INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

ENDOCRINE DISRUPTING CHEMICALS: EXPOSURE IMPACT IN C57BL/6J MICE REPRODUCTIVE PERFORMANCE AND MORPHOLOGY

JOANA RODRIGUES LÓIOS

DOUTORA EDNA RIBEIRO – ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA/ CENTRO DE INVESTIGAÇÃO EM SAÚDE E TECNOLOGIA - H&TRC

DOUTOR PEDRO FAÍSCA – INSTITUTO GULBENKIAN DE CIÊNCIA

Mestrado em **Tecnologias Clínico-Laboratoriais**

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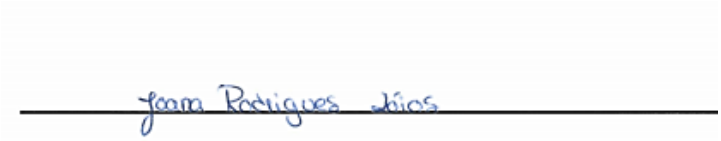
The present work was conducted at the Instituto Gulbenkian de Ciência Campus, located in Oeiras, under the direct supervision of doctors Pedro Faísca (Instituto Gulbenkian de Ciência) and Edna Ribeiro (Escola Superior de Tecnologia da Saúde de Lisboa- Health and Technology Research Center).

Statement of Original Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text. I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.



Joana Rodrigues Lóios

Statement of Copyright

To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science.

in “The evolution of physics”

by Albert Einstein

For my son Miguel,
who has donated to science a most precious gift - his mothers’ time.

Acknowledgments

If I have seen further, it is by standing on the shoulders of giants.

in “Letter from Sir Isaac Newton to Robert Hooke”

by Sir Isaac Newton

There is not enough space in a single page to properly thank all who have helped me achieve this steppingstone in my academic and professional life. You truly are my giants.

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To my sister Margarida Castiço for being the reason I push myself to go further and my mother Maria Helena Ferreira for being the reason I have come so far.

To Miguel, my son, for being my heart, even though walking outside my chest. I hope I can teach you that not even the sky is a limit.

To Pedro, my husband, father of my children, best friend and rock of my life there is no storm too great. Without you maybe I could but it would never be the same.

Abstract

An endocrine disruptor is defined as an exogenous substance or mixture that can alter function(s) of the endocrine system, consequently causing adverse health effects in intact organisms, progeny, or (sub)populations. Previous studies demonstrated that these effects can follow a double hit model and have impact on models of disease, toxicology testing and microbiota assays. However, repercussion on research performed in most research institutes is yet to be scrutinized.

The aim of the present study was to evaluate the presence, and possible impact of Endocrine Disrupting Chemicals (EDCs), in an animal facility at a research institute.

In this dissertation, a list of possible EDCs present in C57BL/6J mice production at Instituto Gulbenkian de Ciência was elaborated, data on reproductive performance (2012 – 2019) was statistically analysed and a reliable, economical, method to obtain quantitative parameters for future endocrine disruptor studies and their impact on research was implemented. Stereological estimations using the *Cavalieri* principle and a physical disector/fractionator combination, in mouse ovary (to asses volume and primordial follicle number, respectively) and testis (for volume, gonocytes and Leydig cell number) prepared for histology techniques in neonate and seven weeks mice, were performed and new baseline values were established.

Data on reproductive performance of the same mice were in accordance to the reported and recommended by the supplier except regarding differences in the timespan the females were productive.

Overall, data obtained in this study should be used as a tool to further evaluate the potential impact of EDCs on different studied models of disease, toxicology testing and microbiota assays.

Keywords: Endocrine Disrupting Chemicals; Reproductive performance; C57BL/6J; Stereology.

Resumo

Um desregulador endócrino pode ser definido como substância, ou mistura exógena, que altera a(s) função(ões) do sistema endócrino e, consequentemente, provoca efeitos adversos à saúde de um organismo saudável, à sua progenia ou (sub) populações. Vários estudos confirmam que estes efeitos podem seguir um modelo de impacto duplo e ter efeitos em vários modelos de doença, testes de toxicologia e ensaios de microbiota. As repercussões nos projetos de investigação a decorrer na maioria dos institutos de investigação estão ainda por analisar.

O objetivo do presente estudo foi avaliar a presença e possível impacto de desreguladores endócrinos, num biotério de um instituto de investigação

Nesta dissertação, foi elaborada uma lista de possíveis desreguladores endócrinos presentes na produção de murganhos C57BL/6J no Instituto Gulbenkian de Ciência, realizou-se análise estatística dos dados do desempenho reprodutivo (2012 - 2019) e foi estabelecido um método fiável, relativamente barato, para obter parametros quantitativos para estudos futuros sobre disruptores endócrinos e seu impacto em investigação. Neste último ponto, estimativas estereológicas utilizando o princípio de *Cavalieri* e uma combinação *physical disector / fracionador*, em ovário (para avaliar o volume e número de folículos primordiais, respectivamente) e testículos (para volume e número de gonócitos e células de Leydig) preparados para técnicas histológicas em ratinhos recém-nascidos e de sete semanas foram realizadas e novos valores de base foram estabelecidos.

Dados sobre o desempenho reprodutivo dos mesmos animais estavam de acordo com os descritos e com as recomendações do fornecedor, exceto no que concerne as diferenças no tempo de produção das fêmeas.

Resumidamente, os dados obtidos neste estudo devem ser usados como uma ferramenta para melhor avaliar o impacto potencial da exposição ambiental a EDCs nos diferentes modelos de doença, em testes de toxicologia e ensaios de microbiota.

Palavras-chave: Desreguladores endócrinos, Performance reprodutiva; C57BL/6J; Estereologia.

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Abbreviation List

2D	Two-Dimensional
3D	Three-Dimensional
AR	Androgen Receptor
AWO	Animal Wellfare Organism
BPA	Bisphenol A
CAS	Chemical Abstracts Service
DDT	P,P'-Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DGAV	Direcção-Geral De Alimentação E Veterinária
DOB	Date Of Birth
DOW	Date Of Weaning
EDC	Endocrine Disrupting Chemicals
EPA List	Danish Environmental Protection Agency
ER	Oestrogen Receptor
EU	European Union
EU REACH SVHC List	European Union Registration, Evaluation, Authorisation and Restriction of Chemicals Substances of Very High Concern List
FDA	Food and Drug Administration
FSH	Follicle Stimulating Hormone
GPCR	G Protein-Coupled Receptor
GSP	Genetic Stability Program
HE	Haematoxylin and Eosin Stain
ID	Identification
IGC	Instituto Gulbenkian De Ciência
IPCP	International Panel on Chemical Pollution

IPL	Instituto Politécnico De Lisboa
ISS	International Society for Stereology
JAX	The Jackson Laboratory
KC	Key Characteristics
LH	Luteinising Hormone
LIEDC	List of Identified Endocrine Disrupting Chemicals
MEK	Mitogen-Activated Protein Kinase
NIEHS	National Institute of Environmental Health Sciences
ORBEA	Órgão Responsável Pelo Bem-Estar Animal
PBBs	Polybrominated Biphenyls
PCBs	Polychlorinated Biphenyls
PGCs	Primordial Germ Cells
POPs	Persistent Organic Pollutants
SHBG	Sex Hormone-Binding Globulin
SIN List	Substitute It Now List
SPF	Specific Pathogen Free
SRC	Steroid Receptor Coactivator
SSCs	Spermatogonial Stem Cells
TEDX List	The Endocrine Disruption Enhance List
TR	Thyroid Receptors
UN	United Nations
UNEP	United Nations Environment Programme
WHO	World Health Organization

1. Introduction

The World Health Organization (WHO) defines endocrine disruptor as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or (sub)populations[1].

