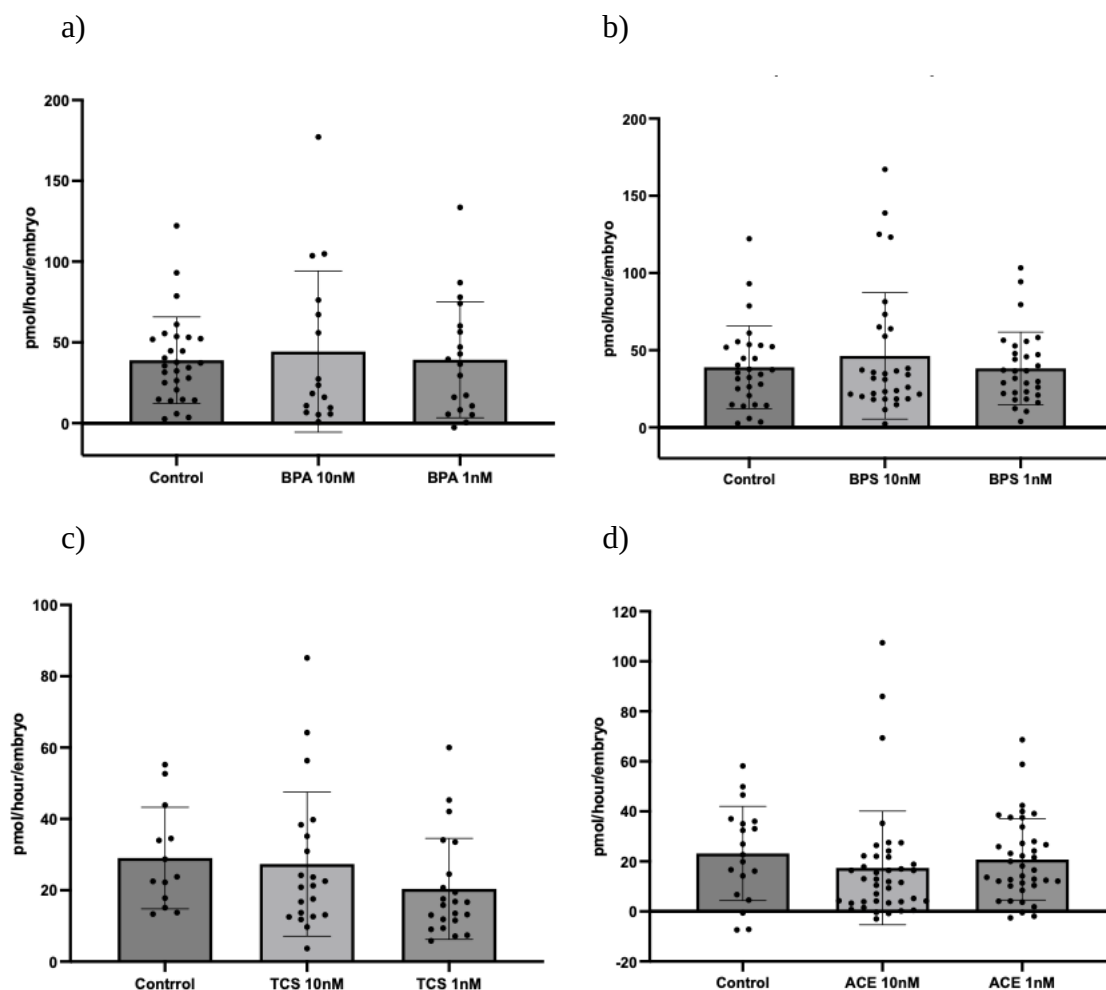
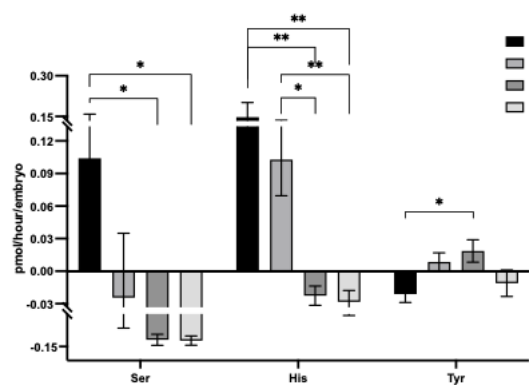


Figure 5.17: The effects of a) BPA and b) BPS, c) TCS and d) ACE on Lactate production of during blastocyst stage exposure. 10 nM or 1 nM of each chemical was used during individual in vitro culture for 24 hours exposure during blastocyst stage. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using One-Way ANOVA with Tukey's post-hoc test or Kruskal-Wallis followed by Dunn's post-hoc test when the data sets passed or not the normality test, respectively *, $p < 0.05$; ** $p < 0.01$; * $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.**

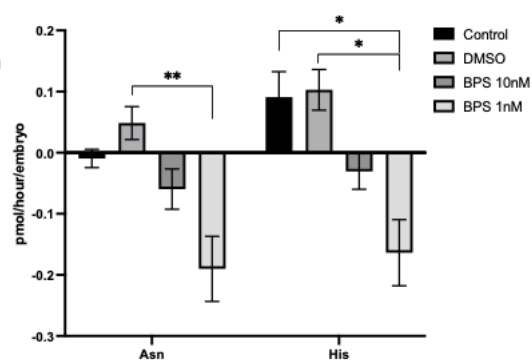
Amino acid turnover by embryos after exposure to environmental contaminants during blastocyst stage was determined. Metabolism of Ser, His and Tyr was significantly impacted by the presence of BPA in the cultured media; Ser and His was released into the medium by untreated blastocyst while the blastocysts consumed Ser and His in the presence of BPA irrespective of the concentration that they were exposed. Untreated blastocysts depleted Tyr whereas embryos treated with 10 nM BPA released this amino acid; a lower dose of BPA had no impact on Tyr handling (fig: 5.18a).

When treated with BPS, blastocysts depleted His from the medium; by contrast untreated embryos released this amino acid. TCS exposure led to alteration of Thr and Trp metabolic profile compared to control group. Thr was consumed by the untreated blastocyst whereas it was released in the media by the blastocyst treated with TCS and via versa in the case of Trp (fig 5.18c). ACE exposure had a marked impact on amino acids turn over during blastocyst stage, with 13 out of 18 amino acids were significantly altered (Asp, Asn, Arg, Ala, Glu, Tyr, Thr, Trp, Met, Val, Leu, Iso, Lys) and the data are presented in figure 5.18d & e.

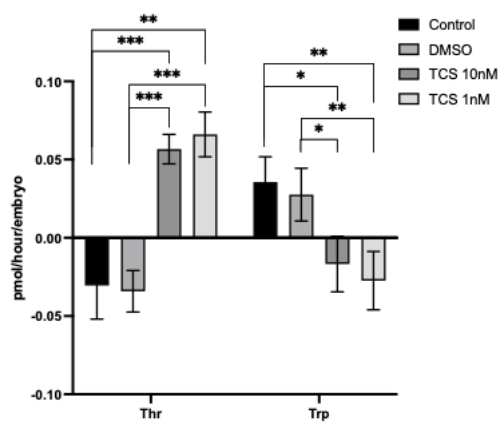
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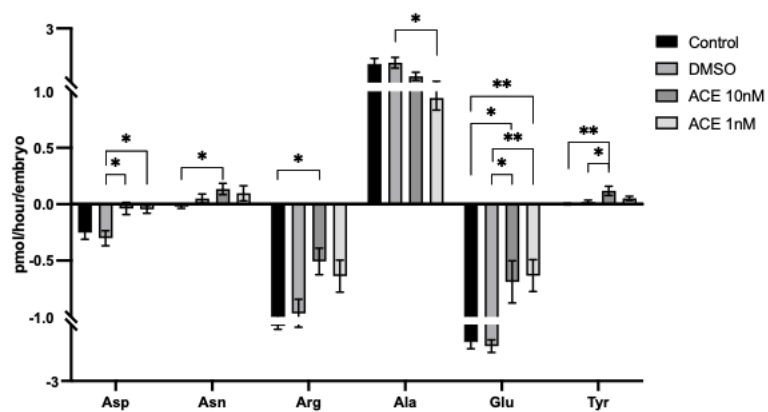
b)



c)



d)



e)

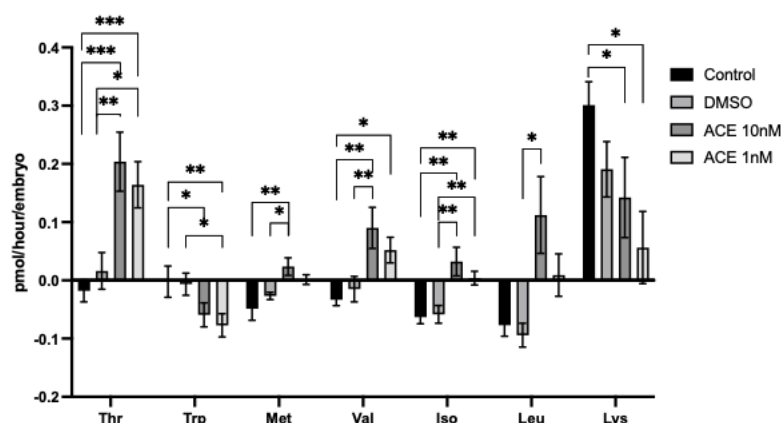


Figure 5.18: The effects of a) & b) BPA and c) BPS, d) & e) TCS and f) & g) ACE on amino acids turnover during blastocyst stage of embryos development. Blastocysts were exposed at 10 nM or 1 nM of each chemical during individual in vitro culture for 424hours. Each graph represent data of at least 3 biological replicates. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using Multiple Mann-Whitney test using Holm-Sidak post-hoc test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

5.4.3 The impact of environmental contaminants on the oxygen consumption rate of bovine blastocysts

Finally, Oxygen Consumption Rate (OCR) of bovine blastocysts was determined. OCR is a measure of the mitochondrial activity of blastocyst. The embryos were generated without the addition of any treatment until the time of the assay. On day 7 of development embryos at early or expanded blastocyst stage were collected and transferred to the Seahorse-XFp analyser (Agilent, Santa Clara, California) the embryos were exposed as indicated by the line graphs in figures 5.19a to 5.21a. Extracellular Flux Analysis (EFA) was conducted as described in Chapter 2, section 2.4.5 and the response of an acute injection of BPA, BPS and TCS are presented in figure 5.19 to figure 5.21, respectively. The OCR of blastocysts exposed to ACE could not assessed due time constraints.

Figures 5.19a, 5.20a, 5.21a indicate the basal OCR, and how the treatments (BPA, BPS and TCS) and subsequent mitochondrial inhibitor addition affected OCR in real time. Subsequently, the OCR response to each treatment was calculated based on the OCR values before and after the addition of each treatment (fig: 5.19b, 5.20b, 5.21b) and finally the

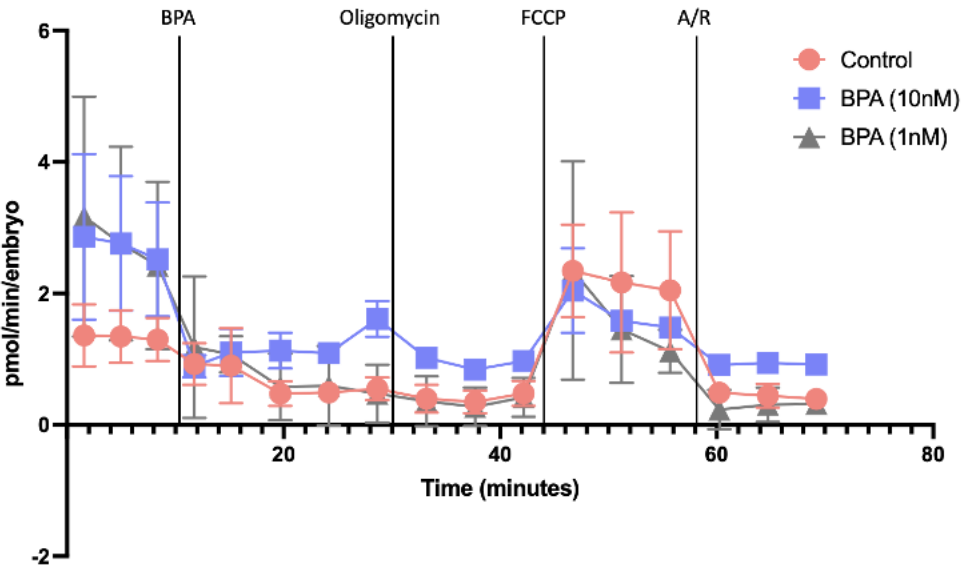
components of the OCR (ATP coupled, proton leak, spare capacity and non-mitochondrial) were quantified as percentage of the basal respiration after the addition of each treatment (fig: 5.19b, 5.20b, 5.21b). All experiments were performed in triplicate.

The OCR of blastocysts was unaffected by the addition of BPA (fig:5.19b) However, when the OCR when the components of oxygen consumption were analysed, there was a strong trend that ATP-coupled OCR was increased in blastocysts treated with 1 nM BPA ($p=0.054$) and non-mitochondrial OCR was decreased ($p=0.077$) when compared to blastocysts treated with 10 nM BPA (fig:5.19c).

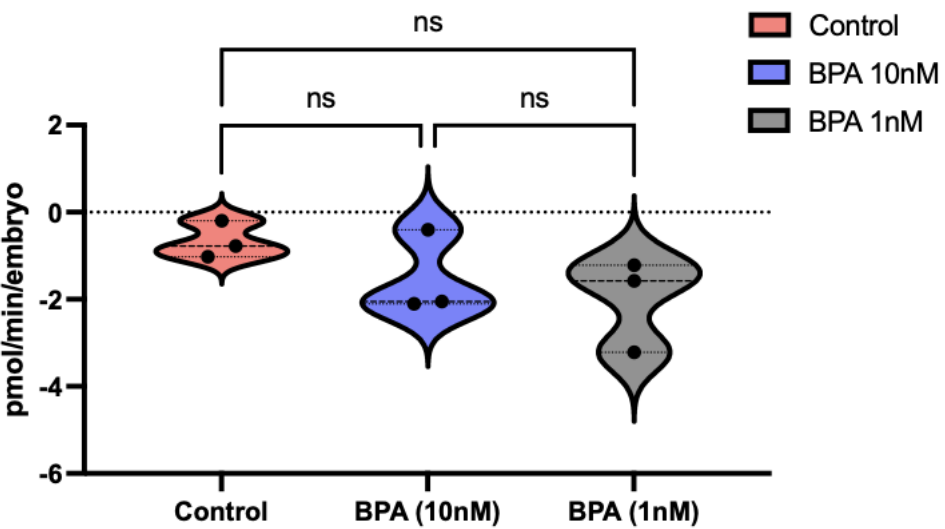
Similarly, the OCR of blastocysts treated with BPS did not differ to untreated group. However, when the OCR components were calculated as percentage of the basal respiration after the addition of BPS results show a significant increase of the ATP-coupled OCR in 1 nM BPS compared to control group (48 ± 6.01 b% vs $22.9 \pm 6.3\%$ $p=0.011$, respectively).

Finally, OCR response to TCS addition did not reveal any statistically significant difference among the groups (fig: 5.21b) However, a statistical significant difference in proton leak rate was identified among the TCS-exposed blastocyst groups with the 1 nM TCS group having significantly lower non-mitochondrial activity rate compared to 10 nM TCS ($25.6 \pm 7.8\%$ vs $46.5 \pm 5.1\%$ $p=0.048$ respectively, fig: 5.21c). OCR was finally assessed on embryos injected with the control vehicle; no significant differences were identified compared to untreated group (fig 5.26_Appendix).

a)



b)



c)

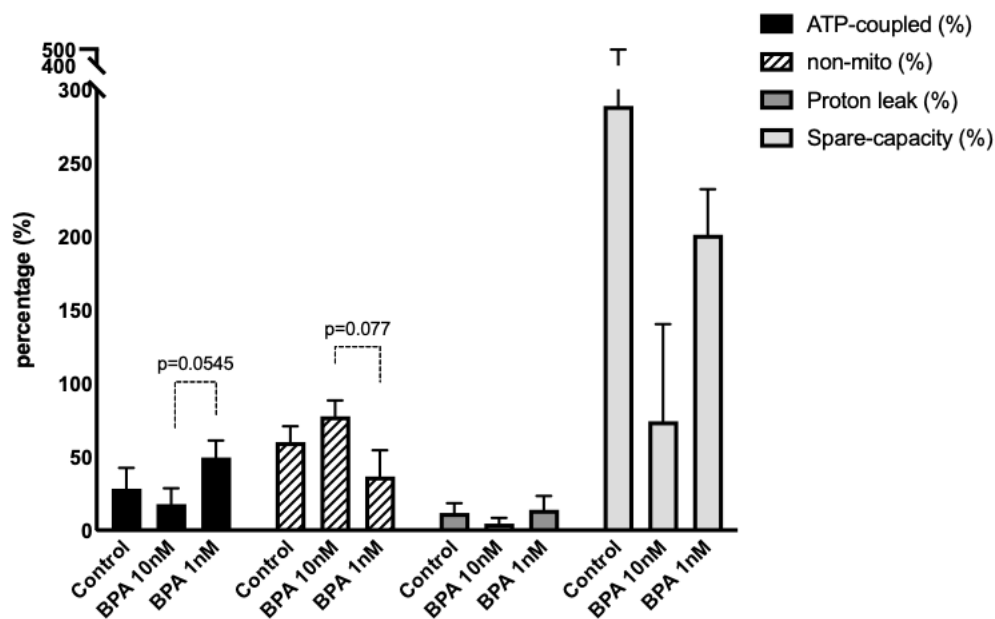
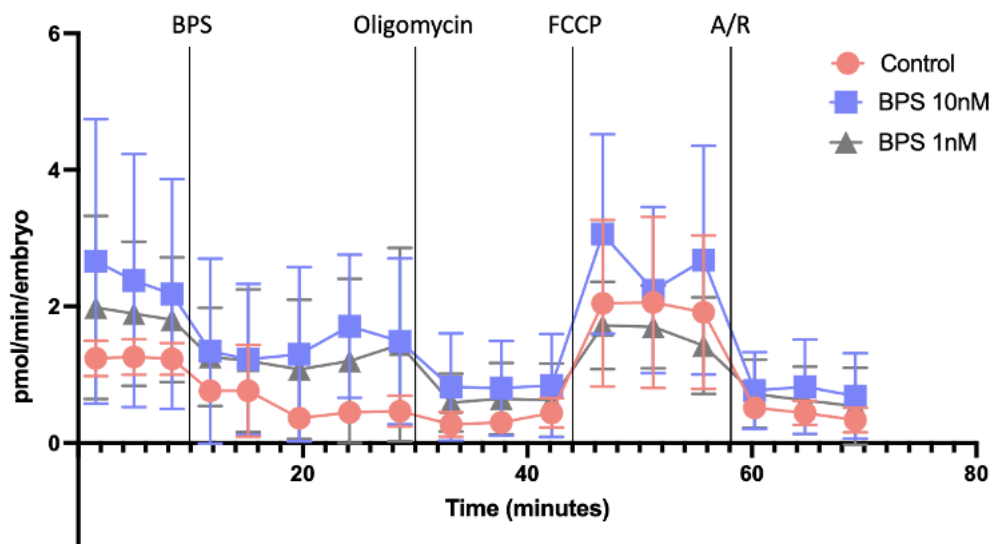
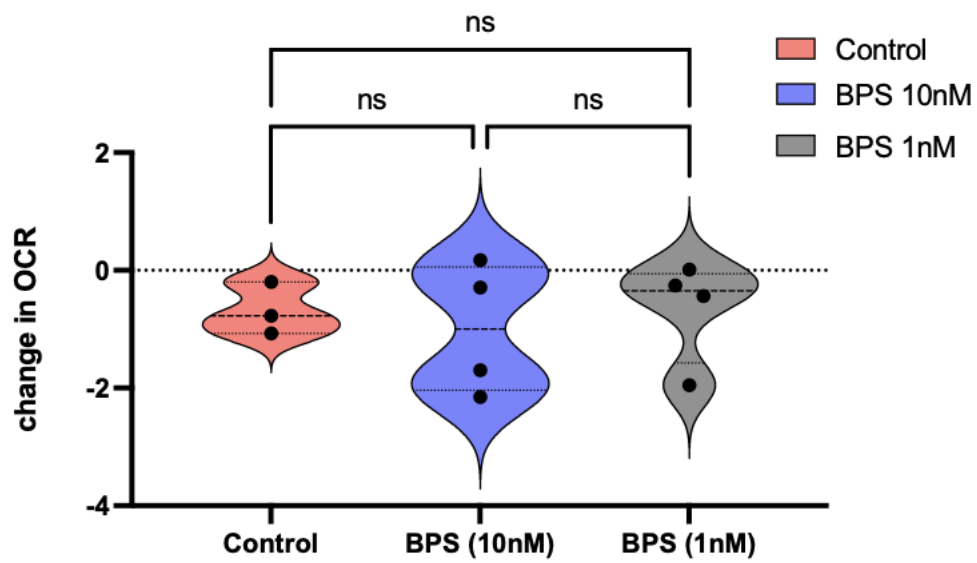


Figure 5.19: OCR of blastocysts in response to acute injection of Bisphenol A (BPA) immediately on D7 of embryo culture a) The timeline of OCR in response to BPA, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPA injection, c) mitochondrial parameters as a percentage of basal OCR after BPA injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented. This graph includes data of 3 biological replicates (OCR, $n=3$).

a)



b)



c)

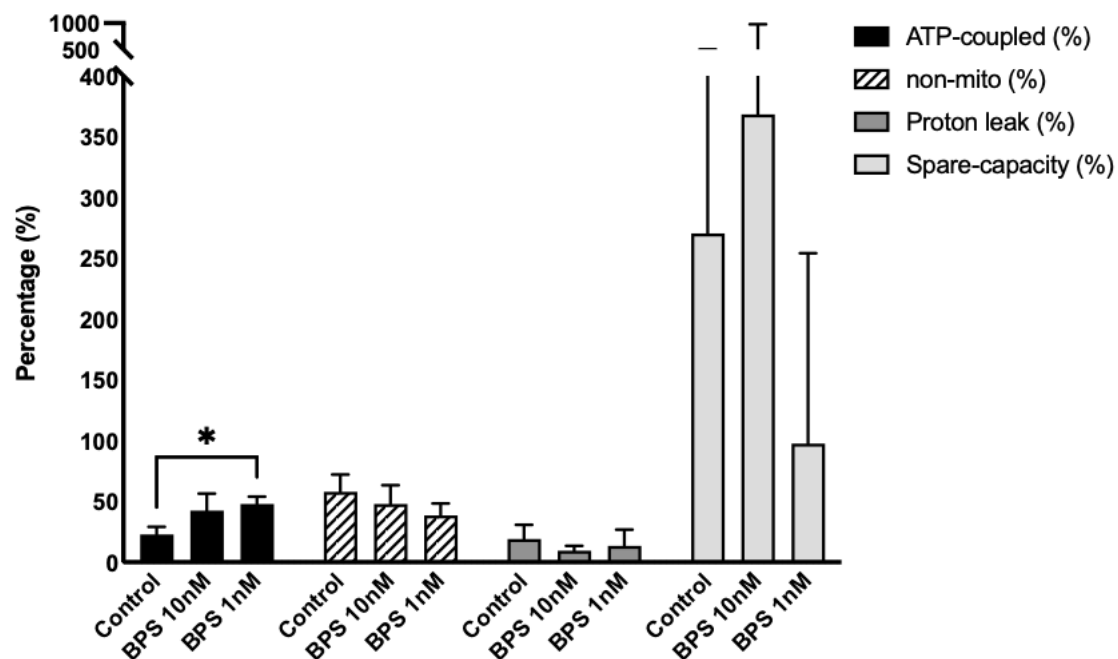
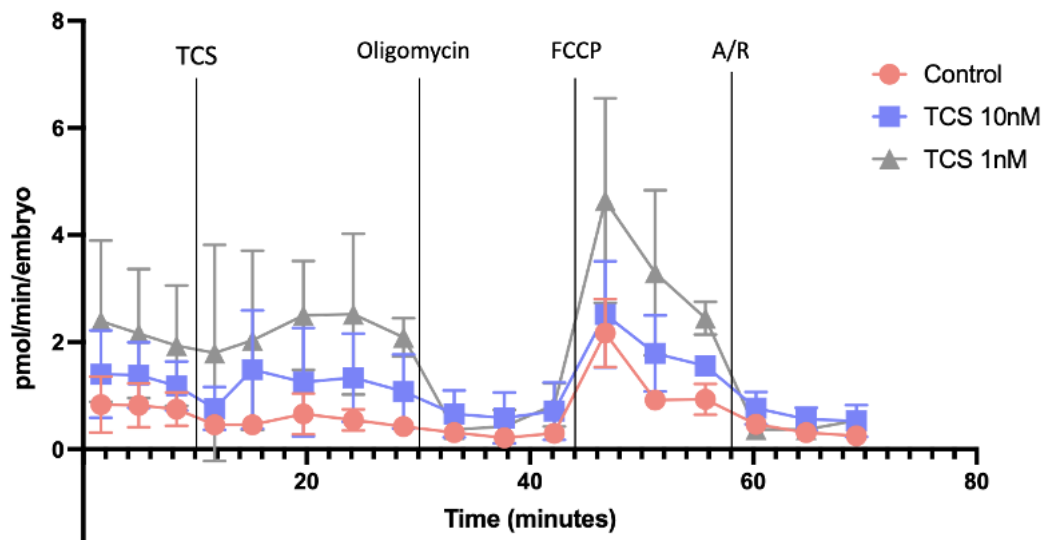
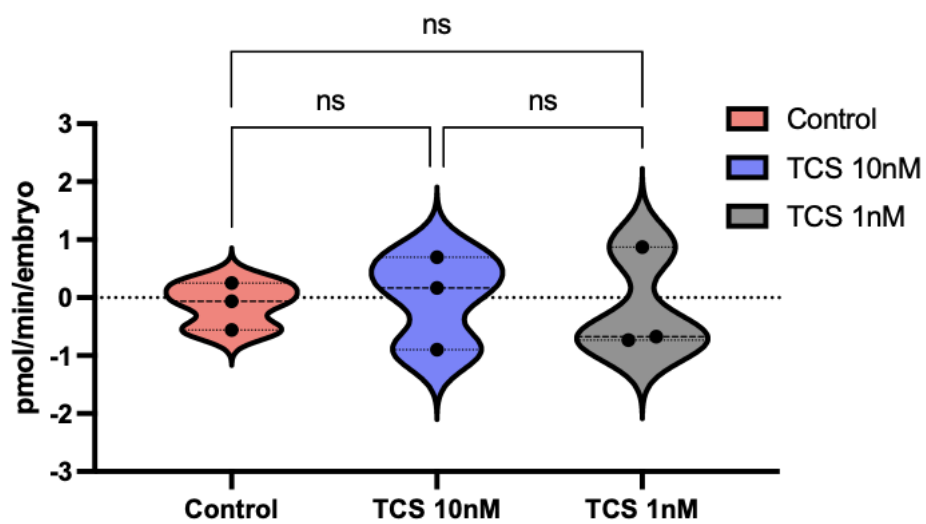


Figure 5.20: OCR of blastocysts in response to acute injection of Bisphenol S (BPS) on D7 of embryo culture
a) The timeline of OCR in response to BPS, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPS injection, c) mitochondrial parameters as a percentage of basal OCR after BPS injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented. This graph includes data of at least 3 biological replicates (OCR, $n=3$).

a)



b)



c)

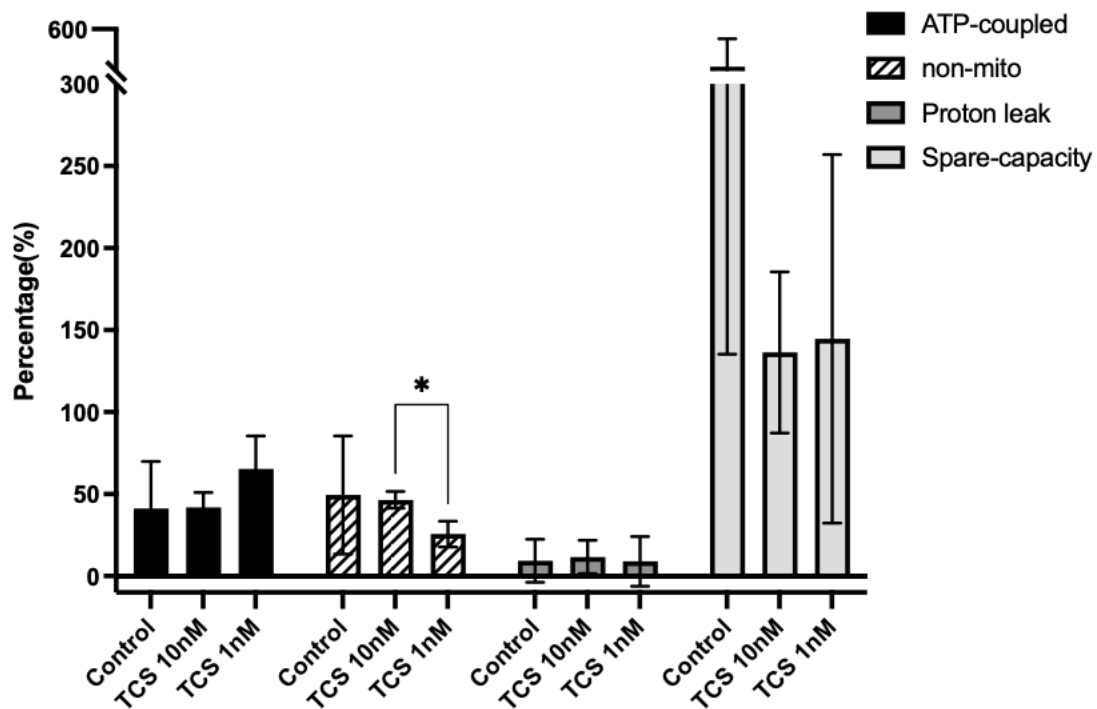


Figure 5.21: OCR of blastocysts in response to acute injection of Triclosan (TCS) on D7 of embryo culture
a) The timeline of OCR in response to TCS, and the mitochondrial inhibitors: oligomycin, FCCP and Antimycin/Rotenone mix as comparison among the different treatment groups b) OCR as response to the BPS injection, c) mitochondrial parameters as a percentage of basal OCR after TCS injection. Differences were detected using b) one-way ANOVA followed by Tukey test, c) two-way ANOVA followed by Tukey test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented. This graph includes data of 3 biological replicates (OCR, $n=3$).

5.5 Discussion

In Chapter 4 the effects of the environmental contaminants of interest (BPA, BPS, TCS, ACE) on oocyte metabolism and steroidogenesis was investigated. To further investigate if onward oocyte developmental competence has been impaired after the exposure to environmental contaminants of interest, the subsequent impact on development and metabolism of the generated blastocyst derived from chemically exposed COCs was investigated in this chapter. Furthermore, since exposure to environmental contaminants can occur at any stage of development, we also investigated the effects of exposure on embryo metabolism during specific time points of development such as cleavage and blastocyst stage.

5.5.1 Exposure to environmental contaminants during IVM impaired the developmental competence of the generated embryos.

Our findings revealed that even though embryos derived from chemically exposed COCs had a cleavage rate similar to the untreated groups there was an impairment on blastocyst rate in most of the cases. More specifically, BPA, TCS and ACE exposure of COCs during IVM led to a significant fall in the proportion the embryos able to reach the blastocyst stage. A 10 nM and 1 nM concentrations were used for all the environmental contaminants during the experiments, because the nanomolar scale is representative, in the majority of the cases, of the levels identified in human bodily fluids (Adolfsson-Erici et al., 2002; Allmyr et al., 2006, 2008; Calafat et al., 2008; Liao et al., 2012; Liu et al., 2017; Han et al., 2018; Lee et al., 2018; Tuzimski et al., 2019; Jin et al., 2020; Zhang & Lu, 2022; Fu et al., 2024) thus allowing comparison among the treatments.

To the best of our knowledge, the cleavage and blastocyst rate of embryos derived from TCS-exposed COCs or from ACE-exposed COCs has not previously been studied. However, a study in parthenogenetically activated porcine embryos, has reported decrease on blastocyst rate after exposure to 50µM and 100µM without any effect on cleavage rate (Kim et al., 2020). Despite the considerable difference of the concentrations that have been used in this study compared to our experiment (50µM and 100µM vs 1 nM and 10 nM respectively) these findings align with our data. Moreover, a study conducted in mouse investigated the effects of cleavage and blastocyst rates of embryos derived from ACE-exposed spermatozoa (Gu et al., 2013). In that

study, spermatozoa were exposed to 500 μ M or 5 mM ACE for 30min and then used for fertilisation and in vitro embryo culture. The data revealed that 500 μ M did not have an effect on cleavage and blastocyst rate, however when sperm was exposed to 5 mM the embryos cleavage and blastocyst rate was significantly decreased, indicating that exposing gametes to these chemicals can affect the fate of future embryos. These results partially agree with our data since statistically significant difference was found only on blastocyst rate. However, care must be exercised when extrapolating these data to the previous study given the effects on sperm compared to eggs and that different animal models that have been used.

It was noteworthy that blastocyst rates were affected when COCs were exposed to the lowest concentration (1nM) of TCS and ACE. In both cases, treating COCs with 10 nM of either compound had no statistically significant effect on blastocyst rates. This pattern of effect may be described as ‘non-monotonic’ dose-response of these chemicals. A non-monotonic dose response is defined by European Food Safety Authority (EFSA) as “A complex relationship between the dose of a substance and its effect, such that instead of a certain response simply increasing or decreasing with dose, the curve may be for example "U" shaped” and can occur in cells, tissue, organs, and organisms in response to nutrients, hormones, pharmacological compounds, and chemicals such as Endocrine Disrupting Chemical (EDCs). Multiple studies have confirmed the non-monotonic dose-response of EDCs (Vandenberg, 2014; Zoeller & Vandenberg, 2015) including TCS and ACE. This suggests that COCs exhibit a non-monotonic dose response. However, it is important to mention that due to the variability between the biological replicates and the fact that only 2 different concentrations were investigated, more biological repetition and higher range of concentrations should be used in the future to strengthen these conclusions.

When COCs were treated with 10 nM BPA the blastocyst rate was significantly diminished. Exposure to a lower concentration also had a marked reduction in blastocyst rate although this did not quite reach statistical significance ($p=0.054$). By contrast, COC exposure to BPS did not affect the cleavage nor the blastocyst stage of development. The findings regarding the Bisphenols align with other studies that conducted in bovine (Ferris et al., 2016; Sabry et al., 2021; Saleh et al., 2021). Similarly, to our experiment, two studies were published where bovine COCs were exposed to BPA or BPS during IVM, and their developmental competence was determined by assessing cleavage and blastocyst rates. In agreement to our finding the blastocyst rate of embryos derived from BPA-exposed COCs was significantly reduced and

meanwhile the cleavage and the blastocyst rate of the BPS group remained unchanged (Sabry et al., 2021; Saleh et al., 2021). However, in both studies the cleavage rate of the BPA derived embryos was significantly reduced a finding that is opposite to our findings. This difference might be a result of the different doses that have been used during their study (0.05 mg/ml) which is such higher dose compared to the one used in our experiment (10 nM = 2.3 ng/ml).

The effects of BPS on oocyte developmental competence using cleavage and blastocyst rate were also examined in ovine model (Desmarchais et al., 2020). In this study, concentrations from 1 nM to 10 μ M (in a 10-fold increase) were used. In contrast with our results, 1 nM concentration led to a significant increase of the cleavage rate (70%) compared to their untreated group (55%). However, in agreement with our results no difference on cleavage stage was identified in 10 nM BPS group. Furthermore, they found a significant decrease in blastocyst rate when the embryos derived from the 10 nM BPS-exposed COCs finding which comes in contrast with our results. However, in this study the blastocyst rate of embryos derived from COCs exposed to 10 nM BPS was reduced by 6.9% while in our results the decrease is 12.16% compared to the untreated groups. The fact that the blastocyst rate is not statistically significant different compared to control group, in our experiment, is due to the high variability among the biological replicates as the SD in this group is $\pm 8.2\%$. Finally, another aspect that could lead to these differences is the use of different animal models (bovine vs ovine).

It is important to reflect on the high variability in the untreated groups in figure 5.2c and 5.2d compared to the untreated groups in figure 5.2a and 5.2b. The blastocyst rates of the control group in TCS and ACE graphs had notable variability compared the ones in BPA and BPS. One possible explanation could be the period of the experiments, as they were conducted in 2021 during summertime, which was a particularly hot summer. According to literature, heat stress can impact the high production cattle. For this reason, a possible explanation could be that the ovaries and subsequently the COCs used for these experiments could come from heated- stressed animals (Alves et al., 2013; Ahmed et al., 2017; Abdelatty et al., 2018). However, despite the fact that could be a reason for the big variability among the biological replicates within the experimental group, the fact that the same batch of ovaries were used each time for the untreated and treated groups, enable comparisons and conclusions to be drawn with some confidence that the differences we find out among the experimental groups are real and not biased by this factor.

5.5.2 Chemical exposure during different time points of development influences the glucose, pyruvate consumption and lactate production of embryos

In this chapter the effects of environmental contaminants on embryo metabolism was also investigated. The embryos were exposed in three time points during 1) in vitro maturation 2) cleavage stage (day 2 and day 3) and 3) blastocyst stage of development. At these stages, depletion of glucose and pyruvate, lactate production and amino acids turnover was quantified. In these experiments, the chemical treatment lasted for 22-24 hours and caused changes in metabolic status of the embryos. However, there are differences on how the metabolic status of these embryos changed based on the compound and the time point of exposure.

As reported earlier, when oocytes were treated with environmental contaminants, subsequent blastocyst development was compromised. However, those embryos that did arise from COCs treated with either BPA, TCS or ACE consumed significantly more pyruvate (fig: 5.4). Further analysis shows that this increase on pyruvate consumption is result of the embryos that remained alive until the end of the assay (fig: 5.6). In the case of ACE group increase in glucose consumption was identified as well (fig 5.6). Meanwhile no statistically significant difference was shown when the embryos were exposed to BPS during this developmental stage.

Interestingly, significant increases of glucose and pyruvate consumption was found when the embryos were exposed to BPS during the blastocyst stage. Since the concentrations (10nM and 1nM) and the time of exposure (approx. 24 hours) was the same in both developmental points we can assume that the BPS effect on embryo development is time dependent. In addition, the lower dose of BPS (1nM) had a notable impact on pyruvate consumption, again suggestive of a non-monotonic action of the chemical. Meanwhile, BPA exposure during the blastocyst stage did not affect the glucose, pyruvate and lactate consumption/production suggesting again a time sensitive window for BPA exposure. These data align with the results of Choi et al., 2016 during which embryos were exposed to 1 ng/ml and 10 ng/ml of BPA on blastocyst stage. It seems that the 1ng/ml BPA (4.3 nM) did not affect nor the glucose consumption neither lactate production, glucose consumption was only increase by the high dose (10 ng/ml) of BPA (Choi et al., 2016).

Regarding, TCS and ACE effects, similarly to previous observation, it seems that pyruvate consumption was statistically increased when the embryos were exposed at blastocyst stage

suggesting a consistency on the way of action of those two chemicals. However, due to the fact that two different actions could lead to the same result further investigation needs to be done as well in the future in order to identify if the increase on pyruvate consumption noticed on blastocyst derived from treated COCs and after direct exposure during blastocyst stage is happening due to the same mechanism of action.

Finally, the glucose, pyruvate and lactate consumption/release were investigated after direct exposure to environmental contaminants of interest during the cleavage stage. Glucose consumption was increased in all cases of exposure and increase on pyruvate consumption was also noticed in the case of BPA, BPS, ACE. One explanation for this is that cleavage embryos got into a “hyperactivated mode” by consuming more nutrients (glucose and pyruvate) compared to untreated groups.

Embryo viability and developmental competence has been correlated with pyruvate consumption and more specifically with an optimal range of consumption (Guerif et al., 2013). In that work, embryos at the cleavage stage which consumed a mid- range of pyruvate appear to have greater blastocyst rate compared to those ones which consume very high or very low amount of pyruvate. As described above increased pyruvate consumption has been identified after the environmental contaminant’s exposure during different time points of development suggesting increased stress during development with possible lower developmental potential. This effect has been described as the “Goldilocks hypothesis” for the embryos during preimplantation development (Leese et al., 2016) suggesting that embryos with higher developmental competence are those which their metabolic status is within an optimal range and the embryos with too high or too low metabolic action are stressed embryos. Based on this principle we could hypothesise that also the increased glucose consumption is also related with increased stress of embryos due to the chemical exposure which also could result on lower ability of these. High glucose consumption could also be related with increase in glycolysis pathway. Through glycolysis, glucose is converted to pyruvate which subsequently either converted to acetyl-CoA by enzyme PDH and enters the mitochondria or is converted to lactate by LDH enzyme and exit the cell. Since the lactate production was not significantly increased, we could speculate that the increase of glucose intake could correspond to increased mitochondrial activity. However, in order to identify if there was an impairment on mitochondrial activity due to the chemical exposure further analysis should be done in the future including genetic analysis.

At this point is important to mention that embryos in all experimental groups followed the pattern of consuming pyruvate and glucose and producing lactate; a pattern which have been confirmed in multiple studies on the pre-implantation embryo (Choi et al., 2016). However, variability across the measurements will be result from the fact that the embryos were selected based on day of development (Day 2 and Day 3) rather than stage of development. This mean that Day 2 and Day 3 embryos include stages of 2-cell to 4-cell embryos and 4-cells to 8 cells respectively when the exposure started and over the 24 hours of incubation into individual culture led to a variable progression (2 cells to 16-cells embryos). Similarly, exposure to blastocyst stage included early to hatched blastocyst.

5.5.3 Amino acids turnover was influenced by the presence of environmental contaminants during different time points of development

Since the embryo exposure to environmental contaminants of interest led to nutrients imbalance in our next step the amino acid turnover was assessed. The consumption/ release of 18 amino acids were quantified. The turnover of blastocysts derived from chemically exposed COCs, and embryos exposed on day 2-3 cleavage, and blastocyst stage was assessed. Amino acids are essential components for the protein synthesis within the embryo. However, most of the amino acids through complex biosynthetic/ biochemical pathways contribute also in other biological processes such as in energy production through TCA cycle and ATP production, the synthesis of nucleotides, also can protect from oxidative stress and regulate the pH and osmolality of the surroundings (Sturme et al., 2008).

The amino acid turnover was significantly influenced by the presence of the environmental contaminants irrespective of the developmental time point the embryos were exposed. However, the specific pattern of the amino acid usage that was significantly influenced in each developmental stage differed among the treatments, and in some cases, concentrations of the chemical that have been used. Data with the amino acids significantly impacted by the presence of environmental contaminants are presented in figures: 5.7, 5.14 and 5.18

Overall, based on the number of amino acids with statistically significant difference among the treated and untreated groups it may be concluded that environmental contaminants exposure

had a greater impact on embryo metabolism when the embryos were exposed directly rather than when blastocysts were exposed as oocytes during IVM. A summary of the amino acids with statistically significant differences per environmental contaminants per time of exposure is presented in table 5.2.

When COCs were exposed to BPA during IVM, the resulting blastocysts released arginine (Arg) and glutamine (Gln) (fig:5.7). Arg and Gln are both present in periconceptual environment and their importance have been highlighted for embryo development. More specifically, glutamine is the amino acid that is routinely added to any commercial media for in vitro embryo production. Gln can contribute to ATP production, as it can be converted to glutamate and subsequently to α -ketoglutarate which is metabolised through the TCA cycle for energy production (Wu et al., 2000). In addition, the importance of Arginine in reproduction and embryo development has been highlighted (González et al., 2012; Leese et al., 2021; Martin et al., 2003; Van Winkle et al., 2006; Van Winkle & Ryznar, 2019), due to its participation on nitric oxide (NO) synthesis, a critical molecule for embryo signalling pathways (Van Winkle, 2001).

When oocytes were exposed to BPA and BPS, the resulting blastocysts exhibited differential tryptophan handling. Tryptophan has been associated with normal embryo implantation and decidualization in mice (Chen et al., 2024). In our data sets Trp was one of the commonly affected amino acids by the presence of the environmental contaminants when the embryos were exposed during blastocyst and cleavage stage. Both Trp and leucine (Leu), were affected by ACE and TCS exposure (table 5.2), are the preferable substrates for amino acid transport system System B^{0,+}, a system important during implantation in rodents and human (Martin et al., 2003; Van Winkle et al., 2006; González et al., 2012; Van Winkle & Ryznar, 2018). Histidine (His) was also influenced by BPA, BPS and TCS when present during either the blastocyst or cleavage stage. Histidine has been reported to assist in the preparation of the uterus for the implantation. His is converted through histidine decarboxylase, an enzyme which is expressed in the uterus, to histamine, which subsequently act as a local signalling factor during implantation (Wood et al., 2000).

Based on our experiments, the cleavage stage of development appeared to be the time point most sensitive to environmental contaminants since 8, 4, 9 and 10 out of 18 amino acids were significantly influenced by the presences of BPA, BPS, TCS, and ACE respectively. Among

the amino acids that were impacted, Threonine (Thr) was consistently influenced by all four different treatments. Thr was also influenced by TCS and ACE after exposure during the blastocyst stage. Thr metabolism plays an important role in bovine blastocyst development (Najafzadeh et al., 2018) as it is converted through threonine dehydrogenase (TDH) to glycine and acetyl-CoA (Van Winkle, 2021) which is oxidised for energy production through the TCA cycle.

Among the 4 different environmental contaminants used during these experiments ACE caused the greatest impact on amino acid metabolism after exposure during embryo development, as 13 and 10 out of 18 amino acids were significantly different on blastocyst and cleavage stage respectively. One of the common amino acids that significantly changed was methionine. Methionine plays a crucial role in the synthesis of nucleotides and eventually to DNA methylation through the methylation cycle (Lightfoot et al., 2005; Gilbody et al., 2007). Other amino acids which were commonly influenced by ACE after exposure during the embryo development were asparagine (Asp), arginine (Arg), glutamate (Glu), isoleucine (Iso), Thr and Leu. In a study published in 2016 the importance of amino acids as regulators of mTORC1 activity was investigated. The authors suggested that amino acids activate the mTORC1 by two separate steps, the priming, which is regulated by Asp, Glu, Arg, Thr, Gly, Ser, Ala, and secondly the activation step which was regulated primarily by Leu but also by Met, isoleucine (Iso) and valine (Val) (Dyachok et al., 2016). mTORC1 signalling pathway is an essential pathway for cell growth, metabolism, and autophagy. Its role has been studied in detail in different systems and organisms among them the female reproductive system (reviewed by (Guo & Yu, 2019) It seems that it participates in processes in the ovary such as folliculogenesis, meiotic maturation and steroid production by the ovarian cells (Guo & Yu, 2019) and early reproductive events such as embryogenesis (Land et al., 2014).