In several studies, Endocrine Disrupting Chemicals (EDCs) have been associated with reproductive effects, neurobehavioral and neurodevelopmental changes, metabolic syndromes, bone and immune disorders, and cancer[2]. However, the impact these ubiquitous substances have on the animal production in research settings is still lacking. Keeping in mind that a properly controlled environment is key for validation of several models of disease, toxicology testing and microbiota assays increasing the information in this area of knowledge is paramount.

Exposure to EDCs in a research animal facility context is expected to be in such low doses that fertility or fecundity are not affected but, although the information is systematically collected by production staff, the results were yet to be analysed to confirm it and, as far as it was possible to check, no tests are routinely performed.

The present project aimed to list the known EDCs present in the production of C57BL/6J mice at Instituto Gulbenkian de Ciência (IGC), statistically analyse the data on reproductive performance from 2012 to 2019 and implement a reliable, and relatively inexpensive, method to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute. In this last point, stereological estimations using the physical disector/ fractionator combination and the *Cavalieri* principle, were applied to mouse ovaries (to estimate primordial follicle number and volume respectively) and testes (to estimate gonocyte/ Leydig cell number and volume) prepared for histology techniques in two distinct time points – neonates (maximum one day) and seven weeks.

The results of the present study may be a breakthrough in the methods applied to research animal production and maintenance, regarding EDC environmental exposure. Careful selection, and comprehensive information, about all items that contact with these animals may prove to be key in guarantying the controlled environment fundamental for research - the basis of human progress.

2. Literature Review

2.1. Endocrine Disrupting Chemicals

2.1.1. Historical perspective

In 1938, with the production of the first synthetic non-steroidal oestrogen: diethylstilbestrol (DES), began the silent history of EDCs. This compound was prescribed to pregnant women between the 1950s–1980s to prevent miscarriages and/or premature delivery. Parturient women, who had experienced threatened or previous miscarriage, were routinely exposed to DES. However, only forty years after was the link between young women diagnosed with vaginal and cervix clear cell adenocarcinoma and their DES-exposed mothers established [3–6].

In 1958, while DES was being administered to pregnant women with no cause to suspect its deleterious role, endocrinologist Roy Hertz commenced investigating the potentially harmful part of hormones in cattle feed lots. These hormones, carried by faecal excretion, leaching in environmental matrices such as soil and water, can reach the human body through the food chain, with serious consequences in reproductive function, development and growth. For the first time, the concept “steroid cycle” was introduced and theory of “bio-accumulation” was foreshadowed [7,8].

Biologist Rachel Carson, in 1962, with the “Silent Spring” book, raised pertinent questions about anthropogenic repercussions in nature. Pointing out the current concepts of bio-accumulation (the concentration of an EDC increases in an individual over time) and of bio-magnification (from one trophic level to the next within the food chain, the EDC concentration increases), Rachel Carson defended that the chemical compound p,p’-Dichlorodiphenyltrichloroethane (DDT) and other pesticides entered the food chain and accumulated in fatty tissues of animals and humans, causing genetic damage and cancer [8,9]. (Figure 2.1)

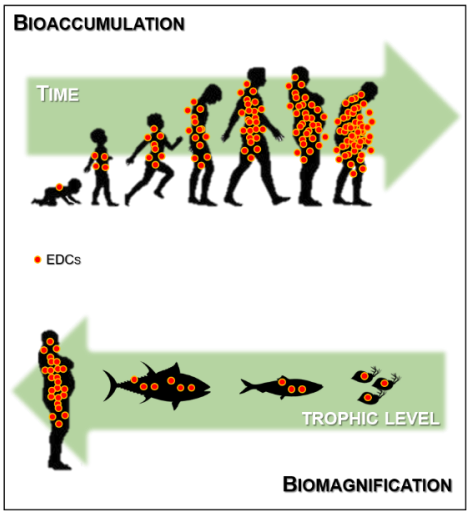


Figure 2.1 - Bioaccumulation and Biomagnification Diagram

Red dots signify EDCs accomulating trough time in a single individual and up trough trophic leves

The impact of Rachel Carson’s “Silent Spring” publication caused ripples throughout the scientific community stimulating researchers to increase the knowledge on EDCs.

In 1966, the National Institute of Environmental Health Sciences (NIEHS) research aimed to “reduce the burden of human illness by understanding how the environment influences the development and the progression of human disease”, and, in 1971, they established DES as a transplacental carcinogen whose toxicity involves the activation of oestrogen receptors, causing adverse effects in the offspring, up to the third generation, without necessarily affecting the mother[10].

In the same year, the United States of America Food and Drug Administration (FDA) issued a drug bulletin urging medical physicians to stop prescribing DES (FDA Drug Bulletin, November 1971).

In Wingspread Conference Centre in Racine, Wisconsin in July 1991 the scientific community convened and began using terms such as “endocrine disruption” and “endocrine disruptors” marking it a turning point in the development of the field of endocrine disruption. Theodora Colborn and colleagues coined these terms and, in a Consensus Statement, described many observations acquired by studies on EDCs, defining concepts such as “the critical window of exposure of susceptibility”, potential of bioaccumulation and long latency between exposure and manifestation of effects.

A consensus was achieved that a wide variety of man-made chemical had reached the environment, joined the already existing natural ones and had the potential to disrupt the endocrine system of animals (humans included).[11] With this new knowledge, time and committed research came the recognition that anthropogenic ubiquitous Endocrine Disrupting Chemicals can be found in every individual and ecosystem tested so far[1,12].

In the following years after the Wingspread Conference, studies on humans and animal models were conducted on several EDCs, and, in 1998, during the Rotterdam Convention on the Prior Informed Consent Procedures for Certain Hazardous Chemicals and Pesticides, more than 150 countries ratified a list of several different chemicals that included known EDCs (United Nations Environment Programme - UNEP 1998). The list was updated in 2001 (UNEP;2001), during the Stockholm Convention, with the introduction of several persistent organic pollutants (POPs).

In 2002, the WHO released a Global Assessment of the State of the Science of Endocrine Disruptors where animal and human case studies were scrutinized and in 2009, the Endocrine Society released its first scientific statement on EDCs with the following key points[2]:

- “The evidence for adverse reproductive outcomes (infertility, cancers, and malformations) from exposure to endocrine-disrupting chemicals is strong, and there is mounting evidence for effects on other endocrine systems, including

thyroid, neuroendocrine, obesity and metabolism, and insulin and glucose homeostasis."

- "Effects of endocrine-disrupting chemicals may be transmitted to further generations through germline epigenetic modifications or from continued exposure of offspring to the environmental insult."[12].

In sum, WHO defines endocrine disruptor as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or (sub)populations[1]. In line with the WHO definition, scientific consensus is that EDCs are either man-made or naturally occurring chemicals (phytoestrogens), present in the environment (air, water and soil), food sources, personal care products, and manufactured products. These chemicals include components of plastics, such as bisphenol A (BPA), phthalates, and other compounds such as dioxins, polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), pesticides including p, p'-Dichlorodiphenyltrichloroethane (DDT) and its metabolites, heavy metals, industrial chemicals, fuels and many others[13,14].

In 2016, a United Nation (UN) List of Identified Endocrine Disrupting Chemicals (LIEDC) was elaborated, as part of an EDC report prepared and published by the International Panel on Chemical Pollution (IPCP) commission by the UN Environment. This list aimed to aggregate several existing lists such as:

- The Endocrine Disruption Exchange List (TEDX List)
- Substitute it Now List (SIN List)
- Danish Environmental Protection Agency EDC List (Danish EPA List)
- European Union Registration, Evaluation, Authorisation and Restriction of Chemicals Substances of Very High Concern List (EU REACH SVHC List)

The UN published list claimed that the chemicals in it have gone through at least one "thorough scientific assessment" and identified as a known or potential endocrine disrupting chemicals. The list was included in an EDC report prepared and published by the International Panel on Chemical Pollution (IPCP) commission by the UN Environment[15] .