At this point it is important to clarify that due to the observational nature of the experiments, we can only speculate why the amino acid metabolism was impacted by the presence of the environmental contaminants. All the aforementioned information above has been gathered from the literature and differences could be identified based on the different systems or models that have been studied. In order to identify the mechanism of action of each environmental contaminant on the amino acid metabolism further experiments should be conducted including for instance gene expression of amino acid transporters or exploring cell signalling pathways in the presence of these environmental contaminants. Overall, we can conclude that exposure

to environmental contaminants caused significant changes in amino acids turn over, these differences were dependent on the chemical, the administrated dose, and the timing of the exposure.

Table 5.2: Amino Acids with statistically significant differences per environmental contaminants per time of exposure

	<i>Exposure during IVM</i>	<i>Blastocyst stage exposure</i>	<i>Cleavage stage exposure</i>
<i>Bisphenol A</i>	Gln, Arg, Trp	Ser, His, Tyr	Thr, Trp, Val, Asp, Glu, Asn, Gln, Arg
<i>Bisphenol S</i>	Trp, Lys	Asn, His	Asn, Gln, Thr, Trp
<i>Triclosan</i>	Leucine	Thr, Trp	His, Leu, Trp, Thr, Arg, Glu, Gln, Gly, Ser
<i>Acetamiprid</i>	Tyrosine	Asp, Asn, Arg, Ala, Glu, Tyr, Thr, Trp, Met, Val, Iso, Leu, Lys	Arg, Asp, Asn, Glu, Gly, Iso, Phe, Leu, Met, Thr,

5.5.4 Environmental contaminants of interest influence the OCR consumption and its components

As discussed earlier, pyruvate and amino acids turn over were significantly affected by the presence of environmental contaminants during embryo development which could be an indicator of mitochondrial dysfunction. To assess this possibility, Extracellular Flux Analysis (EFA) was used to identify any change on mitochondrial activity by using Oxygen Consumption Rate (OCR) as marker.

OCR is a key indicator of mitochondrial activity, and it has been used to assess ATP production in mitochondria through oxidative phosphorylation and electron transport chain reaction, a process that use oxygen as the main substrate. Many different techniques have been used to determine OCR during preimplantation development among them is Extracellular Flux Analysis which has been previously used on bovine oocytes and embryos (Muller et al., 2019).

The OCR of blastocyst was measured during the exposure to BPA, BPS and TCS. There was no significant difference on OCR in response to the treatments. However, when the basal respiration after the treatment was further analysed to its components as percentage of the basal OCR (ATP-coupled, non-mitochondrial, proton leak, and spare capacity) some differences were identified.

Exposure to BPA lead to a reduction of ATP-coupled OCR rate, with a concomitant increase in non-mitochondrial activity. Meanwhile when the embryos were exposed to low dose of BPS a statistically significant increase on ATP-coupled OCR compared to untreated group was identified. Furthermore, low dose of TCS increased the ATP -coupled OCR however this increase was not statistically significant. A significant difference was identified on non-mitochondrial OCR among the two different doses of TCS with 1 nM (low) dose being significantly lower than 10 nM dose.

These data suggest that mitochondrial activity was influenced by the presence of the treatments. This impairment and the requirement of higher ATP-linked OCR in response to EDC treatment may explain the increased pyruvate uptake observed. Increased oxygen consumption and mitochondrial effects after exposure to the environmental contaminants of interest has been previously reported in porcine oocytes (Žalmanová et al., 2023) and embryos (Kim, Park, et al., 2020) as well as in other models such as epithelial cells (Teplova et al., 2017), liver and skeletal muscle (Moon et al., 2012; Teplova et al., 2017; Ahmed et al., 2020).

However, these data sets need to be interpreted with caution due to the fact that they consist of 3 biological replicates with one technical replicate per experimental group. This can explain the high variation per experimental group. Furthermore, one concerning point is that in all experimental groups the ATP-coupled OCR was lower than the non-mitochondrial OCR. Even in the control groups the ATP-coupled ranged from 28.2 to 40.1% whilst non-mitochondrial OCR ranged from 49.5 to 59.9%. Previously has been reported that around 60% of the basal OCR is ATP-linked and approximately 20% originate from non-mitochondrial respiration. Non-mitochondrial OCR could originate from cell surface oxygen which is utilised through electron transport at the membrane facilitating aerobic glycolysis, a concept reported in cancer cells and tumours (Herst & Berridge, 2007). Aerobic glycolysis adaptation (Warburg Effect) during preimplantation embryo development has been previously described and even though is not the most efficient way to generate energy (only 2 ATP molecules are generated per cycle)

allow the embryo to regulate other processes such as REDOX regulation and assist rapid cell proliferation (Krisher & Prather, 2012).

Since in our data sets an increased OCR connected with non-mitochondrial activity have been noticed as explained above this could be linked with preference towards glycolysis even in the presence of oxygen, this fact could explain the higher requirements of glucose after exposure to BPS and TCS during blastocyst stage. However, on the other hand should have led to higher production of lactate which did not happen in our case. For this reason, further investigation needs to be done in the future regarding the OCR during blastocyst stage in order to be able to come up with a clear conclusion.

In summary, the data generated in this chapter has shown that exposing oocytes or embryos to multiple EDCs leads to reduced developmental potential. Those embryos that are able to develop exhibit significant metabolic disruption, indicative of compromised embryo physiology.

Chapter 6:
General Discussion

6.1 Summary of findings

The aim of this thesis was to identify the impact of a range of environmental contaminants on periconceptual environment and early reproductive events. The events studied included creation of the periconceptual environment, oocyte maturation and preimplantation embryo development, embryo physiology and metabolic function.

Our established *in vitro* oviduct model was used to assess the impact of environmental contaminants on creation of the periconceptual environment. In this model, oviduct epithelial cells were cultured in the Transwell system, which better imitates the physiological oviductal environment was used when compared to traditional 2D cell culture approaches. Using this system, the cells were able to generate an intact monolayer of polarised epithelium, after when, the cells are cultured in an air -liquid interphase (ALI) on the top of a semi-permeable membrane. This arrangement permits the cells to be in contact with the basal compartment – analogous to the blood stream - and with the apical phase – which imitates the lumen environment. Crucially, when cultured in an ALI, bovine oviduct epithelial cells (BOECs) create a fluid *de novo* on the apical surface. Using this model, we were able to show that exposing the basolateral surface of the BOECs to environmental contaminants triggered a modification on cell physiology. The impact on BOEC function was identified by profiling the metabolic changes on important molecules such as glucose, pyruvate, lactate and amino acids in oviduct fluid (ivDOF) composition and by assessing the expression of transporters of these molecules.

Next, *in vitro* production in bovine embryos was used to assess the impact of environmental contaminants on oocytes i) during *in vitro* maturation and ii) the subsequent impact on developmental competence and metabolic modifications of the produced blastocysts. Finally, the impact of the embryo metabolism after direct environmental exposure was assessed on different time points of development. Modifications on steroid hormone production during *in vitro* maturation, glucose, pyruvate, lactate and amino acids consumption and production as well as mitochondrial function during oocyte *in vitro* maturation and embryo development were assessed to determine the metabolic profile of chemically exposed oocytes and embryos.

6.1.1 The impact of environmental contaminants exposure on BOEC function and secretions

In Chapter 3, BOEC function after environmental exposure was studied, by assessing proliferation, the energy substrates consumption and release, and formation of tight junctions using transepithelial resistance. Furthermore, using the Transwell system the effects of environmental contaminants exposure on BOEC secretions were determined.

The result revealed that BOEC function was modified after exposure to the environmental contaminants under investigation. However, each environmental compound triggered different responses. BPA significantly decreased pyruvate and increased lactate concentration in the media over after long exposure. TCS, BPS and ACE had an acute effect on pyruvate concentration after 24 hours of exposure which was eased in the next 48hours. Glucose remained unchanged to during the 7 days of exposure. Differences in cell proliferation were also confirmed during the first 24 to 72 hours of exposure, during the proliferating phase of BOEC culture. However, these differences eased from 96 hours to 168 hours of culture when the cell culture was confluent. Finally, no significant differences were identified on TEER measurements suggesting that even during exposure to environmental contaminants BOEC were able to create tight junction forming and intact monolayer. Together these data suggest that oviduct epithelial cells have a metabolic response to exposure to the chosen environmental contaminants, but that this is independent of cell growth or proliferation.

Next the effects on BOEC secretion after environmental exposure through the basal compartment of the Transwell which imitates the blood stream were assessed. Glucose, pyruvate, lactate, and amino acids were quantified in the fluid secreted by oviduct cells after exposure to environmental contaminants over the course of 7 days. The presence of environmental contaminants had a great impact on ivDOF composition the first 24hours. TCS affected the composition through modified glucose, pyruvate, arginine, and lysine transport/secretion to ivDOF during the first 24hour of exposure. BPA and BPS, despite some fluctuation, did not have a significant impact on glucose, pyruvate and lactate transfer into the ivDOF. However, BPA had a notable effect on amino acids composition of in vitro derived oviduct fluid, as 15 out of 18 amino acids were significant different to untreated groups over the first 24 hours. In most of the cases the amino acids were affected by the 10 μ M BPA (high dose) concentration, and their concentration was significantly reduced compared to control or

control-vehicle group. BPS also impacted amino acid composition in the ivDOF as 4 out of 18 amino acids (Asp, Ala, Arg, and Lys) were significantly reduced compared to untreated groups. Arg and Lys concentration was reduced irrespective of the BPS concentration. Finally, ACE exposure changed the composition by altering transport of pyruvate, aspartate, glutamate, and arginine compared to control groups. Pyruvate, Asp, Glu concentration increased whilst Arg concentration decreased in the ivDOF during the first 24 hours. Interestingly, all the aforementioned differences above were eased after 48hours (from 48 to 168 hours) of exposure, pointing a buffering effect from the epithelial cells.

Based on these results, the gene expression of the transporters of the affected molecules was investigated. The result revealed that despite the decrease of glucose concentration in the ivDOF after TCS exposure, the expression of the glucose transporter GLUT1 (SLC2A1) was unchanged. TCS exposure significantly decreased the expression of SLC7A2, the cationic amino acid transporter which transport arginine and lysine through the y^+ system. SLC7A2 expression was also affected by the presence of ACE suggesting that this could be the possible cause of Arg reduction in the ivDOF. A trend of decrease was also observed for SLC1A4 transporter expression after ACE exposure. SLC1A4 is responsible for the transport of acidic amino acids (Asp, Glu) through the ASC system (Gao et al., 2009). This observation aligns with the fact that Asp and Glu presence in ivDOF was significantly influenced by ACE. Finally, SLC7A1 and SLC7A4 expression were both increased in the presence of bisphenols. SLC7A1 and SLC7A4 along with SLC7A2 belong to the cationic amino acids transporters which as mentioned above transport arginine and lysine through the y^+ system (Gao et al., 2009; Forde et al., 2014). Arg and Lys concentration was significantly influenced by the presence of both bisphenols in the ivDOF.

Overall, we can conclude that the environmental contaminants influenced the function and the ivDOF composition of BOEC by altering the concentration of essential molecules such as glucose, pyruvate, and amino acids. The altered amino acid composition seems to be a result of impaired gene expression of the related amino acids transporters. The significance of these results is connected to the crucial role of these nutrients in ensuring embryo survival and development during the preimplantation period.

6.1.2 The effects of environmental contaminants on bovine COCs function and metabolic activity

In Chapter 4 the modifications caused to bovine Cumulus-Oocyte-Complexes (COCs) function and metabolic activity after exposure to environmental contains of interest were investigated. The same panel of contaminants (BPA, BPS, TCS, ACE) as in Chapter 3 was used, however concentrations in the nanomolar range (10 nM and 1 nM) were used for every case of exposure. This better represents levels of these compounds as has been found in the vast majority of studies in human bodily fluids (Adolfsson-Erici *et al.*, 2002; Allmyr *et al.*, 2006, 2008; Arbuckle *et al.*, 2015; Azzouz *et al.*, 2016; Calafat *et al.*, 2008; Fu *et al.*, 2024; Han *et al.*, 2018; Jin *et al.*, 2020; Lee *et al.*, 2018; Liao *et al.*, 2012; Liu *et al.*, 2017; Tuzimski *et al.*, 2019; Zhang & Lu, 2022). The consumption/release of energy substrates, as well as steroid hormone production, maturation status and mitochondrial function during COCs in vitro maturation was assessed. Finally, the pronuclei formation was assessed after environmental exposure during the fertilisation process.

The data from this study revealed that environmental contaminants of interest influence the steroid hormone production of COCs during IVM. 17 β -estradiol was significantly decreased by bisphenols whilst TCS and ACE exposure had no effect. Meanwhile, progesterone production was significantly increased in the presence of low TCS concentration (1nM) but remained unchanged in every other case of exposure. Further to these observations, the consumption/release of pyruvate and lactate was assessed. The bisphenols had no effect on pyruvate nor on lactate metabolism. However, there was notable fluctuation among the biological replicates in some experimental groups, especially in 1nM-BPS group meaning that the results need to be interpreted with caution due to potential variability in biological responses and further replication and validation of the findings are necessary to ensure reliability. TCS and ACE prompted oocytes to deplete pyruvate from the IVM media however, in contrast to controls, which released pyruvate. The release of lactate was unaffected by the presence of TCS or ACE.

Based on the pyruvate results, the nuclear status after exposure during in vitro maturation process and the mitochondrial activity of COCs or CEO was assessed. The result revealed that the maturation rate was not significantly influenced by any of the contaminants. However, changes in mitochondrial activity were detected after acute exposure of COCs or CEOs to TCS

or BPA, BPS, ACE respectively. OCR response to the treatment was not significantly impacted by the presence of bisphenols. However, a trend of increase in non-mitochondrial oxygen consumption was observed in response to BPS exposure. Meanwhile, OCR was significantly decreased when the COCs and CEOs were exposed to 10 nM dose of TCS and ACE respectively. When the OCR components (ATP-coupled, non-mitochondrial, proton leak and spare capacity) were analysed a proportion of overall OCR after the TCS addition, it was discovered that ATP-coupled OCR was significantly reduced, and non-mitochondrial activity was significantly increased. Despite the fact that OCR was reduced after 10 nM- ACE exposure when OCR components were analysed no significant differences were identified.

Finally, after exposure during the fertilisation process, pronuclei formation was assessed and it was observed that BPA significantly decreased the formation of 2 pronuclei with a further strong trend of increased oocytes unfertilised. The rate of zygotes with more than 2 pronuclei was significantly increased compared to control group when exposed to BPS irrespective of the administered dose. The low concentration of ACE also led to the same observation. Finally, a strong trend of increase on the zygotes with multiple pronuclei and a strong trend of decrease on the zygotes with 2 pronuclei was revealed after TCS exposure.

Overall, we can conclude that environmental exposure during in vitro maturation led to changes in function and metabolic activity of the oocytes. However, the impact of each environmental contaminants was different. TCS had the greatest impact as it changes the steroid hormone production by affecting progesterone production, and the metabolic activity by shifting the pyruvate status and the mitochondrial activity of the COCs. Finally, exposure during the fertilisation process led to increased zygotes rate with abnormal number of pronuclei.

6.1.3 The effects of environmental exposure to preimplantation embryo development

Following the results of Chapter 4, in the first part of Chapter 5, the impact on embryos derived from chemically-exposed-COCs was assessed by investigating their developmental competence and metabolic activity. In the second part of Chapter 5 the impact on preimplantation embryo metabolism after direct exposure to environmental contaminants of interest was assessed.

Initially, the cleavage and the blastocyst rate of embryos derived from chemically-exposed-COCs during IVM was investigated. Cleavage rate was not significantly impacted in any case of exposure. However, the blastocyst rate of embryos derived from BPA, TCS or ACE-exposed COCs was reduced suggesting that exposure during IVM decrease oocyte developmental potential. Next the metabolic activity of the produced blastocysts was assessed by quantifying the energy substrates consumed or produced. Neither glucose consumption or lactate production by blastocysts was not significant influenced as a result of originating from oocytes treated with any of the environmental contaminants. However, pyruvate consumption was significantly increased in blastocyst derived from BPA, TCS, or ACE-exposed COCs. Finally, the amino acid turnover of the produced blastocyst was also influenced, and the results are presented in figure 5.7.

In the next part of the chapter the effects on early stages embryos metabolism after direct exposure was investigated. Glucose consumption was significantly increased on D2 and D3 embryos when they were exposed to BPA or TCS. Meanwhile, a strong trend of increase in of glucose consumption was noticed after exposure to TCS on blastocyst stage. BPS did not impact the glucose consumption during the cleavage stages, but it did on blastocyst stage with glucose being significantly increased after exposure to 1 nM of BPS. Pyruvate consumption was also significantly impacted by the presence of BPS or ACE during cleavage and blastocyst stage exposure. However, the presence of BPA significantly increased the pyruvate consumption on cleavage stage embryos but not those on blastocyst stage embryos. The opposite happened after TCS exposure as pyruvate consumption remained unchanged during cleavage stage whilst increased on blastocyst stage irrespective of the administrated dose compared always to the control group. Regarding lactate production, it seems that was significantly decreased after exposure to 10 nM of BPA during cleavage stage, in the rest cases of exposure remained unchanged. Finally, the amino acid turnover was assessed during cleavage and blastocyst stage after environmental contaminants exposure and in figure 5.14 for cleavage stage embryos and in figure 5.18 for the blastocyst stage embryos.

In the final part of Chapter 5 by using OCR as measurement, the mitochondrial activity of blastocyst was assessed during acute exposure to BPA, BPS or TCS however due to the low number of biological and technical replicates per experimental group, no clear conclusion can be made at this point. The results are presented from Figure 5.19 to 5.21.

Overall, it seems that exposure to the panel of environmental contaminants either during IVM or after direct exposure during embryo culture resulting on embryos with impaired metabolism. It seems that the consumption of essential energy substrates such as glucose, pyruvate and amino acids, was significantly influenced. However, each environmental contaminant impacted the metabolic activity differently depending on the dose and time of exposure.

6.2 – The effects of environmental contaminants on early reproduction.

If we consider each contaminant in turn, it is possible to estimate the impact of exposure on the early events of reproduction. In the presence of BPA, the BOEC metabolic activity was modified as the concentration of pyruvate released in the media was significantly decreased. Furthermore, the amino acid composition in the ivDOF was modified. Subsequently, the exposure of oocytes to BPA during IVM did not only decrease 17 β -estradiol production but also interfere with their developmental competence as the blastocyst rate of embryo derived from BPA-exposed oocytes was also decreased. Apart from the fact that fewer blastocysts were produced from BPA-exposed oocytes, the metabolic activity of these embryos was also modified, as they were consuming higher amount of pyruvate. Direct exposure to BPA during early stages of development had also great impact on embryos metabolic activity, as cleavage stage embryos seems to consume higher amount of pyruvate and glucose. Finally, amino acid turnover was significantly impacted after indirect or direct exposure of the embryos during cleavage and blastocyst stage. Taking into consideration that the oviduct cells released less amount of pyruvate and amino acids composition was modified in the ivDOF combined with the fact that BPA-exposed embryos required higher amount of energy substrates could suggest that the oviductal environment becomes insufficient to support their development.

Regarding BPS exposure, the results revealed that BOEC secretions were modified as amino acid composition was significantly influenced. In addition, 17 β -oestradiol production of the oocytes was decreased in the presence of BPS. Apart from an increase of tryptophan and lysine consumption no other subsequent significant metabolic changes were identified in blastocysts derived from BPS-exposed oocytes. However, when the embryos were directly exposed to BPS during cleavage or blastocyst stage higher amount of glucose and pyruvate were consumed. Furthermore, amino acids composition was significantly modified irrespective of the stage of exposure. Finally, mitochondrial activity of blastocyst was influenced by BPS exposure as the

ATP-coupled OCR was significantly increase. Oxygen consumption has previously been suggested as a marker for embryo quality (Lopes et al., 2007). It seems that BPS- exposed oocytes have the ability to counterbalance the negative effects of BPS and produce embryos without major effects on their metabolic activity whilst direct BPS exposure reduce embryo quality by affecting energy substrates and oxygen consumption. One explanation of these findings could be that effects of BPS exposure are time dependent with early stages of embryo development being more sensitive in the presence of BPS.

Comparing the effects of BPA and BPS exposure to oviduct environment and oocyte maturation it seems that the former had a greater impact than the latter. More amino acids were impacted by the presence of BPA than BPS. Also, BPA seems to decrease the developmental competence of the exposed oocytes by reducing the blastocyst rate and changing their metabolic activity, whilst the developmental rates and metabolic activity was not influenced on embryos derived from BPS-exposed oocytes. However, both bisphenols had a great impact on metabolic function after direct exposure during cleavage and blastocyst stages of development. The metabolic profile of embryos exposed to bisphenols shows that they use higher amount of energy substrates during their development. One interpretation of these data could be that embryos increase their metabolic activity trying to counterbalance the adverse effects of bisphenols effects. The “hyperactivated mode” in terms of embryo metabolic activity (Brison et al., 2004; Guerif et al., 2013) have been associated with lower developmental potential (Leese et al., 2022).

In response to TCS, the oviduct environment is modified as there was a significant difference on glucose concentration and amino acid composition in BOEC secretions. Higher production of progesterone, a shift in pyruvate metabolic status and impaired mitochondrial activity was identified in COCs exposed to TCS. These metabolic changes of the COCs lead to reduced blastocyst rate and even those that have become blastocysts had impaired metabolic profile as they were consuming significantly higher amount of pyruvate and producing lower amount of lactate. TCS had also great impact on metabolic activity on early embryos after direct exposure. Glucose and pyruvate consumption was significantly increased after exposure during cleavage and blastocyst stage, respectively. Amino acids turnover was influenced by the presence of TCS irrespective to the time point of exposure. Considering DOHaD hypothesis (Barker, 2007) any modification on periconceptual environment could have a great impact on embryo development leading to impaired health on the later stages of life as adults. Furthermore,

embryos with higher metabolic activity in terms of pyruvate consumption (Guerif, et al., 2013) amino acids turn over (Houghton et al., 2002; Brison et al., 2004) oxygen consumption (Lopes et al., 2007, 2010) has previously been correlated with lower developmental ability (Leese et al., 2022). By taking into consideration the above we could speculate that oviductal environment becomes incompetent to support early reproductive events and oocyte and embryo developmental competence is impaired after exposure to TCS.

Finally, BOEC secretions were also affected by ACE exposure as the amino acids' composition was modified. Furthermore, ACE changed the metabolic status of pyruvate during IVM and decreased the OCR of the COCs which led to decreased developmental competence of the oocytes as the blastocyst rate of the produced embryos was also decreased. Moreover, metabolic function of the embryos was affected as pyruvate consumption was increased and amino acid turnover was modified after indirect or direct exposure to ACE at any point of development. As has been previously mentioned pyruvate and amino acids are essential molecule used as energy substrate during early stages of development. High levels of pyruvate consumptions (Guerif et al., 2013) as well as modified amino acids turnover has been previously correlated with impaired developmental ability. Combining the effects of ACE on oviduct environment, oocyte, and embryo metabolic function we could suggest that ACE affect early events of reproduction. The implications of these impacts in the longer term are not known but may relate to adverse effects on pregnancy outcomes or on health in later stage of development.

6.3 Strength and limitations

This scientific work contains a number of strengths, but, like any work also has limitations. Among the strengths are the validated methods that have been used to investigate the impact of environmental contaminants. Each method has been tested previously and published in peer reviewed studies. The validity of the bovine oviduct epithelial cells collection, isolation from primary tissue as well as the robustness of BOEC culture in a Transwell system and the production of in vitro derived oviduct fluid (ivDOF) has been established previously by our laboratory (Simintiras et al., 2016). In vitro production of bovine embryos, including oocyte isolation from primary tissue, semen preparation, fertilisation and grouped and individual embryo cultured are well established methods as well by our lab (Guerif et al., 2013; Muller et

al., 2019). Furthermore, the quantification of glucose, pyruvate, lactate and amino acids was performed extensively and are widely recognised as the gold standard for measuring the depletion and release of key nutrients (Guerif et al., 2013; Leary et al., 2015; Simintiras & Sturmey, 2017; Muller et al., 2019). Also, the assessment of the mitochondrial function using Seahorse XFp was optimised and established using bovine oocytes and embryos by our laboratory (Muller et al., 2019). Finally, exclusion criteria for each method have been established and followed for each set of analysis. For instance, metabolic assays with standard curve below 98% were systematically excluded, the amino acid quantification was validated in each set of HPLC experiment in order low error levels to be established and the specificity and efficiency of all the primers used for gene expression experiments was assessed and primers with efficiency lower than 90% or higher than 110% were excluded.

In chapter 4 and 5 the strength of the data regarding the metabolic profile assessment comes from the number of oocytes and embryos and the number of biological replicates that have been used per experimental group. The usage of bovine tissue from slaughterhouse during this thesis as material which would be discarded otherwise, should be consider as a strength of this work, as it helps the direction of using less experimental animals in research.

Beyond the strengths of the chosen methods, the work investigated the impact of environmental contaminants at physiologically relevant concentrations. So far, the studies using concentrations in the range of those that have been identified in bodily fluids are very limited. There has been a growing interest in the impact of common environmental contaminants on a multiple body systems. Overwhelmingly, these studies are carried out using in vitro models (Gu et al., 2013; Machtinger & Orvieto, 2014; Ajao et al., 2015; Choi et al., 2016; Ferris et al., 2016; Desmarchais et al., 2020; Kim et al., 2020; Park et al., 2020; Sabry et al., 2021) However, typically, the effects of contaminants are tested at the micro- or even millimolar range – far in excess of concentrations ever detected in biological tissues (Machtinger et al., 2013; Ajao et al., 2015; Kim et al., 2020; Park et al., 2020; Du et al., 2021; Sabry et al., 2021; Darghouthi et al., 2022). This work has identified physiologically-relevant concentrations of the compounds under investigation and ascertained how they impact the cells of the oviduct, the oocyte and the early embryo. The chosen levels of the environmental contaminants in the present work reflect levels common in everyday exposures for humans. Furthermore, bovine has been suggested previously as a suitable model for human IVF (Ménézo & Hérubel, 2002) and to evaluate chemicals on toxicological studies in reproduction (Santos et al., 2014) due to the

biochemical and developmental similarities during the final stages of oocyte maturation, fertilisation and early stages of embryo development. Taking into consideration the reasons mentioned above these results could be extrapolated to humans.

The implications of these impacts in the longer term are not known but may relate to adverse effects on pregnancy outcomes or on health in later stage of development. Future studies could examine the action of these contaminants on embryos morphokinetics and metabolism during the early stages of embryo development and the impact of this exposure on later stages of life.

Importantly, there are a number of limitations regarding this scientific piece of work that should be reflected upon. In chapter three, some experiments were limited by low number of technical replicates. This, coupled with high variability was noticed in some cases, for instance in GPL quantification in ivDOF in Chapter 3 means that the reliability of data may be reduced. Furthermore, in Chapter 3 the assessment of BOEC, regarding their metabolic function, could have been improved by normalising the concentration of GPL against the amount of protein per experimental group.

Regarding Chapter 4 and 5 a common limitation that needs to be considered is the change of the primary tissue collection before and after COVID lockdown. Due to the restriction after COVID lockdown, despite our best efforts, it was not possible to collect and process tissue for transport in optimal conditions. Specifically, because of changes in process in response to COVID restrictions, it was no longer possible to ensure temperature maintenance of ovaries during transportation to the laboratory. This difference could influence the oocytes aspirated from the ovaries and subsequently the zygotes to the blastocyst rates. In Chapter 4 the OCR of COCs or CEOs after exposure to environmental contaminants was assessed, however the investigation could have been improved by assessing the impact of cumulus cells on OCR measurement. In Chapter 5 the metabolic profile of cleavage stage embryos was assessed by investigating the GPL and AA turnover. In GPL quantification the cleavage stage embryos were divided into subgroups by the day of development (Day2 and Day3 embryos) and not by their developmental stage. This may have been important because in each day are included embryos at different developmental stage, for instance on day 2 are included 2-cell and 4-cell embryos and on day 3 are included 4-cell to 8-cell embryos. Similarly, the AA turnover analysis could have been improved if the cleavage stage embryos were divided by developmental stage. Finally, in Chapter 5 the OCR experiment was performed in a limited number of blastocysts

per experimental group and the impact of ACE in blastocyst OCR was not assessed due to the restriction of time and tissue availability.

6.4 Importance of work

This work contributes to the limited literature regarding the effects of environmental exposure to oviductal environment. Indeed, to the best of our knowledge only two studies have investigated the impact of environmental contaminants of interest, BPA and BPS in oviduct epithelial cells (Hung et al., 2018) and oviduct fluid (Téteau et al., 2022) Furthermore, this thesis is focused on modifications caused by the environmental exposure on the metabolic aspect of the oviduct cells and nutrients composition which has not been studied before.

The study of the fallopian tube/oviduct environment in vivo is technically challenging. By using the Transwell culture system which allows the culture of oviduct epithelial cells in air-liquid interface, which imitates the in vivo lumen environment, we had the opportunity to model pathophysiological conditions on the oviduct epithelium. Previous work published by our laboratory has demonstrate the effects of hormonal changes in the ivDOF composition (Simintiras et al., 2016) and the permeability of the epithelial cells to genistein (Simintiras & Sturmey, 2017). Continuing this pipeline, these findings, highlight the importance of studying the effects of pathophysiological conditions such as environmental exposure on the oviduct environment.

Taking into consideration the existing literature which highlight the importance of oviductal environment on supporting early reproductive events (Leese, 2003; Avilés et al., 2015; Li & Winuthayanon, 2017; González-Brusi et al., 2020), this work contributes to the puzzle of how modifications on maternal environment and health could impact the pre-implantation embryo development.

The panel of environmental contaminants that have been selected to be investigated during this work consist of a well-studied compound, BPA, and emerging environmental contaminants such as BPS, TCS and ACE. Even in the case of the well-studied BPA, the literature regarding the metabolic modifications on oocytes (Téteau et al., 2023) and preimplantation embryos (Choi et al., 2016) is limited. Furthermore, as has been mentioned in the previous section most

of the published studies investigate the impact of these environmental contaminants have been using superphysiological concentrations. In contrast with most of the previous *in vitro* studies, during this work the concentrations that have been used during the oocyte and embryo exposure are within the range of the concentration that have previously identified in human bodily fluids, a fact that contributes to the importance of this study.

6.5 Future work

Based on the observations that have been made during this research several questions have been raised and expanded the possibilities for future investigation. A common observation among the chapters was that there were cases where the low concentration of the environmental contaminants had greater impact than the high dose. It would be interesting to explore a wider range of concentrations in the future in order to determine the possible non-monotonic action of these contaminants.

Further to the observation that BOECs are able to create an intact monolayer even in the presence of environmental contaminants but meanwhile the composition of the ivDOF is modified, it would be interesting to visualise the morphology of the monolayer and the proportion of secretory to ciliated cells after exposure. A possible way to demonstrate that is by Transmission Electron Microscopy.

Additionally, the use of Transwell system could be expanded by creating more specific conditions that imitates specific oviductal regions. It is well known that the proportion of secretory to ciliated cells changes along the way of the oviduct (Ford et al., 2021). and the oviduct fluid composition depends on the stage of the oestrous cycle (Gabler et al., 2001). It would be very interesting if we could imitate the proportion of epithelial cells of the ampulla, the oviduct region where fertilisation take place in order to identify the metabolic changes caused by the environmental contaminants' exposure.

During the work on Chapter 3, by using the Transwell system we model exposure through the blood stream and explore the metabolic changes on ivDOF composition. However, a question that remained to be answered is the permeability of the epithelium monolayer to these contaminants. Permeability of the oviduct epithelium to genistein has been demonstrated by

our laboratory before (Simintiras & Sturmey, 2017) and also the fact that environmental contaminants have been found in bodily fluids (Adolfsson-Erici et al., 2002; Allmyr et al., 2006, 2008; Arbuckle *et al.*, 2015; Azzouz *et al.*, 2016; Calafat et al., 2008; Fu et al., 2024; Han et al., 2018; Jin et al., 2020; Lee et al., 2018; Liao et al., 2012; Liu et al., 2017; Tuzimski et al., 2019; Zhang & Lu, 2022) suggesting that they are circulated through the blood stream to tissues. However, examining the transport of environmental contaminants across the oviduct epithelium would inform us of the proportion that can be transferred which subsequently would provide useful information about the concentrations that embryos could directly been exposed during preimplantation development.

During Chapter 3 there was found that ivDOF composition was modified by the environmental contaminants and we were able to quantify these modifications. The obvious question that has been raised from this experiment is how these modifications in ivDOF could impact the embryo development. In the future research these modifications could applied on in vitro embryo culture to study the metabolic impact. Furthermore, in extension to this experiment bovine embryos could be cultured directly in modified ivDOF and the metabolic impact to be investigated. Finally, modifications of expression of imprinted genes have been previously correlated with altered culture conditions (Manipalviratn et al., 2009; Owen et al., 2009). Based on this fact, the modified environment could be correlated with modifications in imprinting in the embryo, which open another perspective for future work.

In Chapter 4 the impact of environmental contaminants exposure on COCs function and metabolism was investigated. However, based on the observational nature of the experiments the mechanism of action of these contaminants was not identified. Future work could focus on the molecular changes that caused the observed modifications on oocyte metabolism. Furthermore, during this chapter, we were focused on the maternal perspective, and how the female gametes were influenced by the exposure and the impact on their developmental competence however would be interesting to investigate the paternal perspective in the future.

In chapter 5 we show that embryos generated by chemically exposed COCs have modified metabolic profile and lower developmental probability. These observations raised the question of further investigating their developmental potential. It would be interesting to identify the implantation rates and pregnancy outcomes of these embryos by conducting embryo transfer experiments. Embryos could be generated in vitro using the desired experimental modifications

and then implanted into healthy cows. This approach would inform us whether the modified metabolic profile of the exposed embryos that have been identified in chapter 5 could have a long-lasting impact on the health of the offspring. Furthermore, would be interesting to expand the investigation regarding the metabolic profile of the exposed embryos by studying other important metabolic pathways, such as β -oxidation and fatty acid metabolism. Additionally, changes in amino acids metabolism indicated a possible involvement of the mTOR pathway which could be a subject for future investigation.

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Appendices

Chapter 2

Listed of chemicals used

Chemical substance	Supplier	Product Number
Adenosine 5'-triphosphate disodium salt hydrate 99% (ATP)	Sigma - Aldrich	A26209
Alanine	Sigma - Aldrich	A-7627
Amino Acid standard solution	Sigma - Aldrich	AAS18
Amphotericin B	Invitrogen	15290026
Antibiotic Antimycotic (Ab-Am)	Fisher Scientific	11580486
Antimycin A	Sigma - Aldrich	A8674
Aprotinin from bovine lung	Sigma - Aldrich	A6279
Arginine	Sigma - Aldrich	A-5131
Asparagine	Sigma - Aldrich	A-4284
Aspartate	Sigma - Aldrich	A-4534
BO-IVC - BOVINE EMBRYO CULTURE MEDIUM	IVF Bioscience	71005
BO-IVM - BOVINE OOCYTE MATURATION MEDIUM	IVF Bioscience	71001
BO-IVF - BOVINE FERTILISATION MEDIUM	IVF Bioscience	71004
Bovine Serum Albumin (BSA)	Sigma - Aldrich	A7030
BO-SEMENPREP- SEMEN PREPARATION MEDIUM	IVF Bioscience	71003
Calcium chloride dihydrate (CaCl₂.2H₂O)	Sigma - Aldrich	C7902
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma - Aldrich	C2920
Chloroform, for HPLC, stabilized with amylene	Fisher Scientific	C/4966/17
Cysteine	Sigma - Aldrich	C-1276
D-ABA	Acros Organics	142140050
D-Glucose	Sigma - Aldrich	G6152
Dithiothreitol	D0632	D0632
DMEM	Sigma - Aldrich	
EDTA	Sigma - Aldrich	ED4SS
Embryo-tested water	Fresenius Kabi	
Epidermal Growth Factor from murine submaxillary gland	Sigma - Aldrich	E4127
EPPS	Sigma - Aldrich	E9502
Essential Amino Acids	Sigma - Aldrich	M7145
Fetal Bovine Serum (FBS)	ThermoFischer Scientific	10270106
Fibroblast Growth Factor from bovine pituitary	Sigma - Aldrich	F3133
Fluorescein	Sigma - Aldrich	F6377
Glacial Acetic Acid	Sigma - Aldrich	CH1018
Glucose standard, 5.0mmol/L, 30ml	Analox instruments LTD	GMRD-010
Glutamax 100x	Thermo-Fisher Scientific	35050061
Glycine	Sigma - Aldrich	G6388
Hank's Balanced Salt Solution	Lonza	LZBE10-543F
Heparin sodium salt	Sigma - Aldrich	H3393
HEPES	Sigma - Aldrich	H3375
HEPES sodium salt	Sigma - Aldrich	H3784

Hexokinase/Glucose-6-phosphatase (H3K/G6P)	Roche	10127825001
High-Capacity cDNA Reverse Transcription Kit	Thermo-Fisher Scientific	4368814
Histidine	Sigma - Aldrich	H-8125
Hydrazine sulphate	Sigma - Aldrich	H3376
Isoleucine	Argos Organics	166170250
Isopropanol, for HPLC	Fisher Scientific	P/7507/17
Lactate standard 5.0mmol/L 25ml	Analox instruments LTD	GMRD-079
Leucine	Argos Organics	125121000
L-Glutamine	Sigma - Aldrich	G7513
L-Lactate Dehydrogenase (L-LDH), from rabbit muscle	Roche	10127884001
Lysine	Sigma - Aldrich	L-5626
M199	Sigma - Aldrich	M0650
Magnesium sulphate	Sigma - Aldrich	M2643
Magnesium sulphate heptahydrate (MgSO₄.7H₂O)	Sigma - Aldrich	M7774
Menopur/Merional (75IU)	Ferring Pharmaceuticals Ltd	
Methanol for HPLC	Fisher Scientific	10675112
Methionine	Sigma - Aldrich	M-9625
Mineral oil	Sigma - Aldrich	M8410
Monobasic potassium phosphate (KH₂PO₄)	Sigma - Aldrich	P5655
NAD, Grade II, free acid	Roche	10127981001
NADH Disodium salt	Roche	10128023001
NADP	Roche	128 040
Newborn Calf Serum (NCS)	Sigma - Aldrich	N4762
Nutrient Mix F12	Sigma - Aldrich	N8641
OIL - OVERLAY OIL	IVF Bioscience	51003
Oligomycin	Sigma - Aldrich	O4876
O-phthaldialdehyde	Sigma - Aldrich	P0532
OPU - OVUM PICK-UP MEDIUM	IVF Bioscience	51001
Penicillin G	Sigma - Aldrich	P4697
Penicillin/Streptomycin	Sigma - Aldrich	P0781
Percoll	Sigma - Aldrich	P1644
Phenylalanine	Argos Organics	130310250
Phtaldialdehyde Reagent (OPA)	Sigma - Aldrich	P0532
Poly-L-lysine	Sigma - Aldrich	P1524
Potassium chloride (KCl)	Sigma - Aldrich	P5405
Proline	Sigma - Aldrich	P-0380
Rotenone	Sigma - Aldrich	R8875
Serine	Sigma - Aldrich	S-4500
Sodium bicarbonate (NaHCO₃)	Sigma - Aldrich	S5761
Sodium Chloride (NaCl)	Sigma - Aldrich	S5886
Sodium DL-lactate solution	Sigma - Aldrich	L1375
Sodium hydroxide	Fisher Scientific	S/4920/60
Sodium pyruvate	Sigma - Aldrich	P4562
Streptomycin	Sigma - Aldrich	S1277
SYBR Green PCR Master Mix	Fischer Scientific	10187094
Tetrahydrofuran (THF)	Sigma - Aldrich	34865
Threonine	Sigma - Aldrich	T-8625
Trizol	Thermo-Fisher Scientific	15596026

Trypan Blue	Sigma - Aldrich	T8154
Trypsin	Sigma - Aldrich	59427C
Tryptophan	Sigma - Aldrich	T-0254
Tyrosine	Sigma - Aldrich	T-1020
Valine	Sigma - Aldrich	V-0500
WASH - OOCYTE AND EMBRYO WASH MEDIUM	IVF Bioscience	51002
b-mecraptoethanol	Sigma - Aldrich	M6250
(L)-Glutamate	Sigma - Aldrich	G-1251
(L)-Glutamine	Sigma - Aldrich	G8540

List of consumables

Consumables	Supplier	Product Number
96-well PCR plates, Skirted, Low Profile	StarLabs	E1403-5200
MicroAmp Fast Opriical 96-well reaction plate	Applied Biosciences	4346907
THINCERT CELL CULTURE INSERT FOR 12 WELL PLATES, TC, STERILE, TRANSLUCENT MEMBRANE (PET), PORE DIAMETER: 0,4 µm	Greiner Bio-One	665640
Falcon 60 mm x 15 mm Petri dish 20/Pack	Corning	351007
Nunc 4-well dishes for IVF, Nunclon Delta Surface	ThermoScientific	176740
Tissue Culture Flask, 25cm Adherent Surface, Non-Pyrogenic, Non-Cytotoxic, Sterile	Sarstedt	83.3910
Tissue Culture Flask, 75cm Adherent Surface, Non-Pyrogenic, Non-Cytotoxic, Sterile	Sarstedt	83.3911
Tissue Culture Plate, 6 Flat Well, Adherent Surface, Non-Pyrogenic, Non-Cytotoxic, Individually Wrapped Sterile, Red	Sarstedt	83.3920
Tissue Culture Plate, 12 Flat Well, Adherent Surface, Non-Pyrogenic, Non-Cytotoxic, Individually Wrapped Sterile, Red	Sarstedt	83.3921
Tube Conical Skirted Base 50mL, 115x28mm Polypropylene Screen Printed Sterile	Sarstedt	62.559.001
Tube Conical Base, 15mL, 120x17mm Polypropylene Screen Printed Sterile	Sarstedt	62.554.502
Serological Pipette 5ml Individually Wrapped, Sterile	Sarstedt	86.1253.001
Serological Pipette 10ml Individually Wrapped, Sterile	Sarstedt	86.1254.001
Serological Pipette 25ml Individually Wrapped, Sterile	Sarstedt	86.1685.001
Micro Tube Conical Base Push Cap 0.5ml standard neutral	Sarstedt	72.699
Micro Tube Conical Base Push Cap 1.5ml standard neutral	Sarstedt	72.690.001
Tip Standard 10µl Bagged	Sarstedt	70.1130
Tip Standard 200µl Bagged	Sarstedt	70.760.002
Tip Standard 1000µl Bagged	Sarstedt	70.762
10µl Filter tip, Sterile, Biosphere Plus, DNA-/RNase-/ATP Free	Sarstedt	70.1130.210
20µl Filter tip, Sterile, Biosphere Plus, DNA-/RNase-/ATP Free	Sarstedt	70.760.213
200µl Filter tip, Sterile, Biosphere Plus, DNA-/RNase-/ATP Free	Sarstedt	70.760.211

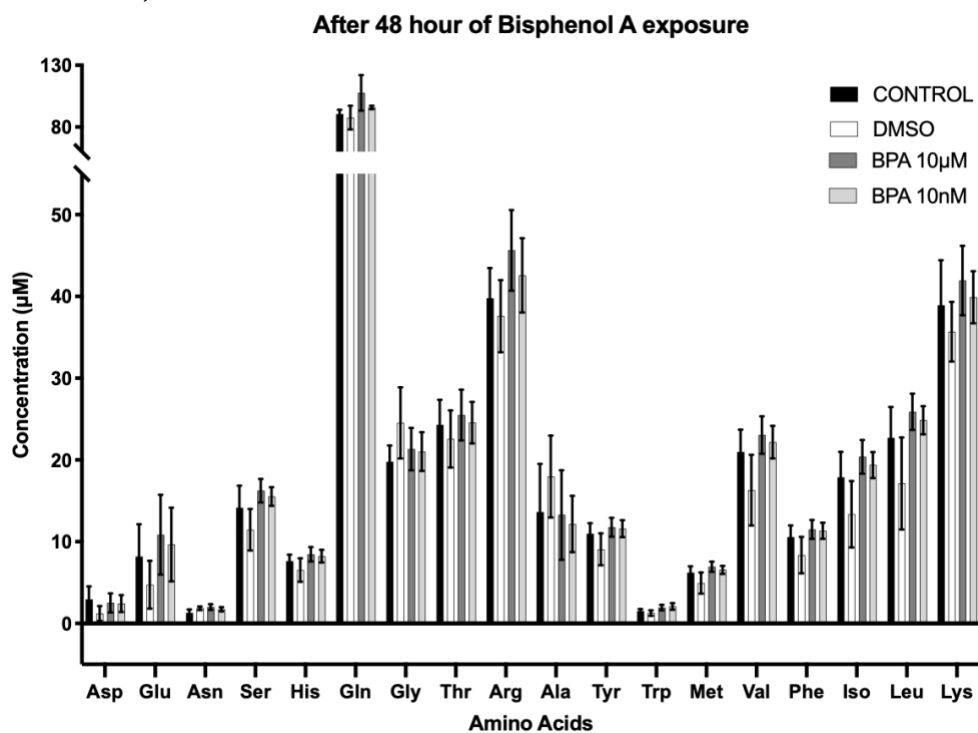
1000µl Filter tip, Sterile, Biosphere Plus, DNA-/RNase-/ATP Free	Sarstedt	70.3050.255
Transfer Pipette 3.5ml Bulb Volume Graduated to 3ml (0.5ml Graduations) Length 155mm Drop Size 35-55µl Individually Wrapped Sterile	Sarstedt	86.1171.001
0.22µm Syringe Filter, PES (Sterile), 33mm, Blue	StarLab	E4780-1226