In May, 2020 a website and searching tool, administered, on behalf of all participating national authorities, by The Danish EPA, aiming primarily to inform stakeholders about the current status of substances identified as endocrine disruptors, or under evaluation for endocrine disrupting properties within the European Union (EU) was placed online. It is the most recent list available[16]. There is no such thing as a perfect list, and in this EDC context, such a complex multi-factorial subject, consensus is almost utopic.

However, a list can help to limit the amount of chemicals under scrutiny, especially in a baseline setting as the present study aims to be.

2.1.2. Mechanisms of endocrine disruption

As previously mentioned, EDCs are, for the most part, synthetic molecules from industrial origin and some natural occurring molecules, present in the environment that promote adverse changes of the endocrine homeostasis in humans and/or animals[2]. These molecules were originally believed to act primarily through nuclear hormone receptors, including but not limited to oestrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors, amongst others[17–19] However, significant scientific advances have demonstrated a vast array of mechanisms of action.

In 2019, Merrill *et al*, in an Expert Consensus Statement, anticipated that chemicals that interfere with hormone action have identifiable key characteristics (KCs) that relate to their ability to interact with key regulatory steps of hormone systems. Ten KCs for EDCs were identified, representing ten categories for the organization of the mechanistic evidence[20]. Being through direct impact on hormone production, receptors, distribution and availability or epigenetic effects, there are many mechanisms in which EDCs may interfere with biological functions. Respecting the ten KCs organization from the Expert Consensus Statement, both the mechanisms and its description are summarized on Table 2.1.

Table 2.1 – Ten Key Characteristics of Endocrine Disrupting Chemicals

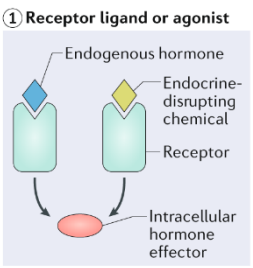
Key Characteristics	
Interaction with or activation of hormone receptors	
	Hormones, as a rule, act trough specific binding to a receptor or receptors[21]. EDCs that inappropriately bind to and/or activate hormone receptors can produce adverse biological effects, for example, inappropriately activating oestrogen receptors (ERα and ERβ) during development. This action results in an increased the risk of infertility in both sexes as well as an increased risk of reproductive tract cancer in women and prostate cancer in men[22].

Table 2.1 (cont.) – Ten Key Characteristics of Endocrine Disrupting Chemicals

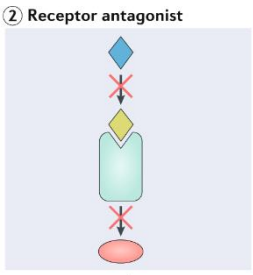
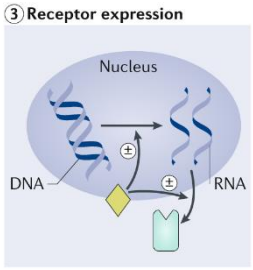
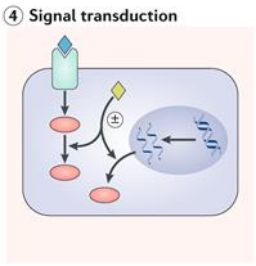
Key Characteristics
Antagonist to hormone receptors
 EDCs can inhibit or block the effects of endogenous hormones by acting as receptor antagonists[14]. While membrane hormone receptors or intracellular hormone receptors antagonism can occur, most exogenous chemical research into antagonization of receptors has been focused on nuclear hormone receptors. Nuclear receptors, acting as ligand-dependent transcription factors by mediating genomic regulatory responses, can be antagonized by some EDCs[23–25]. As androgens are key regulators of male sexual differentiation during foetal development, the disruption of androgen action through androgen receptor antagonism in this phase can permanently demasculinize male foetuses and lead to genital tract malformations[26,27]
Hormone receptor expression alteration
 The physiological and temporal pattern of expression of hormone receptors dictate their response to hormone signals and, in consequence, mediate hormone actions[28,29]. For example, receptor abundance can determine both the concentration of hormones that produces an effect as well as the magnitude of the effect itself and EDCs can modulate hormone receptor expression, internalization and degradation[30,31].
Signal transduction alteration in hormone-responsive cells
 When a hormone binds itself to a receptor, specific intracellular responses are triggered. These responses depend both on the receptor and on the specific tissue properties of the targeted cell. Some EDCs can alter signal transduction mediated through both membrane and intracellular hormone receptors[21]. Some membrane GPCRs, for example, the well-studied G protein-coupled oestrogen receptor (GPER) that bind steroids is amongst the most studied receptors regarding the EDC effects (for example, BPA)[32]. Additionally, EDCs can attenuate or potentiate hormone action through signal transduction, being it by impairing insulin action by reducing insulin receptor substrate 1 [33]; or potentiating ligand-activated transcription and progesterone receptor-mediated transcription in a mitogen-activated protein kinase (specifically MEK1 and MEK2) activity dependent mode[34]. Likewise, ionotropic receptor signalling can be disrupted by EDCs[35–37]. Finally, yet equally important, signal transduction initiated by nuclear receptors can also be affected by EDCs. These effects encompass interactions with coregulatory factors such as activators and repressors, key parts of the molecular machinery defining the downstream response to nuclear hormone receptor activation. The coregulatory factors for the steroid receptor coactivator (SRC) family are among the most studied in exogenous chemical research[38–42].

Table 2.1 (cont.) – Ten Key Characteristics of Endocrine Disrupting Chemicals

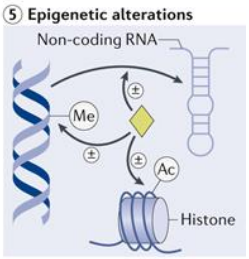
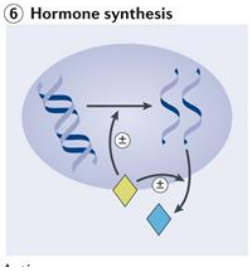
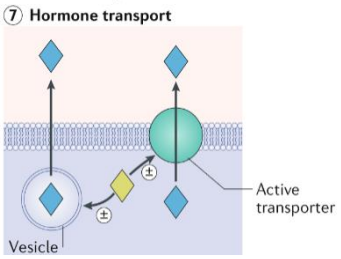
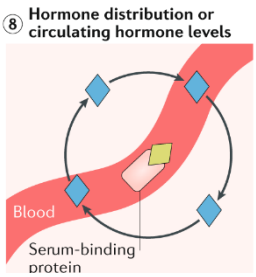
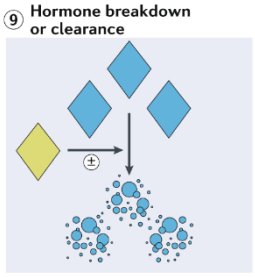
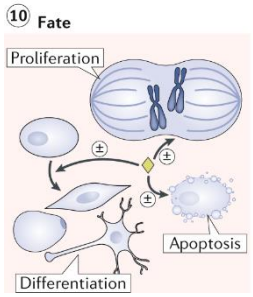
Key Characteristics
Epigenetic modifications in hormone-producing or hormone-responsive cells
 Especially during development and differentiation, hormones can exert permanent effects by modifying epigenetic processes, including DNA and histone modifications and non-coding RNA expression[21] Chemicals interfering with hormone action can induce these epigenetic changes or interfere a hormone ability to induce them (for instance, changing the transcription of hormone-responsive genes or the expression or action of a hormone receptor)[43,44]. For example, some EDCs boost the expression of the DNA methyltransferase DNMT3B to hypermethylate DNA, including ESR2 (which encodes ERβ) in the ovary of developmentally exposed rats (methoxychlor phthalate) or inappropriately demethylates MR DNA in the testis of male mice (di-(2-ethylhexyl) phthalate)[30,45].
Alteration of hormone synthesis
 Feedback mechanisms have an essential role in regulating hormone synthesis. This regulation can occur through both intracellular and distant endocrine feedback mechanisms. Some EDCs are known to affect hormone synthesis, for example blocking the uptake of iodine into thyroid cells, and in so doing so, inhibiting thyroid hormone synthesis (perchlorate)[46]. After transcription and translation, proteins and peptide hormones are wrapped into secretory vesicles where they can be stored. Dissimilarity, steroid hormone synthesis, as well as the transformation of a pro-hormone to hormone, occurs essentially simultaneously with hormone activity[21]. Some EDCs are known to interfere with hormone synthesis blocking, for example, the uptake of iodine into thyroid cells, thereby inhibiting thyroid hormone synthesis or reducing testosterone synthesis in the foetal rat testis, with subsequent testosterone insufficiency[47].
Alteration of hormone transport across cell membranes
 Steroid hormones such as oestrogens, androgens, progestins and adrenal steroids are lipophilic by nature, moving through membranes passively. Other hormones (amine, peptide, protein and thyroid hormones) need to be selectively transported across membranes[48] and both selective and passive transport processes can be disrupted by EDCs. One example of compromised selective transport by an EDC is a low dose BPA, which reduces calcium entry into mouse pancreatic β-cells to reduce insulin secretion from vesicles[49].