List of equipment used

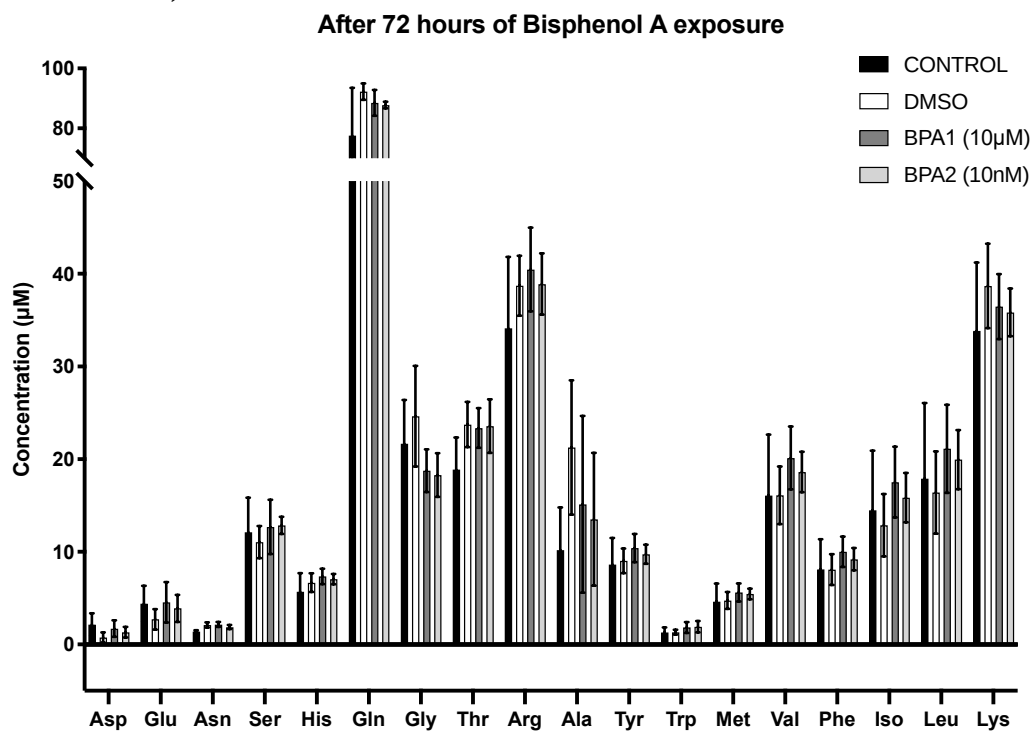
Equipment	Supplier	Use
Nanodrop 2000c	Thermo Sceintific	RNA purity check
EVOM voltohmmetre	World Precision Instruments	TEER measurement
Applied Biosystems 2720 Thermal Cycler	Applied Biosystems	cDNA synthesis
StepOne Plus Real-Time PCR system	Applied Biosciences	qRT-PCR
BMG plate reader FLUOstar Omega Microplate Reader	BMG LABTECH	Glucose, Lactate, Pyruvate assay, ELISA
Agilent 1100/1200	Agilent	High Performance Liquid Chromatography
Class II Cabinet		Cell culture and media preparation
Zeiss Apotome	Zeiss	Oocyte and zygotes staining

Chapter 3

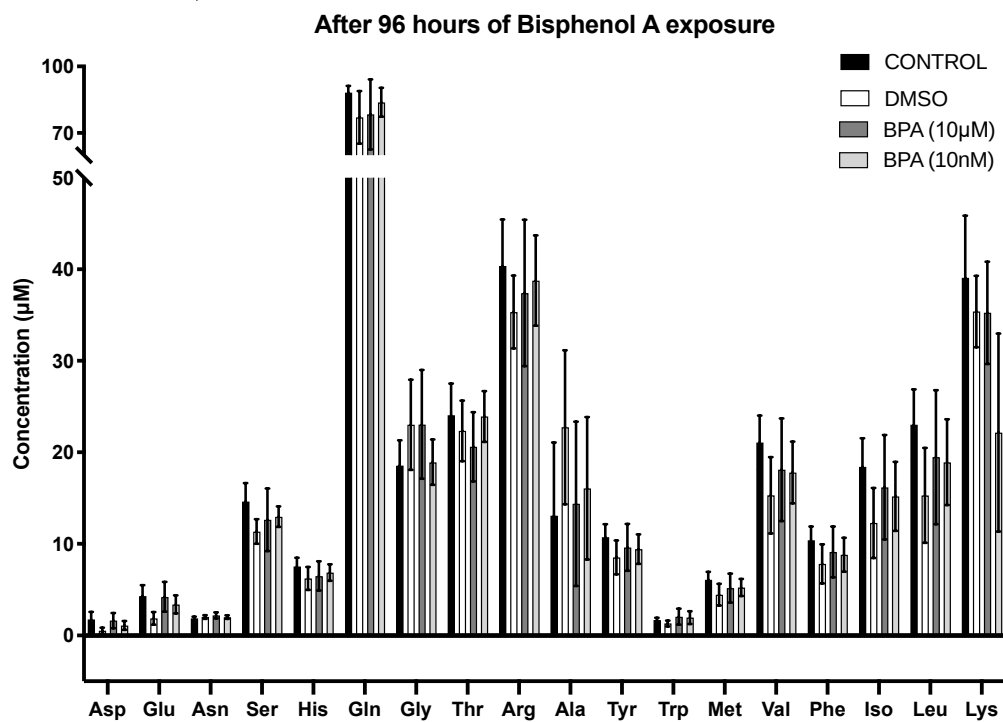
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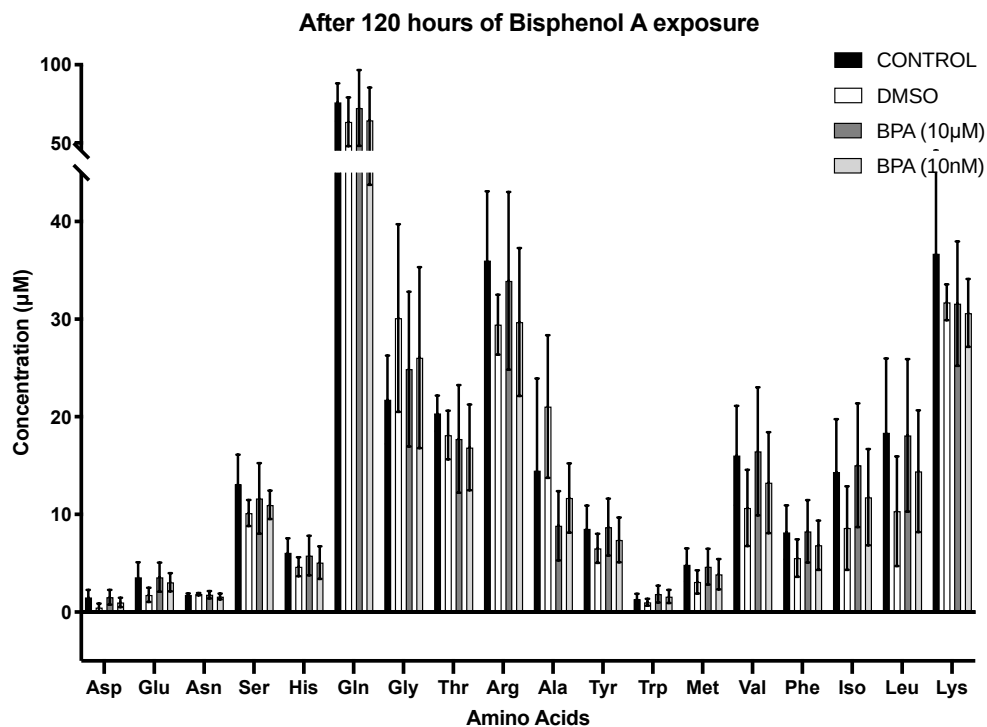
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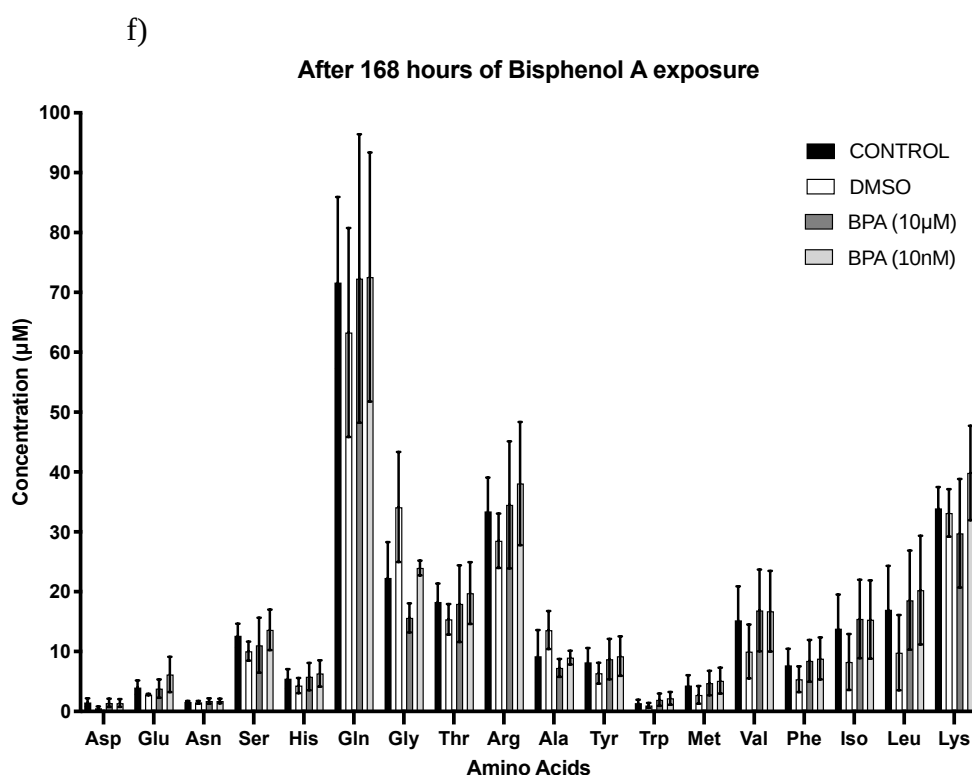
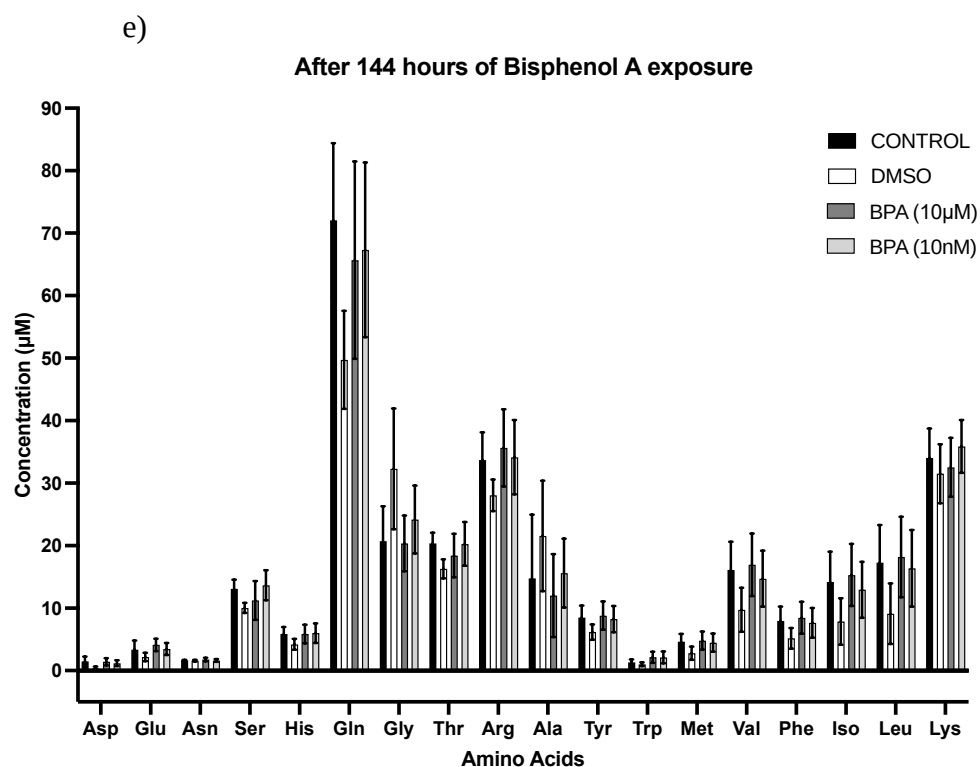
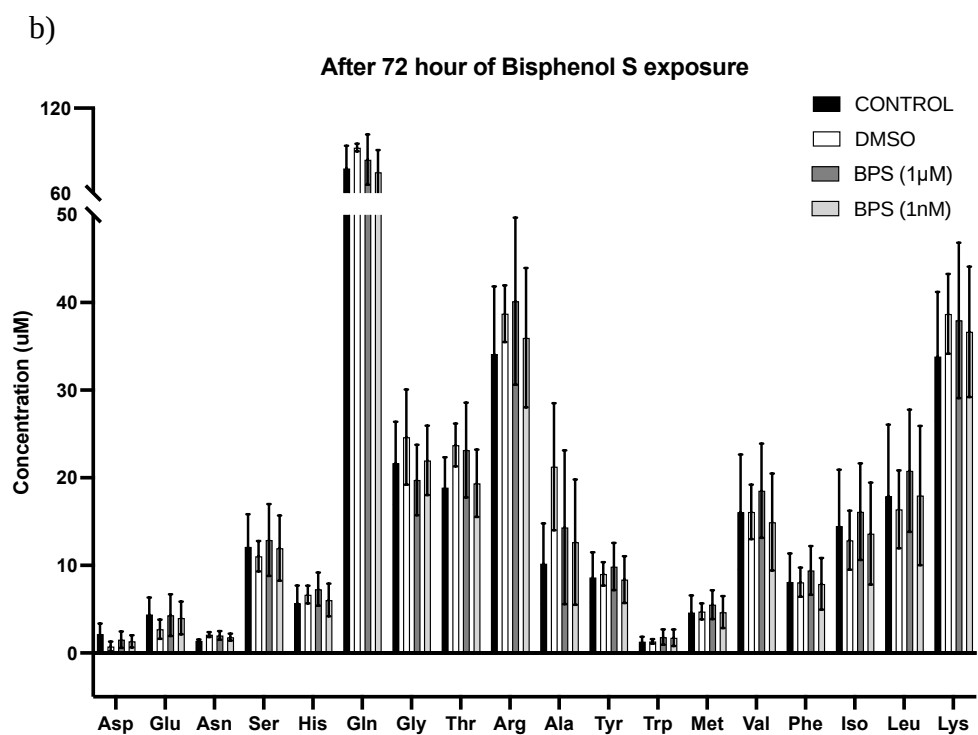
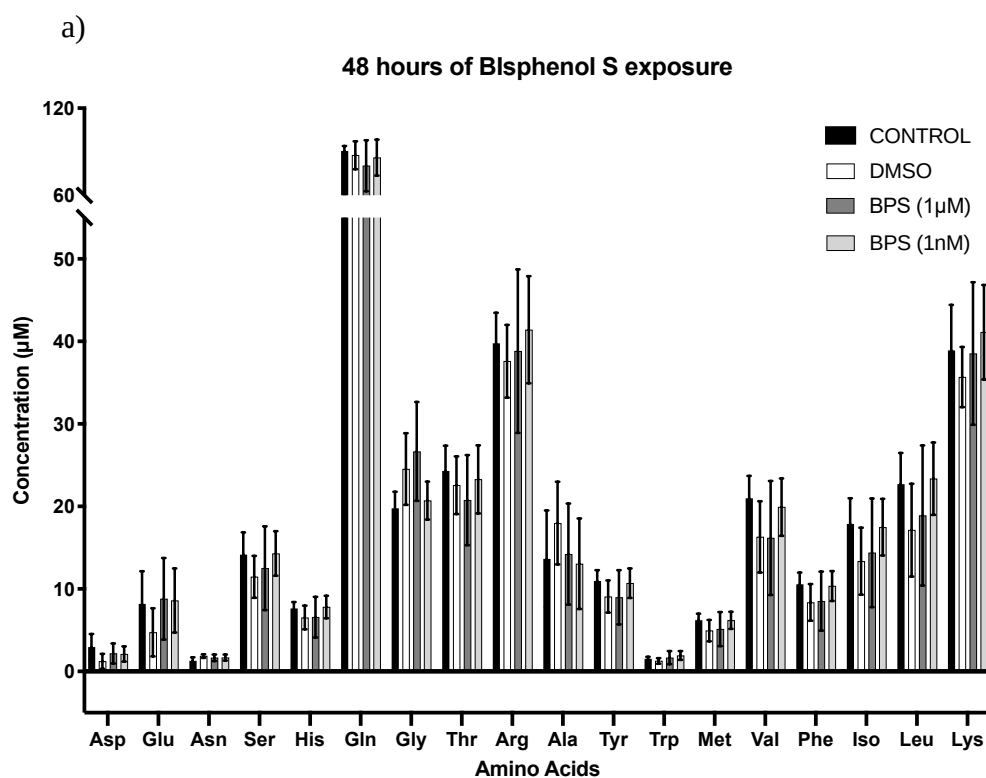
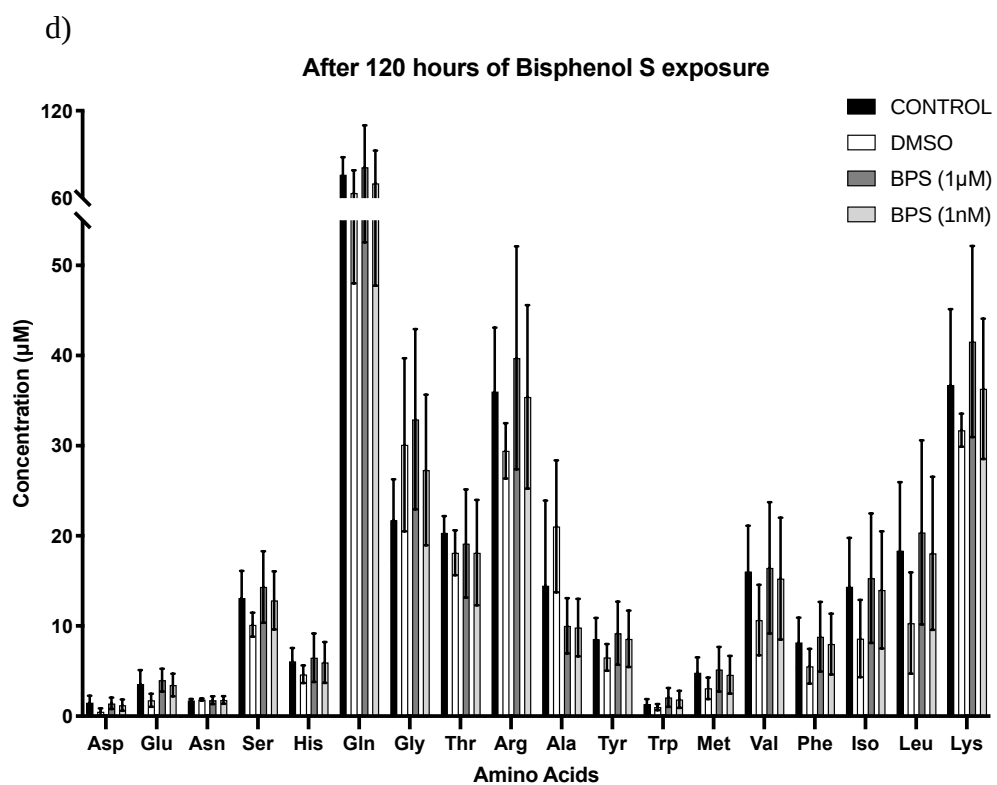
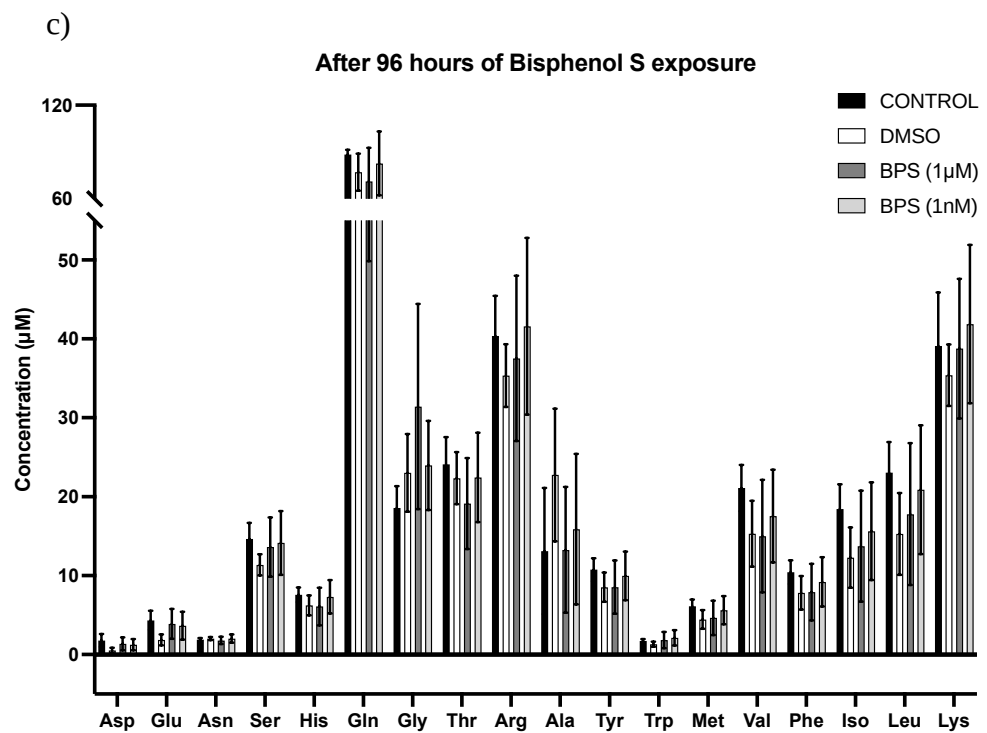


Figure 3.21_Appendix: Bisphenol A impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.