Table 2.1 (cont.) – Ten Key Characteristics of Endocrine Disrupting Chemicals

Key Characteristics
Alteration of hormone distribution or circulating levels of hormones
 Hormones characteristically tend to circulate throughout in the blood in low concentrations (usually in a range of parts per billion and trillion)[50]. Circulating hormones can be either transported unbound to any serum protein, with or without conjugation (such as glucuronidation or sulfation) or bound to various proteins. EDCs can alter hormone bioavailability meddling with the hormones in hormone distribution on-responsive tissues or with hormone circulation, displacing hormones from their serum binding proteins, which can lead to impaired active hormone delivery to target tissues. For example, EDCs such as BPA cause a concentration-dependent reduction in circulating concentration of testosterone in both male rats and men[51,52]. Likewise, DES intravenous administration to men causes a six-fold reduction of total testosterone and a 20% reduction of free testosterone. Oestrogen suffers, also a fivefold reduction simultaneous with an increase in serum concentration of sex hormone-binding globulin (SHBG)[53].
Alteration of hormone metabolism or clearance
 For the numerous hormone types (protein, peptide, steroid or thyroid) there are different inactivation pathways. Protein hormones (such as, gonadotropins) are deactivated by proteases in the blood. However, steroid and thyroid hormones are processed by enzymes that both promote inactivation as well as increase their water solubility to facilitate excretion[21]. EDCs inactivation rates of hormones alter their concentrations and finally their activity. For example, a large number of chemicals activate glucuronidases, increasing thyroid hormone clearance from the blood[54].
Alteration of the fate/outcome of hormone-producing or hormone-responsive cells
 The fate/outcome of hormone-producing or hormone-responsive cells can also be impaired by EDCs. By affecting cellular proliferation, migration or differentiation and/or cell death (being it by inducing apoptosis or necrosis) during development and adulthood can alter tissue structure, its organization and function [55–57]. In adulthood, some endocrine organs tend to have a stable number of cells (such as adrenals and pancreas), whereas others depend on cell growth for normal function (such as the testicles, the uterine endometrium and vaginal lining). EDCs action can compromise the number or location of cells in hormone-producing or hormone-responsive tissues by disrupting or promoting the cell mechanisms previously mentioned. For example, in female mice exposed to oxybenzone during pregnancy and lactation have increased mammary epithelial cell proliferation, which is observed even weeks after exposures cease [58] .

Note: All images in Table 2.1 were adapted from Merrill MA, Vandenberg LN, Smith MT, Goodson W, Browne P, Patisaul HB, *et al. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification*. Nature Reviews Endocrinology 2020;16(1):45–57.

Besides the mechanism of action, there are several concerns that have been proven to be key for a full understanding of the consequences of exposure to EDCs and reviewed in detail [59].

Age of exposure is one of such concerns. Adult exposure to an EDC may have very dissimilar implications from exposure in a developing foetus or in an infant. Point in fact, the field of endocrine disruption has incorporated the terminology “the foetal basis of adult disease” [60] to designate observations that the environment of a developing organism encompassing the maternal environment (eutherian mammals), the egg (other vertebrates), and the external environment, act together with the individual’s genes to determine the propensity of that individual to develop a disease or dysfunction later in life. Some authors extend this concept beyond the foetal period to an early postnatal developmental period arguing that, in this later phase organs continue to undergo substantial development. Accordingly, the terminology used is “the developmental basis of adult disease[14,59].

This concept also implies that there is a delay between the time of exposure and the manifestation of a disorder, meaning that consequences of developmental exposure that may not be immediately apparent early in life nonetheless may be manifested in adulthood or even during aging. This concept is known as latency[59].

Another major concern regarding the effects of endocrine disrupting chemical relates to the epigenetic key mechanism. Evidence suggests that the mechanism of transmission can involve the germline and be non-genomic[61].

As it was previously described in 1971 with DES[10] these transgenerational, epigenetic effects affect not only the exposed individual but also the offspring and subsequent generations being transmitted not due to mutation in the DNA sequence, but through modifications to factors that regulate gene expression such as DNA methylation and histone acetylation[62].

The non-traditional dose-response dynamics of EDCs are amongst the most controversial properties of these chemicals[59]. Apparently negligible levels of exposure, in fact any level of exposure, may cause endocrine and/or reproductive anomalies. Linking this fact to the previously mentioned concepts of transgenerational, epigenetic effects and latency, exposure during a critical developmental window is particularly worrisome[63]. In fact, low doses in some cases, are known to exert more potent effects than higher doses meaning that EDCs may exert non-traditional dose-response curves,

such as inverted-U or U-shaped curves [64,65] as depicted in Figure 2.2.

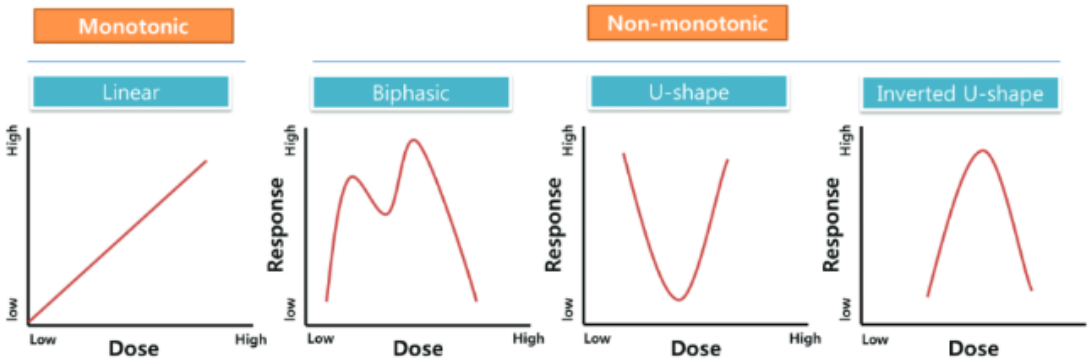


Figure 2.2 - Examples of monotonic and non-monotonic dose response curve

Adapted from Hong, Y.-P., & Yang, Y.-J. (2017). *Low-Dose Exposure to Bisphenol A in Early Life. In Bisphenol A Exposure and Health Risks*. InTech. https://doi.org/10.5772

Finally, another concern while studying and analysing the mechanisms of action and the consequences of EDC exposure is the importance of mixtures[59].

Contemporary amenities have made is so that environmental exposure to EDCs can occur through food, drinking water, air, soil and house dust, or through direct contact with various household materials or consumer products[66].

If individuals and populations are exposed to an EDC, it is likely that other environmental pollutants are involved because contamination of environments is rarely due to a single compound[59,67]. In addition, there is a clear need for further studies to assess additive, synergistic or antagonistic effects of EDC mixtures, however, these studies, in humans, still need to transcend one of its most important methodological limitations. Due to the ubiquitous presence of EDCs, humans are chronically and consistently exposed to these compounds, resulting in a non-existent negative control group, particularly at low doses[68].

Moreover, effects to EDCs have associated cell type specificities[69,70]. As cellular effects may vary depending of the used *in vitro* cell systems, considerable caution should be exerted in extrapolating *in vitro* measures of hormonal activity to *in vivo* models[71].

To overcome these limitations and complement *in vitro* studies, science has traditionally depended on research with experimental animals for detection of important toxic properties of chemical substances, for assessing risks to human and environmental health, and as models of human disease[72,73].

2.2. Animal models of human disease

The use of animals as models for human anatomy and physiology in ancient Greece were the first steps of comparative medicine, originated on the idea that other animal species share physiological, behavioural, and/or other characteristics with humans. This concept has evolved greatly and, nowadays, animal models are employed in nearly all fields of biomedical research including, but not limited to, basic biology, immunology, infectious disease, oncology, and behavioural studies[73].

Although animal modelling, predominantly in rodents, became the required method to establish biological significance, researchers soon appreciated the confounding factor of genetic variability in outbred animals in their research[74].

Individuals like William Castle, Clarence Little, Halsey Bagg, and Leonell Strong, addressed this issue via inbreeding of mice to the point that genetically identical mice became available for experimental research with limited genetic variability from litter to litter and year to year. As more and more inbred strains of mice and rats were developed, it was soon recognized that there were inherent differences between strains in basic biological parameters, as well as in susceptibility to induced and spontaneously occurring diseases[75].

2.2.1. The rise of the C57BL/6

In the early 1920s, Clarence C. Little, later the founder of the Jackson Laboratory (JAX), established a colony from the mating of female 57 with male 52 from Miss Abbie Lathrop's stock.[76] Miss Abbie Lathrop was an entrepreneur and spontaneous scientist that established a breeding animal business to sell, originally, as pets in the early 1900s. Later, the requests to sell to research institutes became one of her main sources of income[77].

The colony established by C.C. Little in 1921 of C57BL mice later gave rise to the C57BL/6 strain in the 1930s and in 1948, these mice, maintained at JAX, became known as C57BL/6J, with “J” for Jackson. Since then the C57BL/6 became the most frequently used mouse strain in biomedical research. The popularity of C57BL/6 inbred mice led to the establishment of many colonies at different vendors and academic institutions around the world[78].

As an unavoidable side note, there is no such thing as an C57BL/6 mouse. An inbred strain is defined as any set of brother-sister mating that occurs for at least 20 consecutive generations. A sub-strain is a branch of the inbred strain that has

significantly diverged from the original founding strain. Genetic drift occurs when natural mutations are maintained within an isolated breeding population. This can lead to distinct sub-strains at different breeding facilities over time[79]. Every time a new C57BL/6 colony is maintained separately from 20 or more generations, it becomes a new C57BL/6 sub-strain with potentially different phenotypes (such as C57BL/6N, C57BL/6NJ, C57BL/6NCrl, and so on)[78,79].

JAX implemented a colony management program maintained by a Genetic Stability Program (GSP), designed to limit cumulative genetic drift, including copy number variations, in most commonly used research strains such as the C57BL/6J, regularly rebuilding inbred strain foundation stocks every few generations from cryopreserved, pedigreed embryos. This Genetic Stability Program is the industry's gold standard to generate lab mice that remain as genetically stable as possible[80].

Likewise, C57BL/6J mice can also be purchased from other commercial vendors such as Charles River (IGC main mice supplier). Charles River frequently reintroduces breeding stock directly from the JAX colony to avoid genetic drift as well[81]. This is also the case in the animals under study in this dissertation.

2.2.2. The characteristics and importance of C57BL/6J mouse

The C57BL/6 mouse strain is the most widely used strain in biomedical research, with nearly 25,000 articles on PubMed documenting its use. Nearly half of these articles cite the use of C57BL/6J. [82] (Figure 2.3)

This strain is not only the most widely used inbred strain but also the first to have its genome sequenced.[83]

Figure 2.3 – C57BL/6J mouse

The Jackson Laboratory. Retrieved October 4, 2020, from https://www.jax.org/

The C57BL/6J strain has a dark brown to black coat, accordingly, being commonly referred to as "Black 6"[76].

With a relatively long-life span (878 ±10 days for males and 794 ± 6 days for females) and being quite fertile (average 6 pups per litter), this strain exhibits resistance to a variety of cancers, but nonetheless expresses mutations to the full[76,84,85].

As the most popular of all inbred strains, the C57BL/6J has been used in virtually all major areas of biomedical research being the most common background in the generation of congenic and transgenic mice for the study of mutant phenotypes[76].

Other characteristics include: a high susceptibility to diet-induced obesity, type 2 diabetes, and atherosclerosis; a high incidence of microphthalmia and other associated eye abnormalities; resistance to audiogenic seizures; low bone density; hereditary hydrocephalus, portosystemic shunts, hair loss associated with over grooming; a preference for alcohol and morphine; late-onset hearing loss; and increased incidence of hydrocephalus and malocclusion[85].

A well-rounded knowledge in the strain characteristics is fundamental for the correct choice and use of appropriate animal models in toxicology studies to reduce or evade factors that could confound studies, introduce preventable variation, or incorrect interpretation of the results[86,87]. Genetic backgrounds establish the metabolic rate of a chemical, mediating how it is absorbed, distributed, metabolized, and eliminated.[88,89] Ultimately, these characteristics may influence how sensitive specific strains are to the same compounds under the same exposure conditions[90].

The C57BL/6J mouse is not used due to a particular sensitivity to endocrine disruption in toxicology studies[91]. However, the fact that it is the most widely used mouse in research should raise questions in the impact that environmental exposure to EDCs has on this strain.

2.2.3. EDC environmental exposure research and C57BL/6J

The endocrine system plays an essential role regulating critical biological functions such as metabolism, development, reproduction, and behaviour in vertebrates. Epidemiological studies link EDCs with reproductive effects, neurobehavioral and neurodevelopmental changes, metabolic syndromes, bone and immune disorders, and cancer[2]. Animal studies show associations with several health effects, including asthma, learning and behavioural issues, early puberty onset, infertility, breast and prostate cancer, Parkinson's disease, obesity, and other diseases[2,72,92]. As it was previously mentioned, EDCs can be found everywhere, in everything and everyone so it is safe to infer that, similarly, facilities that produce, house and test mice for research purposes are no exception. The animal production in research settings has several environmental and natural occurring, chemicals that are known or potential endocrine disruptors. Some studies already suggest that commercial and research laboratories are raising animals in environments that are not properly controlled and defined when it comes to EDC exposure. It is expected that this exposure occurs in low enough doses that fertility or fecundity are not greatly affected but it could still influence other hormone-

sensitive endpoints and a multitude of disease pathways.[20,93] This is yet to be confirmed.

In fact, some studies already report the need to, actively, control the exposure to environmental chemicals that could potentially interfere in EDCs studies in laboratory experiments[94–96].

There are also, several studies that have demonstrated that early life exposure to EDCs alters responsiveness of animals to hormones or carcinogen challenges at puberty or in adulthood[90,97,98].