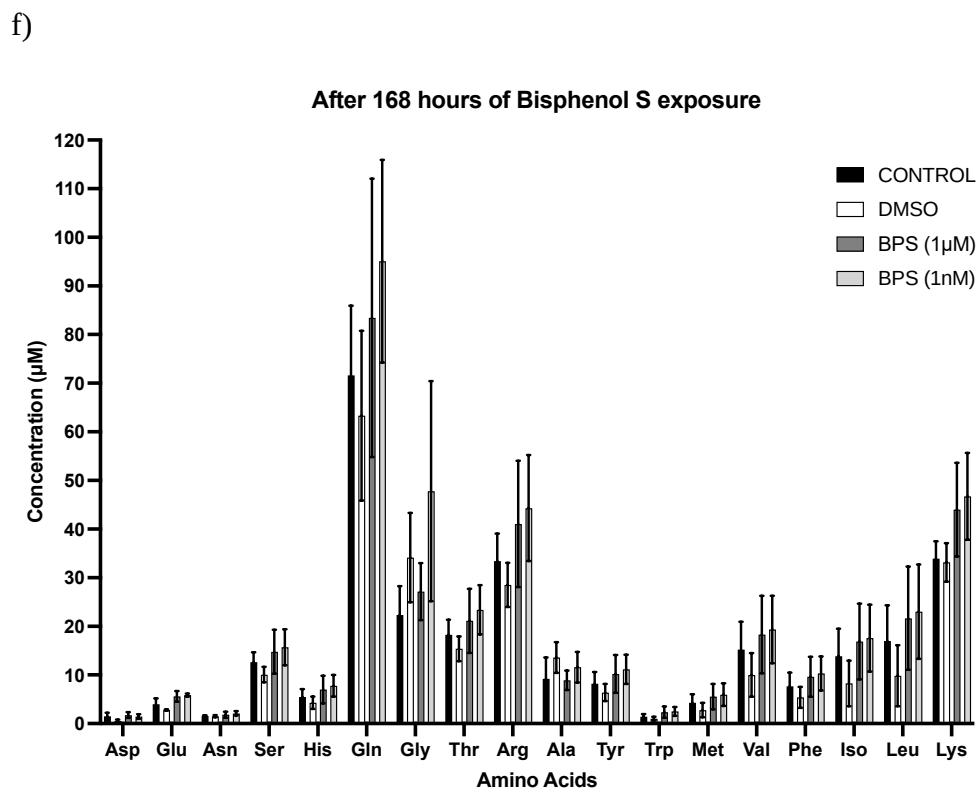
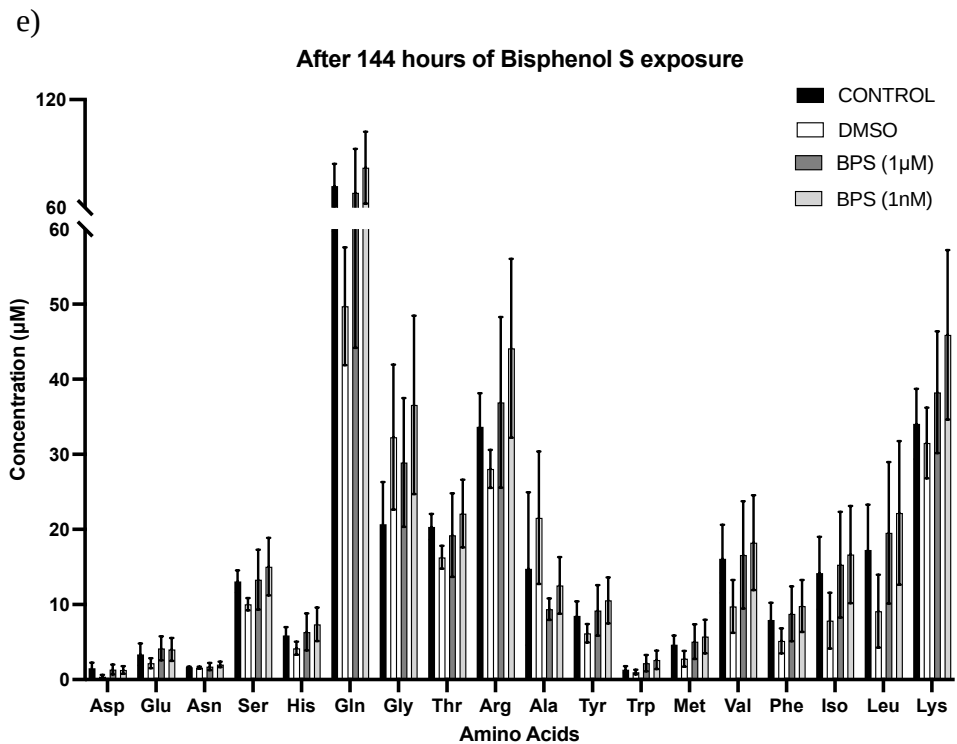
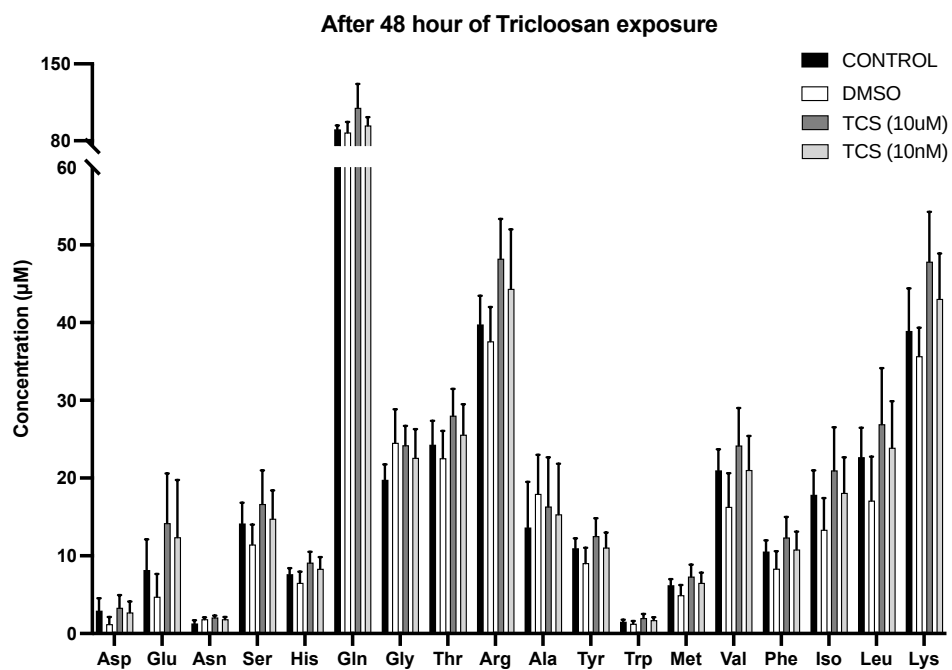
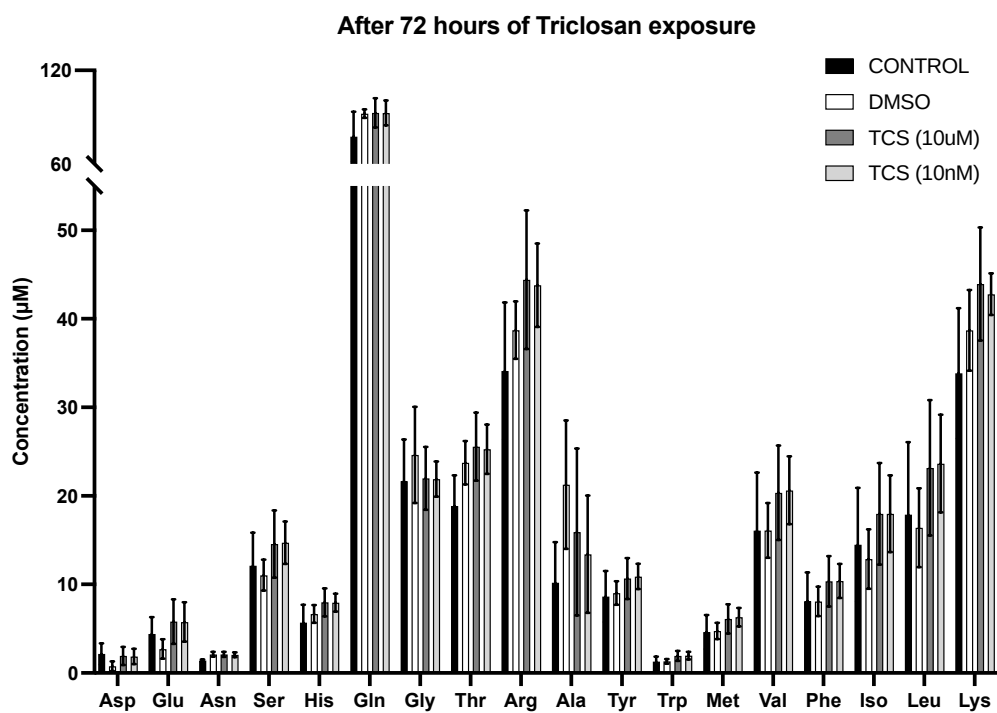


Figure 3.22_Appendix: Bisphenol S impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase. Each graph is result of 3 biological replicates ($n=3$). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Trends are illustrated by a dashed line, with p-value presented.

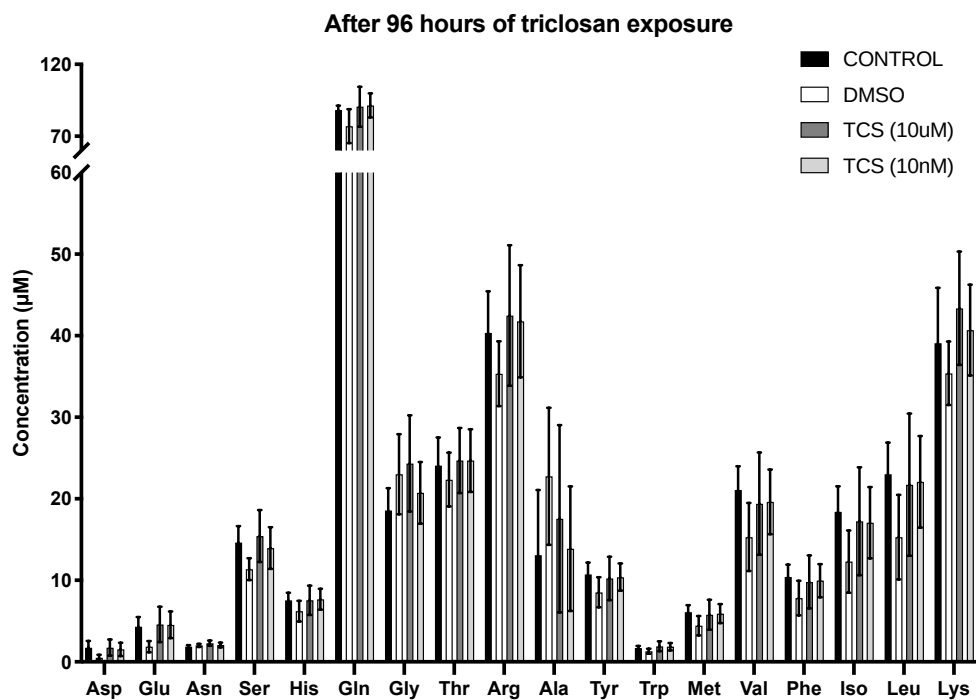
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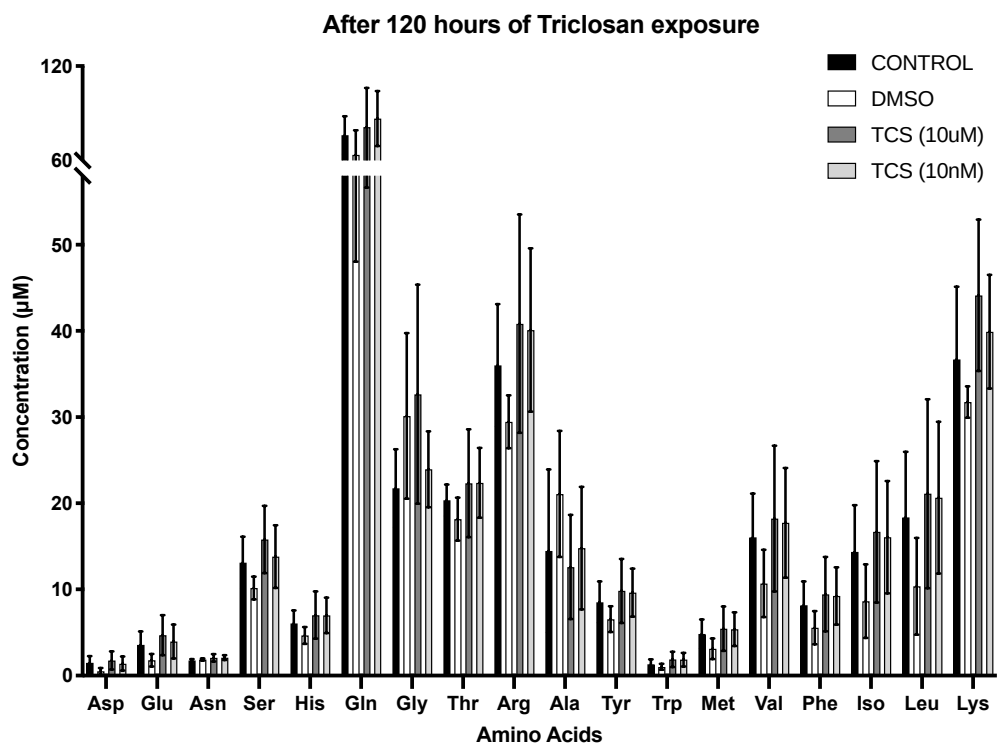
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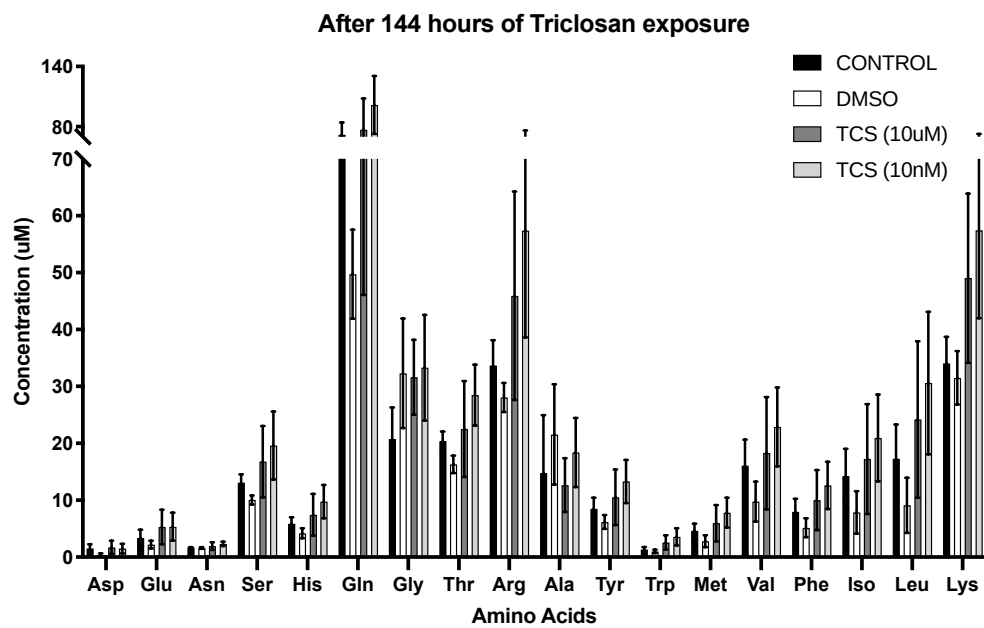
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d)



e)



e)

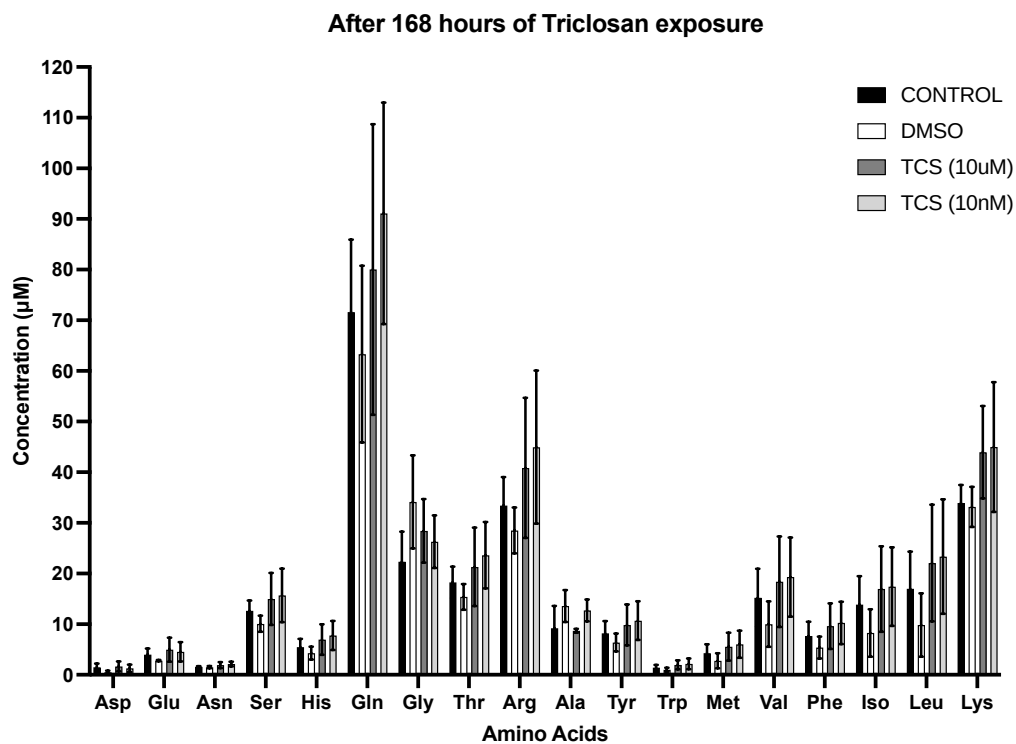
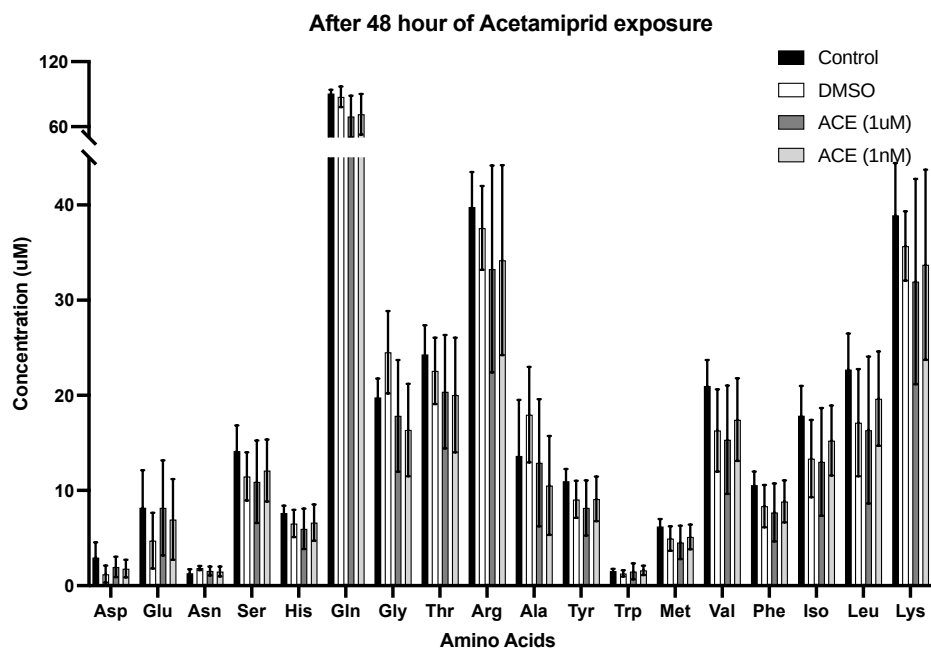
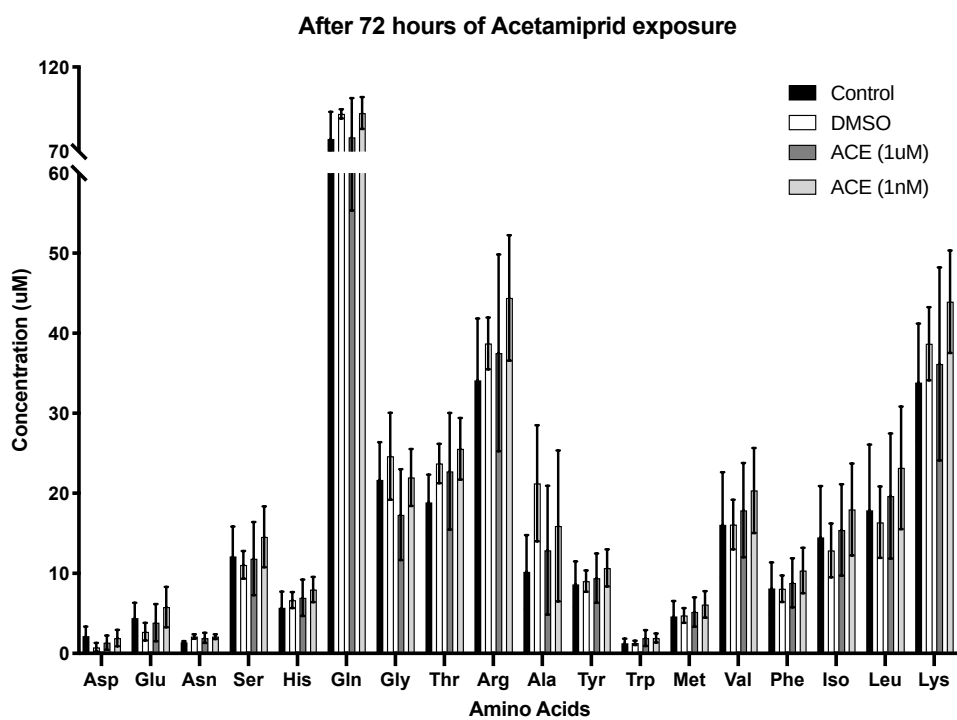


Figure 3.23_Appendix: Triclosan impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

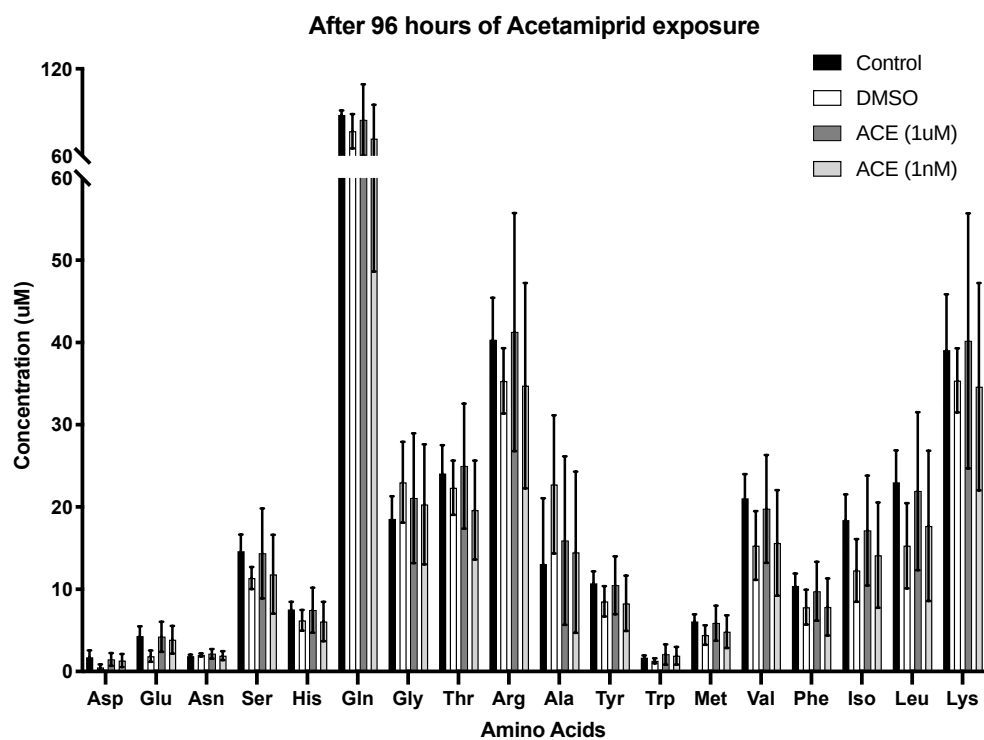
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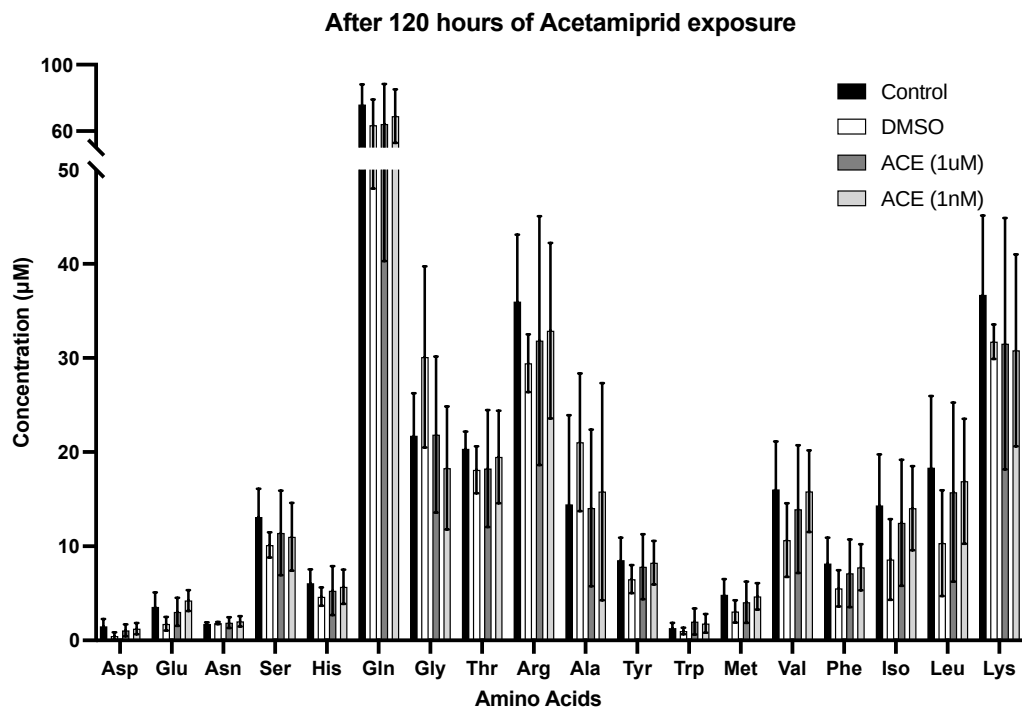
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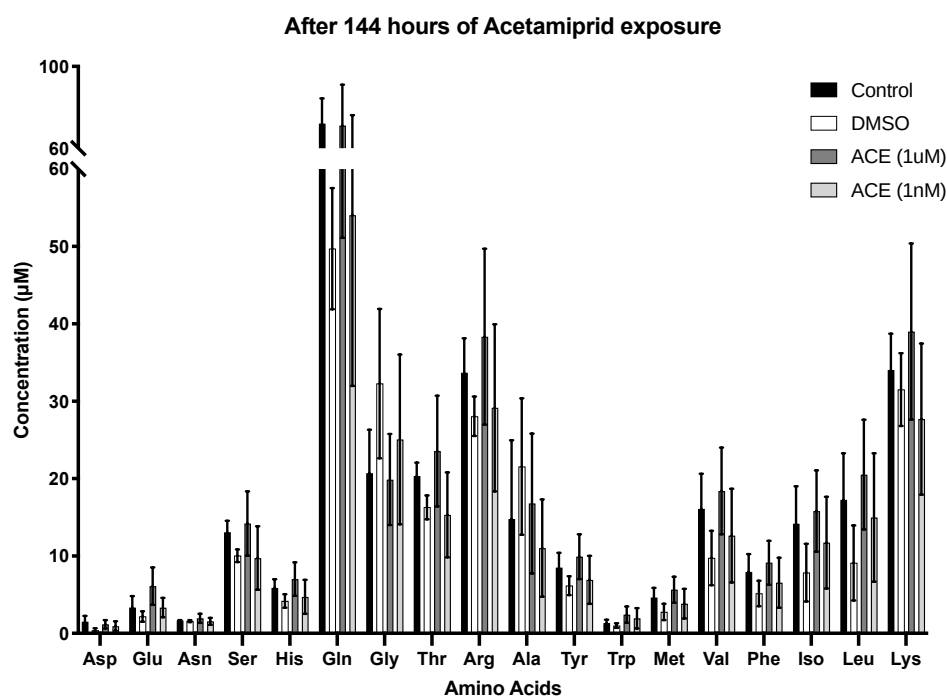
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f)