The double hit model advocates that developmental exposures may not be enough to induce adverse effects, but the consequences of EDC exposures are better visualized when a secondary challenge is experienced[99]. In this case, the uncontrolled EDC exposures that occur in commercial and research animal facilities could represent the first hit and the controlled exposure experienced during toxicity testing would be the second[93].

At the same time, the rise of Epigenetics, bring new questions into the fold. Adding to their hormone-disrupting actions, EDCs can induce long-term changes in gene expression, via perturbation of epigenetic patterning. These epigenetic effects have been well documented, but the exact mechanisms by which they interfere with epigenetic marks need further studies[100].

In recent years, research has also suggested that gut microbiota can be a factor in several pathologies and this microbiota can be influenced by EDCs[101].

The impact of this influence is not yet fully studied and the potential for bias in research is immense.

Specifically, in C57BL/6 some studies have reported that these mice (sub-strain “J” not specified) are more sensitive to oestrogen-induced alterations in oocyte development than other strains typically used (such as CD-1, FVB, or Oct4-GFP mice)[102,103]. Chronic low-dose exposure to a mixture of phthalates and alkylphenols, has also been linked to alterations in testis such as a increased presence of foci or regions of Leydig cells displaying hyperplasia, decreasing intratesticular estradiol levels in exposed C57BL/6J mice[104] .

These specific studies, although very relevant, are toxicology research with controlled exposure to EDCs with no regard to environmental exposure, which has a significant potential to alter the phenotype. The most popular example of phenotype alteration trough environmental exposure is the *agouti* conundrum[105].

Genetically identical adult viable yellow *agouti* (A^{vy}) mice have five different coat colour phenotypes. Yellow mice are hypomethylated at the transposable element upstream of the *Agouti* gene allowing maximal ectopic expression, whereas hypermethylation of this site silences ectopic agouti expression in the *pseudoagouti* animals. (Figure 2.4) Mice that are predominately yellow are also clearly more obese than brown mice[106]. Studies have reported that exposure to BPA induces a shift in frequency towards the yellow coat obese phenotype (hypomethylation)[107]. No obvious pattern such as this was detected for the C57BL/6J but this fact does not imply that there is no effect for the environmental exposure.

Figure 2.4 – Five different coat color phenotypes of genetically identical adult viable yellow agouti (A^{vy}) mice

Adapted from Wolff, G. L., Roberts, D. W., & Galbraith, D. B. (1986). Prenatal determination of obesity, tumor susceptibility, and coat color pattern in viable yellow (Avy /a) mice - The yellow mouse syndrome. In *The Journal of Heredity* (Vol. 77).

Unquestionably, an *a priori* screening of existing EDCs (and their influence) in the animal facilities as well as establishing controls for such an exposure, is the best approach, ultimately, to avoid environmental chemical contamination of animals to be used in laboratory experiments[93].

A good starting point could be the analysis of mouse ovaries and testes. Exposure to EDCs has been associated with less primordial follicles as well as a reduced testicular size[107,108].

2.2.4. Testicular and ovarian morphology of the C57BL/6J

In mammals, reproduction is a complex and delicate process that must occur in congruence with prevailing dietary, physical and social conditions[109].

Its importance to the survival of organisms makes it so that reproduction is a high priority function of all mammalian species including the mice[110].

By definition, sexual reproduction is the creation of a novel living organism by coalescing the genetic information of two entities from different sexes, determining the genetic sex of the embryo (the presence or absence of the Y chromosome), the gonadal sex (testis

or ovary), which in turn converges in the development of the phenotypic sex (secondary sexual characteristics, such as external genitalia)[111].

At embryonic day 7.5 (E7.5) the genital ridge composed of somatic and germ cells begins to form. In mice, primordial germ cells (PGCs) are specified in the proximal epiblast and migrate from the primitive streak to the endoderm, which will form the future hindgut. PGCs then journey along the endoderm to reach the genital ridge at E10.5 to consequently form the embryonic gonad[112,113] (Figure 2.5).

Whether the embryonic gonad will develop into either a testis or an ovary originates from the differentiation of supporting cells (Sertoli in males or granulosa in females) correlating to the genetic sex of the embryo at E10.5[111].

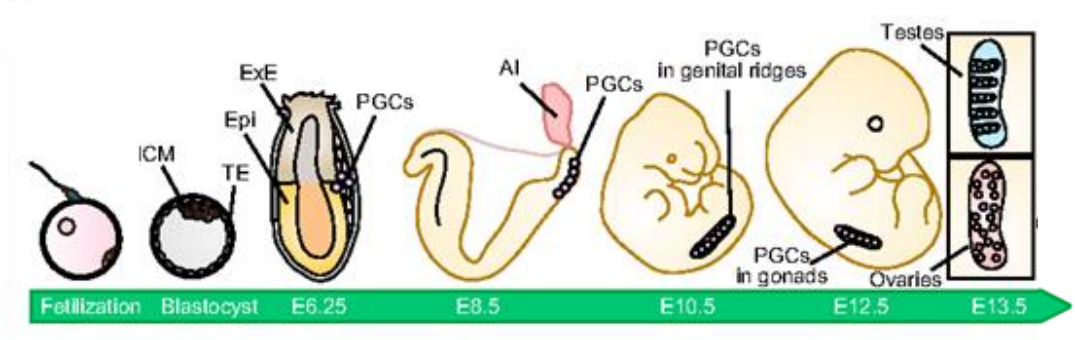


Figure 2.5 – Timeline of sex specification and development of the mouse

Adapted from O'Neill, M., Zhelyazkova, B., White, J. T., Thirumavalavan, N., & Lamb, D. J. (2018). *Developmental genetics of the male reproductive system. In Human Reproductive and Prenatal Genetics* (pp. 3–25). Elsevier. https://doi.org/10.1016/B978-0-12-813570-9.00001-2

The female or male phenotype is governed by the balance of androgens and oestrogens. For example, loss of oestrogen signalling decreases the oestrogen: androgen ratio, resulting in masculinization of female genitalia. On the other hand, increased androgen signalling in a female increases the oestrogen: androgen ratio, resulting in genital tubercle elongation, complete tubularization of the urethra with some degree of labioscrotal fold fusion resulting in clitoromegaly or a pseudo phallus. The gonads correct development is a progression tightly regulated and, if disrupted, can result in sex development disorders such as gonadal dysfunction, infertility and/or ambiguous genitalia[111,114].

2.2.4.1. Mouse Testes

The male reproductive tract is composed by testes (male gonads), a ductal system (epididymis and vas deferens), penis, scrotum, and accessory glands (prostate, seminal vesicles and the bulbourethral gland)[110,115,116] (Figure 2.6).

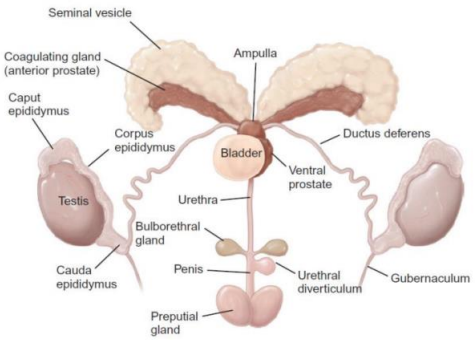


Figure 2.6 – Adult male mouse reproductive tract

Adapted from Cook MJ. 1965. *The Anatomy of the Laboratory Mouse*. www.informatics.jax.org/cookbook

Throughout the embryological development, each individual testis with the primordial duct system, blood vessels, lymphatics and nerves, descend from the posterior wall of the peritoneal cavity to the scrotum. During migration, the testis carries a double lining called the *tunica vaginalis* consisting in visceral and parietal layers separated by serous fluid. This fluid, secreted by the mesothelial cells, acts as a lubricant, allowing the testis to move freely in the scrotal sac[117].