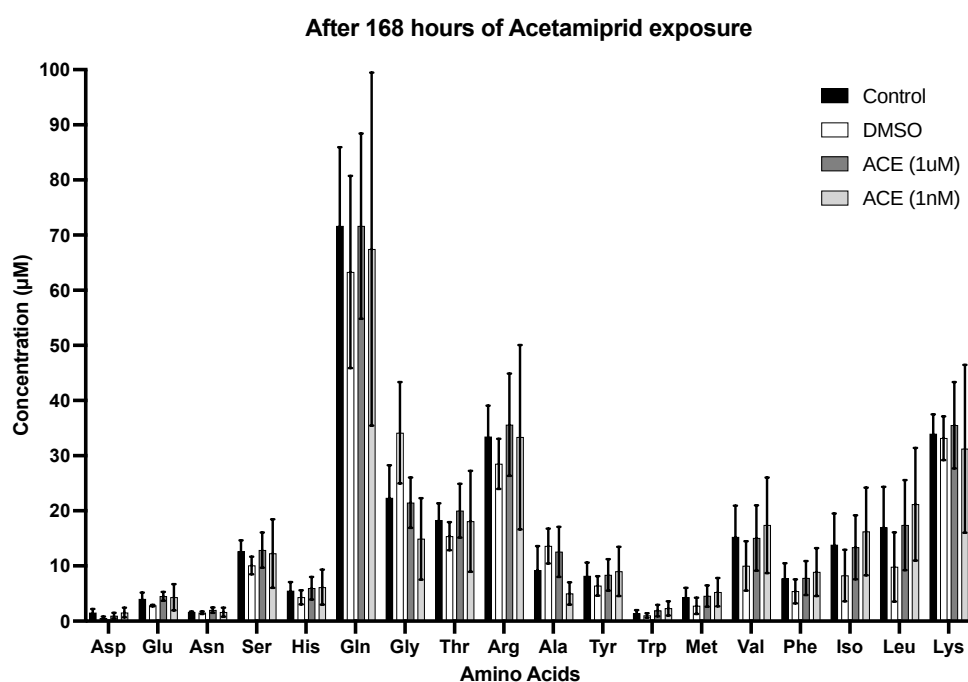


Figure 3.24_Appendix: Triclosan impact on amino acids concentration in the in vitro derived oviduct fluid (ivDOF) after a) 48-hours, b) 72-hours, c) 96-hours, d) 120-hours, e) 144-hours f) 168-hours of BOECs cultured in air-liquid phase. Each graph is result of 3 biological replicates (n=3). Data are expressed as Mean $\pm$ SEM. Differences were determined using Two-Way ANOVA with Tukey's post-hoc test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

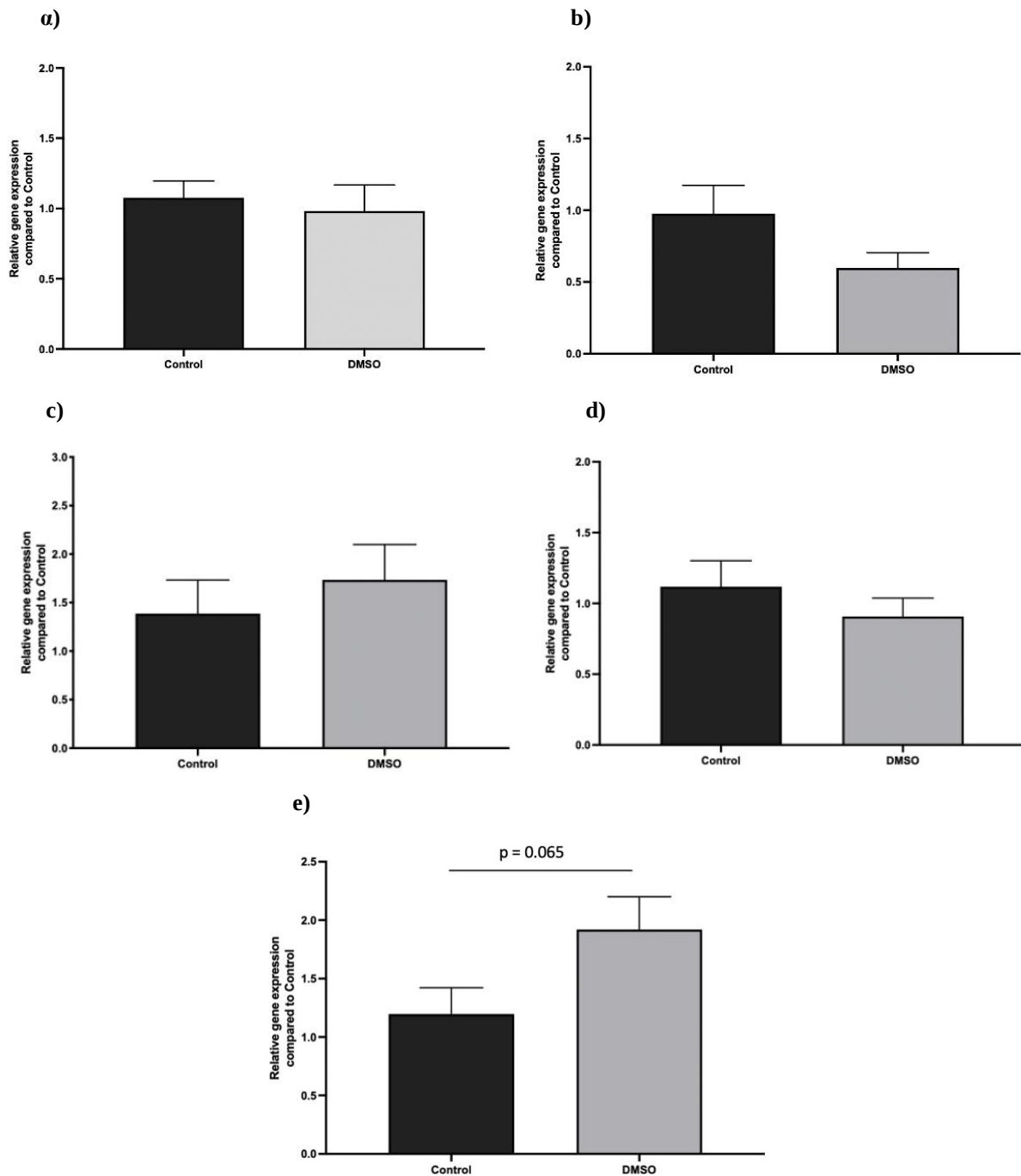


Figure 3.25: Relative gene expression of a) SLC1A4, b) SLC1A5, c) SLC7A1, d) SLC7A2 and e) SLC7A4 amino acid transporters in BOEC exposed to DMSO for 24 hours in air-liquid phase. These graphs represent data of five biological replicates (n=5) for each experimental group. Differences were determined using unpaired t-test. No significant statistical differences were identified among the groups. Only a strong trend of increase on SLC7A4 expression when the cells were exposed to DMSO compared to untreated group.

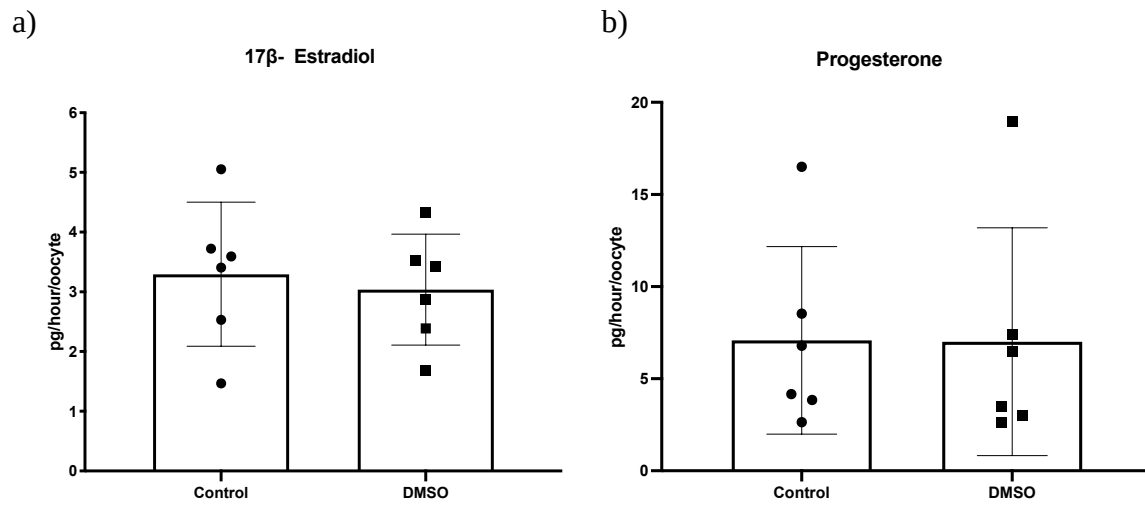


Figure 4.13_Appendix: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to DMSO during in vitro maturation (n=6. each dot represents an individual replicate). Differences were assessed using Welch's t-test for 17 β -estradiol and Mann-Whitney test for progesterone data * p<0.05. Data are presented in mean $\pm$ SD

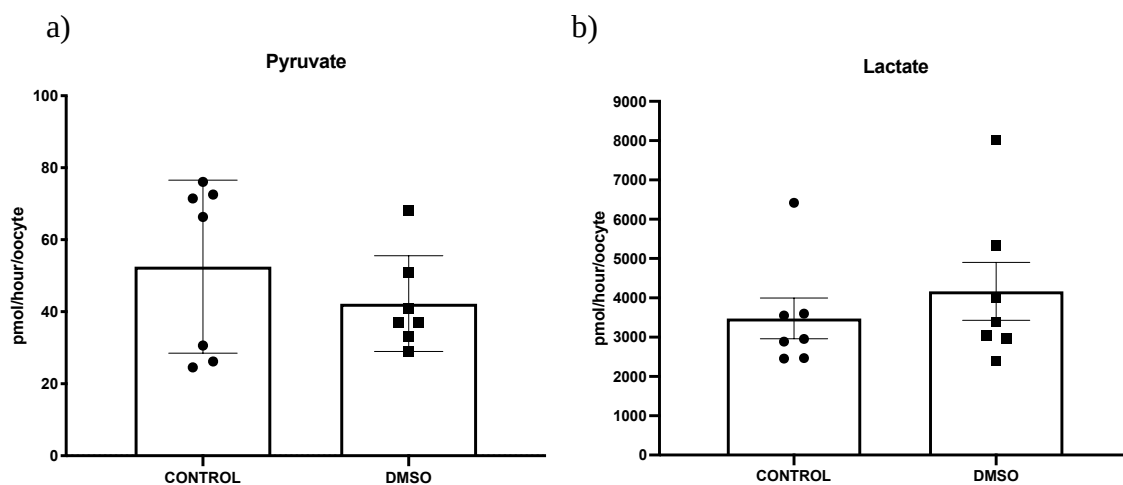


Figure 4.14_Appendix: Release of a) 17 β -estradiol and b) progesterone from bovine COCs exposed to DMSO during in vitro maturation (n=7. each dot represents an individual replicate). Differences were assessed using Mann-Whitney test for both data sets. * p<0.05. Data are presented in mean $\pm$ SD

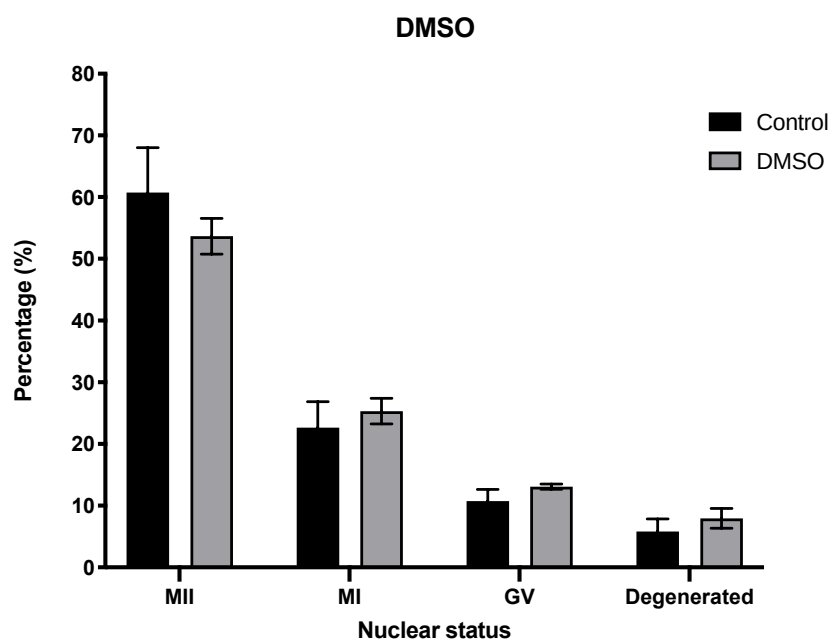
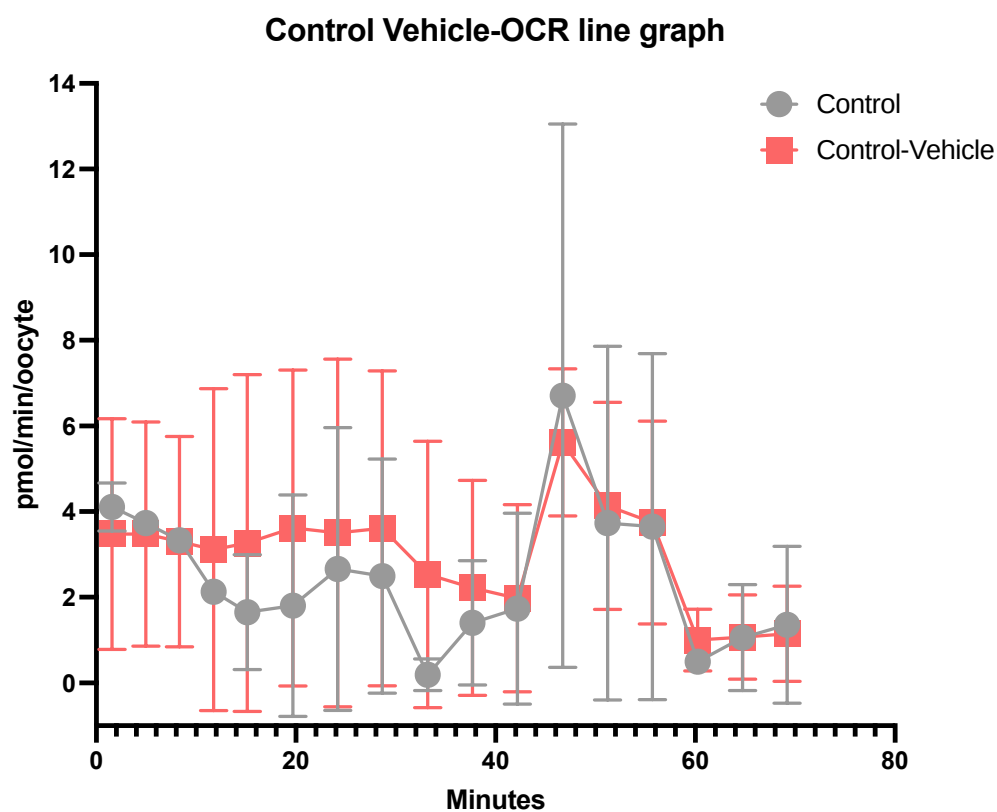
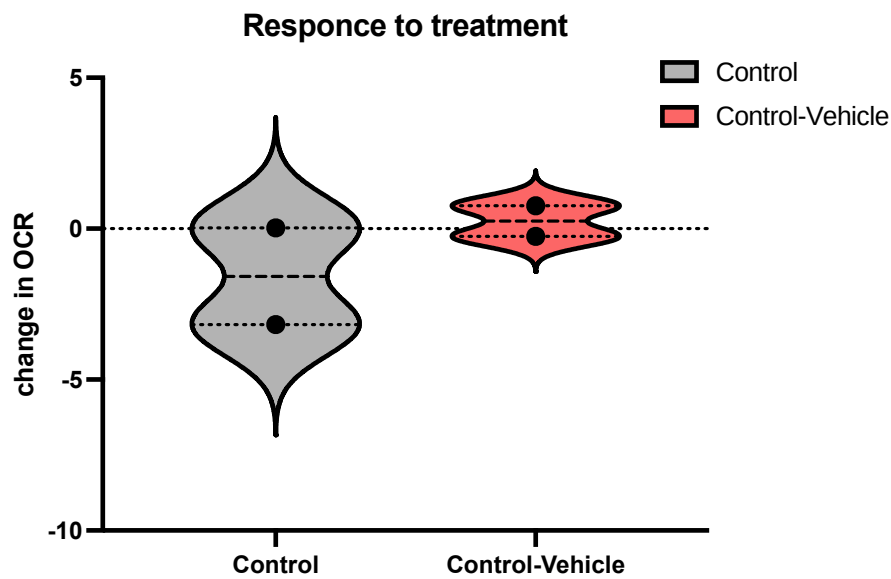


Figure 4.15_Appendix: Nuclear status of bovine COCs exposed DMSO during IVM. MII represent oocytes in metaphase II, MI represent oocytes metaphase I, GV represent oocytes in germinal vesicles stage and **degenerated** group includes oocytes with degenerated nucleus. As the data are presented in percentages, initially they underwent arcsine transformation and subsequently, Two-way ANOVA followed by Tuckey test was performed, * $p < 0.05$, data are presented in mean $\pm$ SD. Three biological replicates were used for the investigation of each environmental compounds ($n=3$).

a)



b)



c)

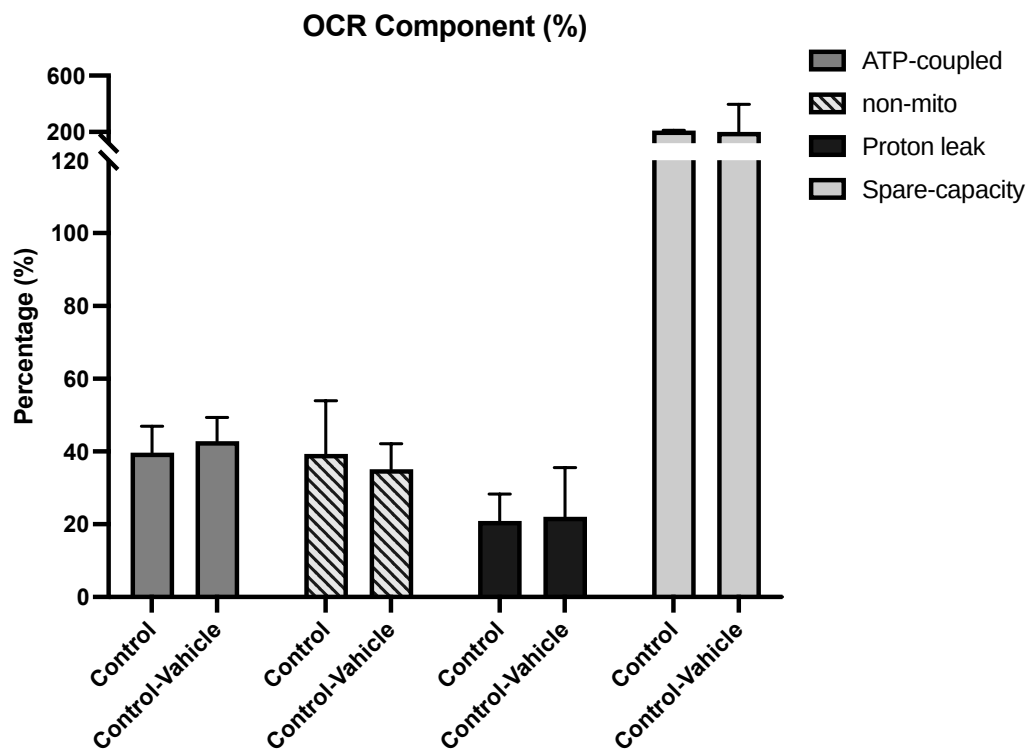


Figure 4.16_Appendix: The impact on OCR immature CEOs acutely exposed to Control-Vehicle immediately after isolation from the ovary. a) OCR in COCs in response to BPA, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the BPA treatment, c) mitochondrial parameters as a percentage of basal OCR after BPA injection. Differences were detected using b) unpaired two-tailed t-test, c) two-way ANOVA followed by Tukey test, * $p < 0.05$ Data are presented in mean $\pm$ SD. These graphs include data of 2 biological replicates (OCR, $n=2$).