Sertoli cells (large, post-proliferative cells that are essential to spermatogenesis) are the first somatic element to differentiate[118,119]. *Sertoli* cells proliferate more actively before birth and, in mice proliferation extends to 2 weeks after birth[120]. Thereafter, the number of *Sertoli* cells per testis is considered stable throughout the life of the animal[121]

After the initial differentiation of *Sertoli* cells has occurred, Leydig cells differentiate in the interstitial space and are denominated foetal Leydig cells[122,123]. Functional activity of foetal Leydig cells decreases during late foetal life and neonatal life and another population of Leydig cells (denominated adult Leydig cells) start to differentiate between 5-15 days *post-partum*[124]. The morphology and some physiological features of adult Leydig cells differ from those of the foetal cells[125,126].

In the adult, intratesticular androgen levels, crucial to germ cell maintenance, are sustained by Leydig cells. These endocrine cells are outside of the protective blood–testis barrier[119].

Leydig cells are the principal cell type found in the interstitial supporting tissue between the seminiferous tubules, occurring singly or in clusters. These cell nuclei are round with dispersed chromatin, with one or two nucleoli at the periphery, and extensive eosinophilic cytoplasm. Leydig cells are responsible for the production of testosterone, hormone not only responsible for the development of male secondary sexual

characteristics at puberty but also essential for the continued function of the seminiferous epithelium[117].

In neonates, testicular gonocytes, also nominated pro-spermatogonia (Figure 2.7), are the foetal/ neonatal precursors of the undifferentiated spermatogonial stem cells (SSCs). Gonocytes are large cells with a spherical euchromatic nucleus, two nucleoli and a surrounding, ring-like cytosol located in seminiferous cords of the testis[127,128].

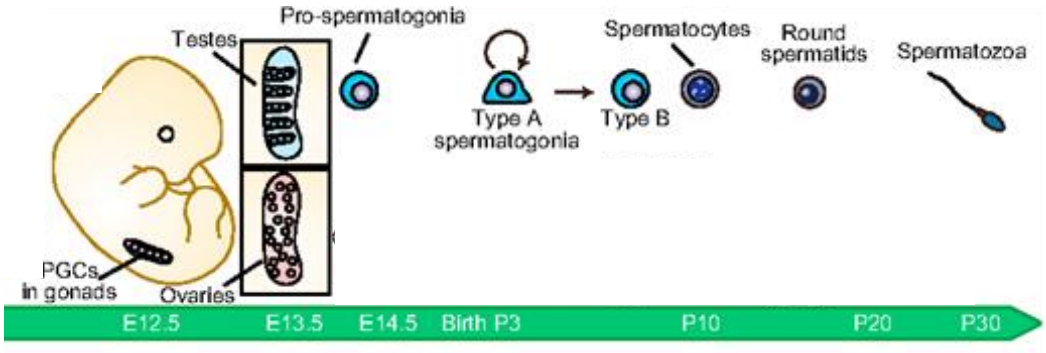


Figure 2.7 – Timeline of mouse male gametogenesis

Adapted from O'Neill, M., Zhelyazkova, B., White, J. T., Thirumavalavan, N., & Lamb, D. J. (2018). *Developmental genetics of the male reproductive system. In Human Reproductive and Prenatal Genetics* (pp. 3–25). Elsevier. https://doi.org/10.1016/B978-0-12-813570-9.00001-2

Following birth in the mouse, which has a short prepubertal period, gonocytes undergo a transition to SSCs or develop directly to type A1 spermatogonia, an early differentiation stage, by day six of life[128].

In adults, the testis encompasses compactly arrayed convoluted loops of seminiferous tubules separated by an interstitium containing Leydig cells, vasculature, macrophages, a protein- and testosterone-rich ultrafiltrate, and supporting stroma. The seminiferous epithelium is formed by basally located *Sertoli* cells supporting successive synchronized populations of maturing germ cells, namely spermatogonia, spermatocytes, round spermatids, and elongating spermatids. Seminiferous tubules are unsheathed by contractile myoid cells and converge, through *tubuli recti*, on the *rete testis*, which is continuous with the efferent ductulus and epididymis[119].

2.2.4.2. Mouse Ovaries

The female reproductive tract is composed by ovaries (female gonads), oviducts (Fallopian tubes), uterus (horns and fundus), cervical region, vaginal vault, and an external vaginal opening[117]. (Figure 2.8)

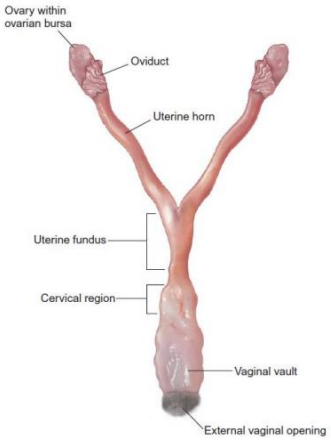


Figure 2.8 – Adult female mouse reproductive tract

Adapted from Cook MJ. 1965. *The Anatomy of the Laboratory Mouse*. www.informatics.jax.org/cookbook

In the present study the focus is centred on the female gonads, the ovaries. Ovaries are paired organs lying on either side of the uterus adjacent to the lateral wall of the pelvis in the mouse[117]. The ovary is a primary functional organ of the female reproductive system, and it plays two major physiological roles being responsible for the differentiation and release of a mature oocyte for fertilization and synthesizing and secreting hormones that are essential for follicle development, menstrual/oestrous cyclicity and maintenance of the reproductive tract and its function as well as the emergence of secondary sex characteristics[129].

The normal development of the ovaries involves several cell types (such as oogonia, theca and granulosa cells) that provide both structure and function, essential for natural reproduction. In the development two major steps can be defined, starting with the expansion of the ovary and establishment of the founding primordial follicle pool and, later, on a second phase (beginning with the onset of puberty) where follicles maturation and release of a fertilizable egg occurs[130].

The first phase of ovarian development, or early ovarian development, occurs predominantly during the embryonic period[117,131].

In the foetal mouse ovary, due to cell division, germ cells form clusters with incomplete cytokinesis[132]. In the neonatal ovary, each surviving oocyte is enclosed in a layer of flattened pre-granulosa cells which, in turn, are surrounded by a basal lamina to form primordial follicles[133]. In mice, early ovarian development finishes on postnatal day two (P2)[130].

Although it has been recently disputed [134–136], it is a widely held view that in mammalians, including the mouse, neonatal ovary contains a finite stockpile of non-growing primordial follicles each of which encloses an oocyte arrested at the diplotene step of meiotic prophase[136].

After birth, cohorts of primordial follicles are regularly recruited to enter into a 3-week growth phase while simultaneously the granulosa cells transform to acquire a cuboidal shape, forming primary follicles as shown on Figure 2.9.