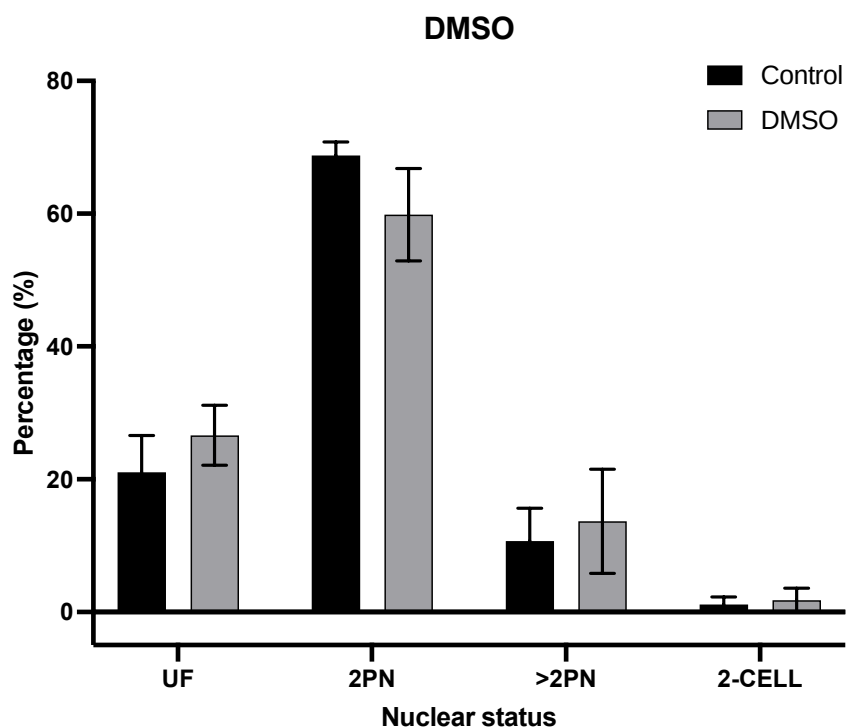


Figure 4.17_Appendix: Pro-nucleus formation of zygotes exposed to DMSO during in-vitro fertilisation. **Uf group, represent** the unfertilised oocytes, **2PN group** represent zygotes with 2 pronucleus, **>2PN group** represent the zygotes with more than 2 PN, and finally, **2-cell group** represent the zygotes that have complete the first cell division. As the data are presented in percentages, initially they underwent arcsine transformation and subsequently, Two-way ANOVA followed by Tuckey test was performed. The results are presented in mean and $\pm$ SD. Three biological replicates were used for the investigation of each environmental compounds (n=3).

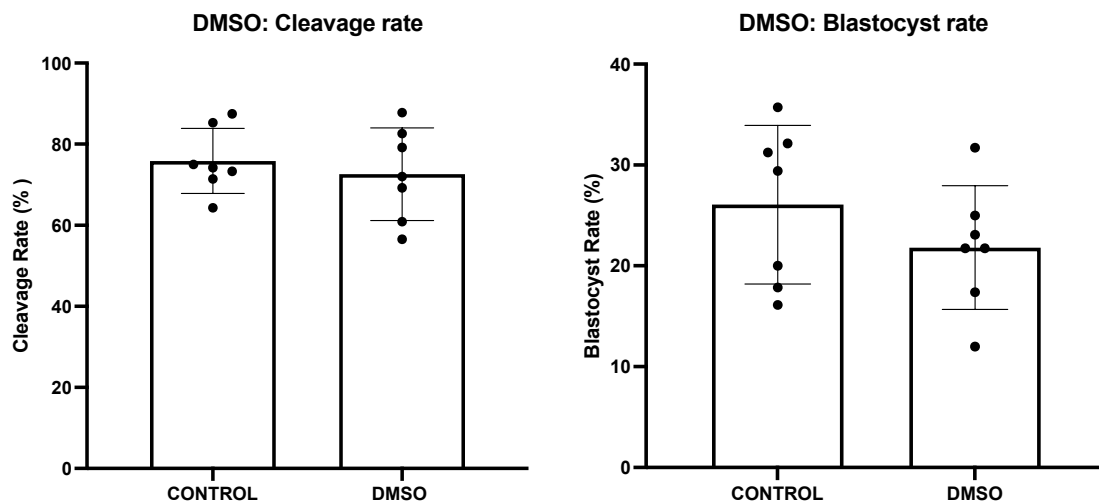


Figure 5.22_Appendix: a) Cleavage and b) Blastocyst rate of embryos derived from DMSO exposed COCs during in vitro maturation. Each graph represent data of at 7 biological replicates with each dot presenting an individual biological replicate (n=7). Data are expressed as Mean $\pm$ SD. Differences were determined using Welch's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

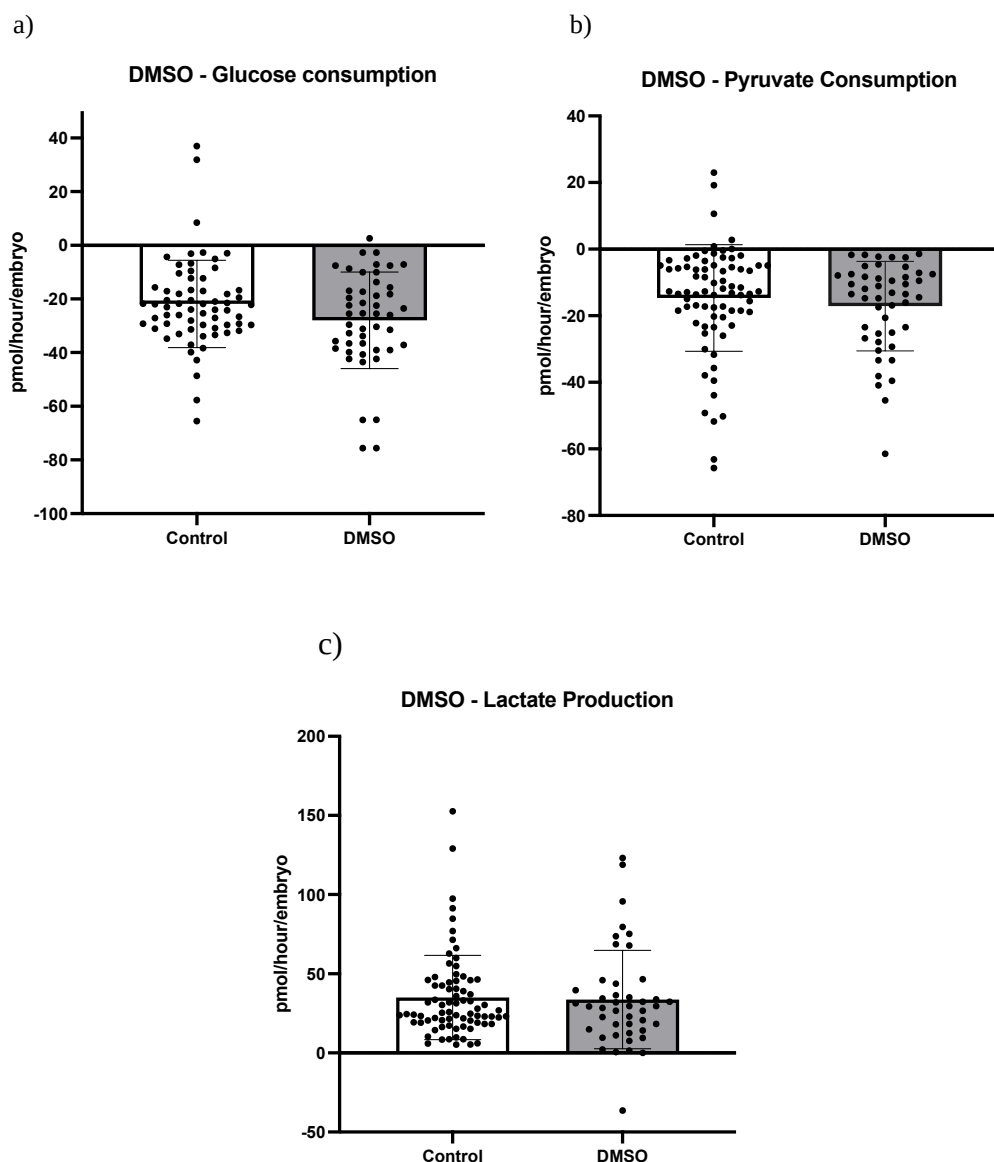


Figure 5.23_Appendix: a) Glucose, b) Pyruvate consumption, and c) Lactate production of blastocyst stage embryos derived from DMSO-exposed COCs during in vitro maturation. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using Mann-Whitney t-test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

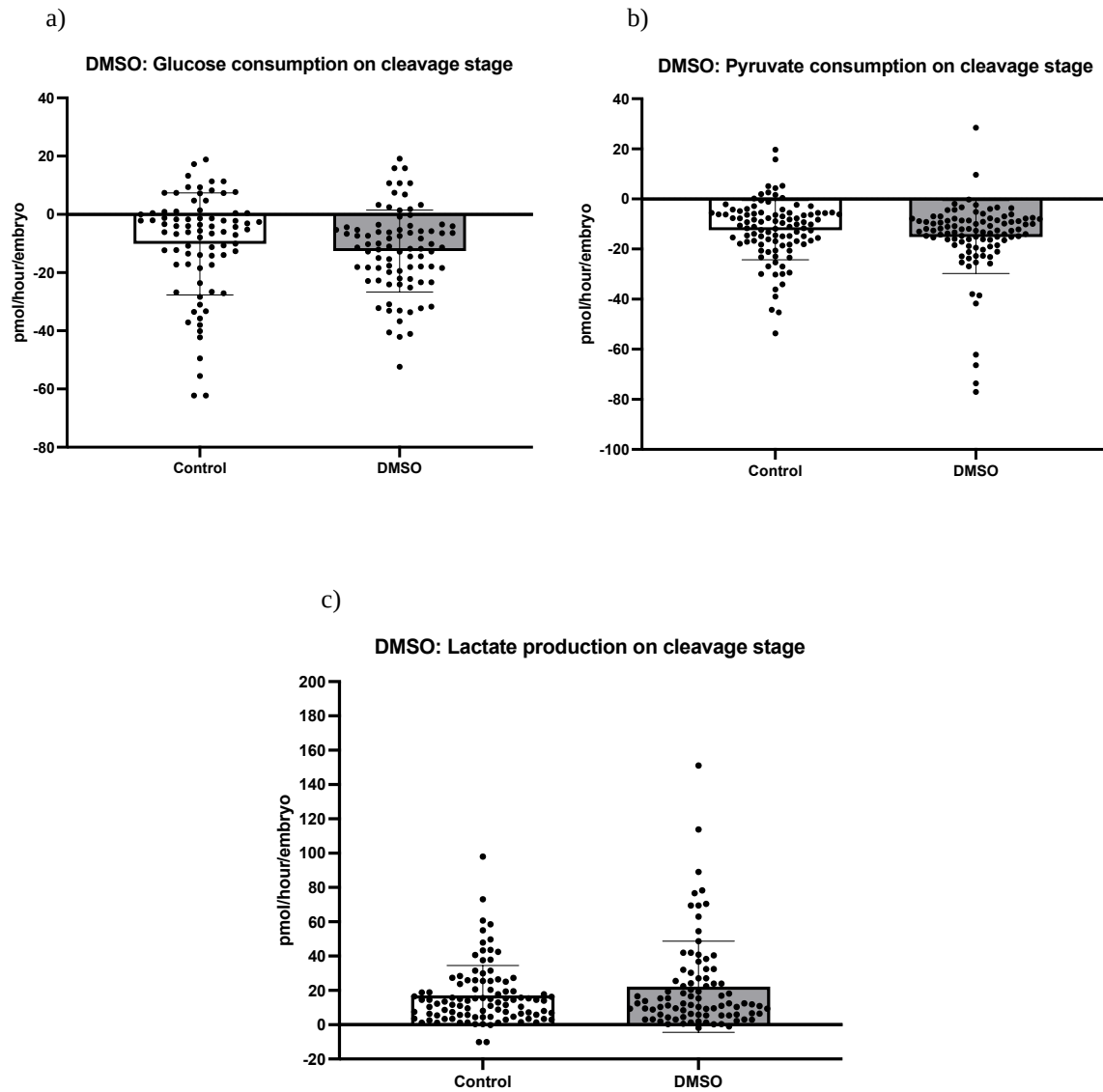


Figure 5.24_Appendix: a) Glucose, b) Pyruvate consumption, and c) Lactate production of embryos exposed to DMSO during cleavage stage. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using Mann-Whitney t-test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

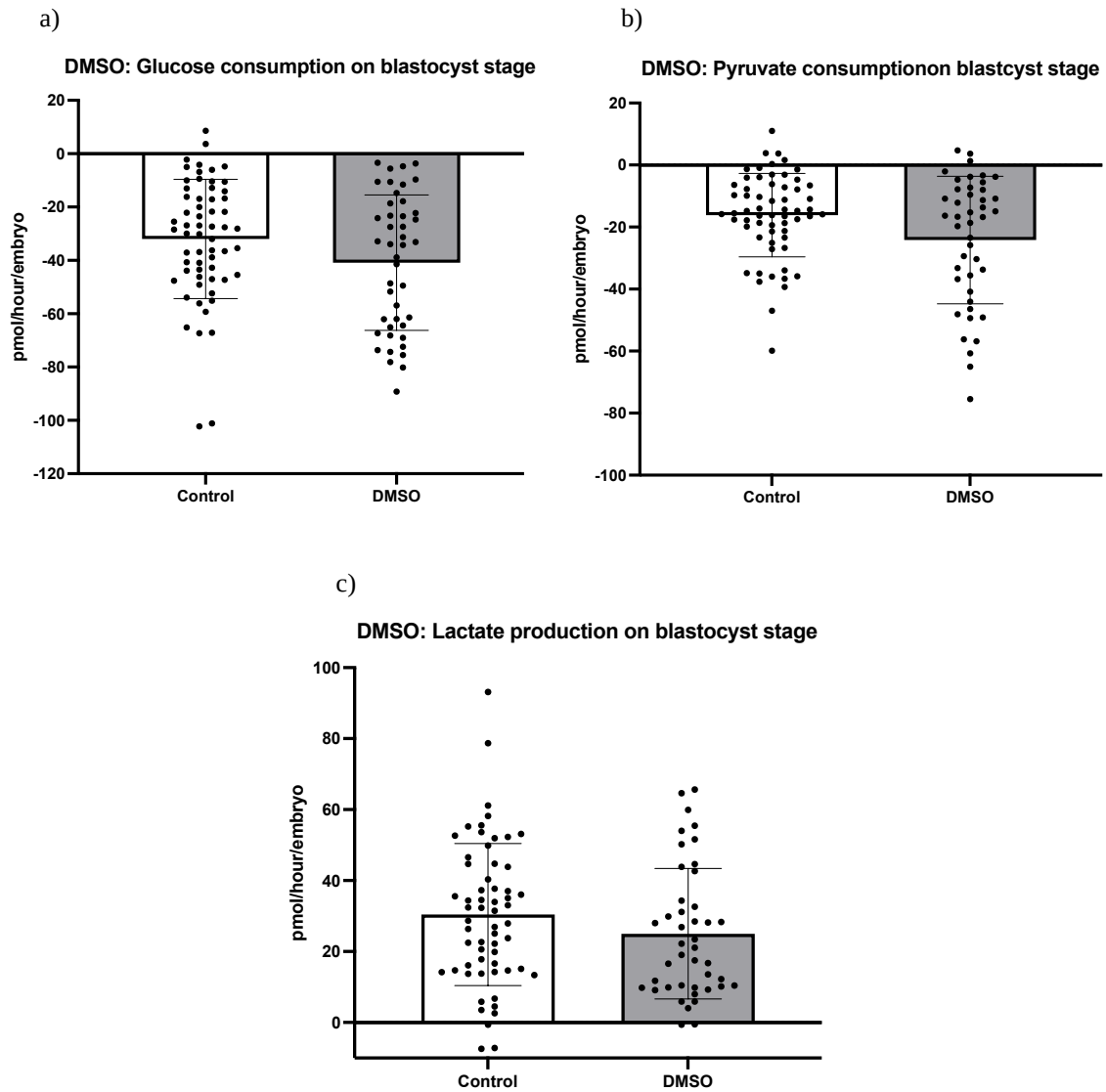
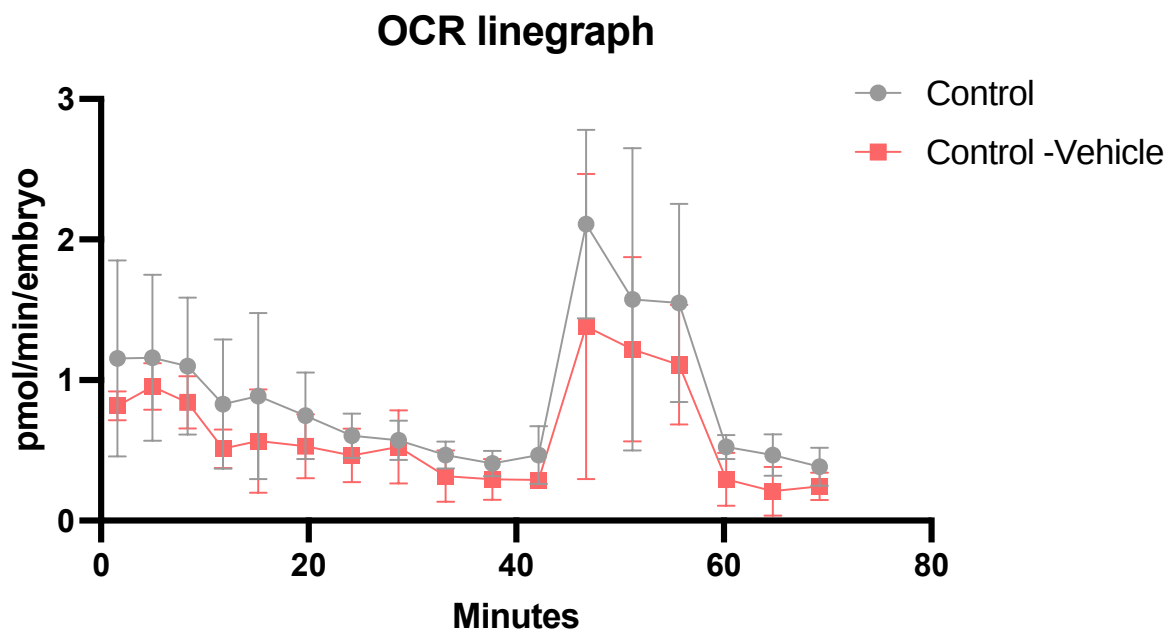
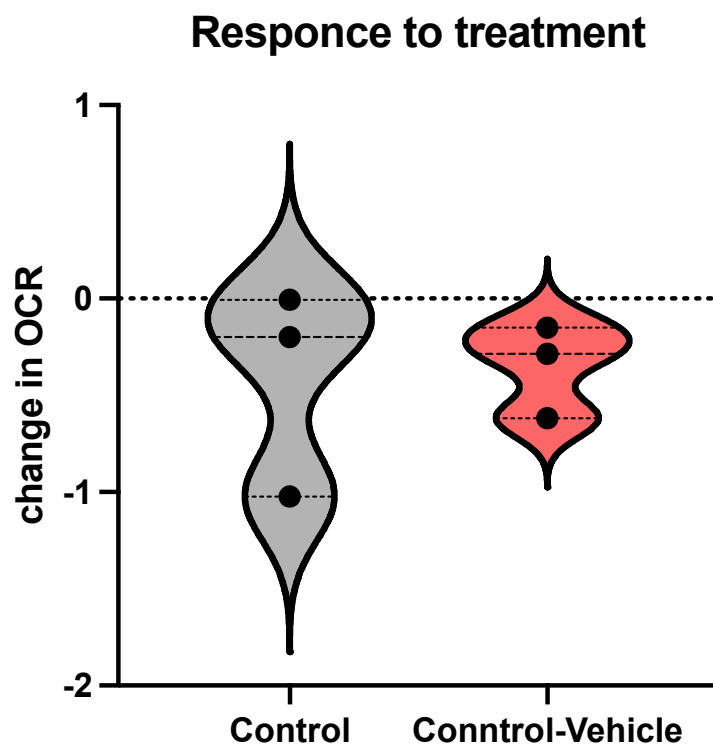


Figure 5.25_Appendix: a) Glucose, b) Pyruvate consumption, and c) Lactate production of embryos exposed to DMSO during blastocyst stage. Each graph represent data of at least 3 biological replicates with each dot presenting an individual embryo. Data are expressed as Mean $\pm$ SD. Shapiro-Walk test was conducted for each data set. Differences were determined using Mann-Whitney t-test *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented.

a)



b)



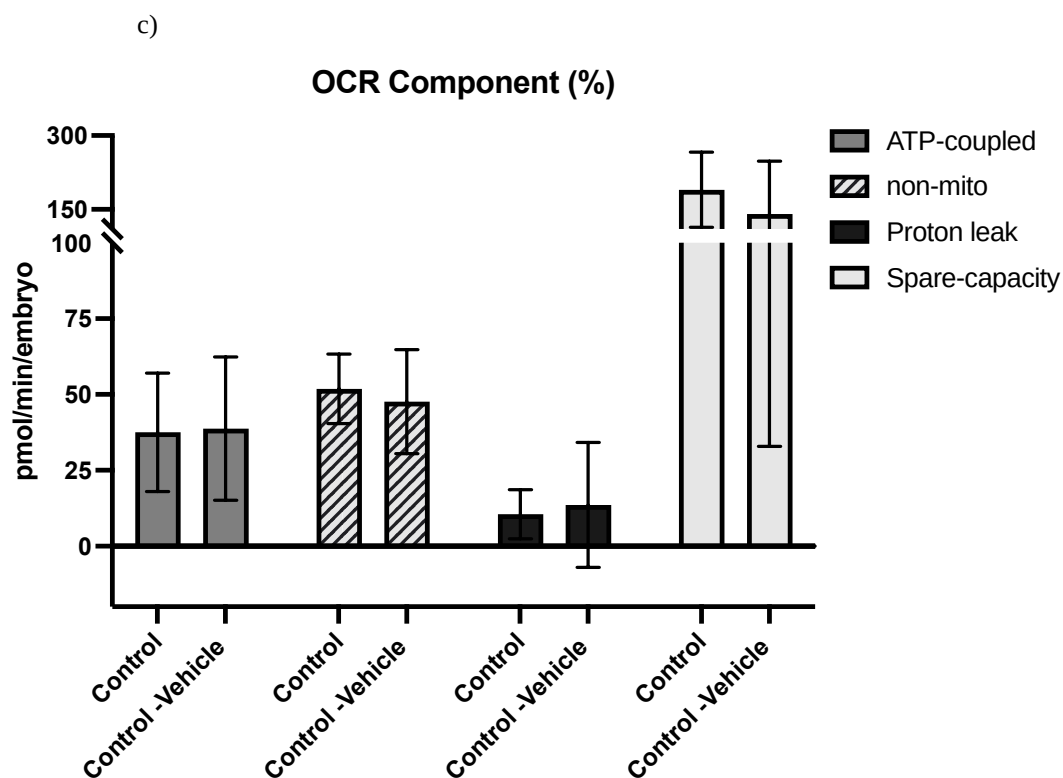


Figure 5.26_Appendix: The impact on OCR blastocysts acutely exposed to Control-Vehicle on D7 of embryo culture a) The timeline of OCR in response to Control-Vehicle, and the mitochondrial inhibitors: oligomycin, FCCP and A/R as comparison among the different treatment groups b) OCR as response to the Control-Vehicle injection, c) mitochondrial parameters as a percentage of basal OCR after Control-Vehicle injection. Differences were detected using b) unpaired t-test, c) two-way ANOVA followed by Tukey test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Trends are illustrated by a dashed line, with p-value presented. This graph includes data of 3 biological replicates (OCR, $n=3$).