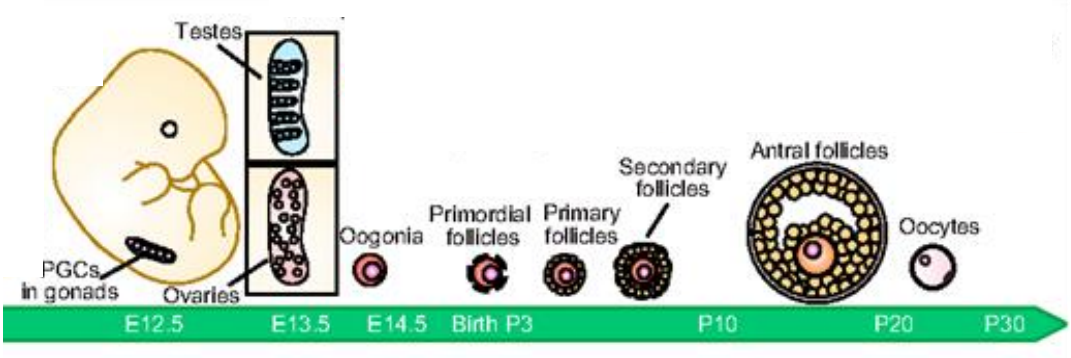


Figure 2.9 – Timeline of mouse female male gametogenesis

Adapted from O'Neill, M., Zhelyazkova, B., White, J. T., Thirumavalavan, N., & Lamb, D. J. (2018). *Developmental genetics of the male reproductive system. In Human Reproductive and Prenatal Genetics* (pp. 3–25). Elsevier. https://doi.org/10.1016/B978-0-12-813570-9.00001-2

The proliferation of granulosa cells results in pre-antral follicles composed of multilayers of granulosa cells surrounding the oocyte within the follicular basement membrane[137]. Ovaries are also endocrine organs that manufacture oestrogen and progesterone. Both functions are controlled by the cyclical release from the anterior pituitary of the gonadotrophic hormones, luteinising hormone (LH) and follicle stimulating hormone (FSH). Oestrogen and progesterone, in turn, regulate LH and FSH production by feedback mechanisms. Therefore, ovulation is coordinated with preparation of the uterus to receive the fertilised *ovum*[117].

After puberty, further growth of follicles is accelerated by gonadotrophins, FSH, leading to the formation of antral follicles[138]. Although oocytes within large antral follicles are competent for maturation, they remain arrested due to their interaction with the surrounding granulosa cells[139]. In response to the LH surge, fully grown oocytes complete the first meiotic division, extrude the first polar body, and become arrested at metaphase II. The LH surge is responsible for ovulation of the oocyte marking the end of folliculogenesis[140].

These processes, as it was mentioned for male gonads, are highly regulated. Disruption in any pathway can lead to adverse reproductive outcomes, such as a smaller primordial follicle pool, incomplete development of follicles, and failure of normal sexual differentiation[130,141].

2.3. Histology and stereology techniques

One very useful, relatively inexpensive, and conventional tool for analysis of EDC induced alterations in organs is histology, allowing for the study of both cells and tissues. Unfortunately, histology, by itself, results in loss of three-dimensional (3D) information. On histology sections solids are converted into profiles, surfaces become lines, linear features are transformed into points, and finally objects become an unpredictable number of sectional profiles[142]. These concepts are better visualized in Figure 2.10.

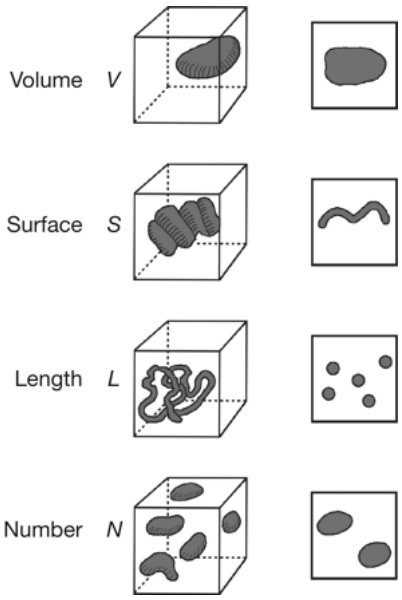


Figure 2.10 – Schematics of 2D profiles of 3D structural information

Adapted from West, M. J. (2012). *Introduction to stereology*. Cold Spring Harbor Protocols, 7(8), 843–851. https://doi.org/10.1101/pdb.top070623

Nevertheless, obtaining significant quantitative statements regarding these structural parameters in three dimensions by sampling and measuring the structural features present in the sections is possible through the application of mathematical relationship equations that consider the pertinent structural parameters that are lost while producing histology sections. Such is the basis for the Stereology methodology, providing meaningful quantitative descriptions of the geometry of 3D structures from measurements obtained on two dimensional (2D) images[143–145].

As further back as ancient Greek and Egyptian civilizations, mankind has sought methods to quantify 3-D objects. However, Stereology as a scientific discipline did not exist until German-born histology Professor Hans Elias organized the first meeting in 1961[146].

Scientists from the fields of biology, geology, engineering, and materials sciences convened in Feldberg, Germany with the purpose of sharing and discussing methods for quantifying 3D objects based on their appearance on 2D sections. The following year the International Society for Stereology (ISS) assembled for its first congress in Vienna, Austria. The ISS continues today with distinction as the largest multidisciplinary collaboration of international scientists with a non-war purpose in human history[142,146].

The timing of the rise of stereology as a scientific discipline overlapped with a several technological innovations such as the increase in accessibility to powerful and

affordable microscopes, novel histological procedures and immuno-based methods for visualization of specific microstructures[146].

In the 70s, as biologists began to favor the more objective stereology approaches over subjective evaluations by experts (well-known from poor inter-observer consistency) mathematicians brought their unique perspectives to the theoretical aspects of stereology, identifying a major flaw in earlier approaches: quantification of biological structures, where essentially nothing fits these classical geometric models, requires stochastic geometry and probability theory[146,147].

After the publication of the *disector* principle[148], Gundersen and colleagues steered stereology towards unbiased methods for Region Volume based on the *Cavalieri* method[149] and Number based on the disector principle[150] making unbiased stereology efficient enough for routine research studies. The possibility to measure structural features such as object number and volume can be used to make quantitative statements about function that are undoubtedly valuable in comparative and experimental research[142].

Determination of the total amount of any structural parameter is both time-consuming and unnecessary; therefore, estimates (approximations with statistically defined margins of error) are enough to achieve the goals of a study. The margins of error of stereological estimates are dependent on the amount of sampling, and the variance due to measurement error. A coefficient of error between 2 and 5% are generally accepted.

As it was previously mentioned, Stereology allows for quantification of 3D structures from measurements preformed on 2D images[143–145].

In this study three main methods were applied and are described briefly bellow. More in depth description will be provided in the methods section.

2.3.1. The *Cavalieri* Method

For volume estimates the *Cavalieri* method indicates that the volume of an arbitrary shaped object can be estimated in an unbiased manner from the product of the distance between planes and the sum of areas on systematic-random sections through the object.[151] Likewise, areas can be determined by point counting, so the volume can be estimated without any traditional measurement. In sum, the organ is serially sectioned with uniform thickness (t). For example, one every 5 sections (T=5t) is assessed to determine area (for example by point counting method: in a square array test system the point area is calculated as the square of the distance between points[151,152].

2.3.2. The disector

As mentioned by Howard and Reed in Unbiased Stereology, a traditional approach for whole sample counting in microscopy has been an exhaustive serial sectioning of the object combined with *a posteriori* reconstruction[152]. However, in 1984, a tremendous breakthrough in stereological counting in 3D was provided by D. C. Sterio with the *disector*[148]. This method is based on a three-dimensional stereological probe (shape that interacts with the object of study), using two sections at a known distance apart (physical disector). The events (interactions of the probe with the characteristic under study) counted are only visible in the first section of the pair (reference), but not observable in the second section (look-up) as depicted in Figure 2.11. This method avoids double counts, and its specific strength is that it is completely independent of the size and shape of the objects being counted[136,148].

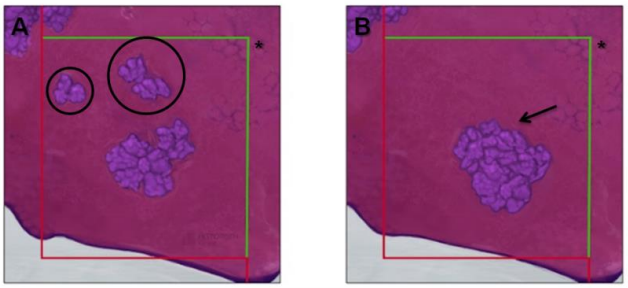


Figure 2.11 – Schematic representation of the physical disector principle

Structures of interest present in A (Reference - black circles) and absent in B (Look-up) within the counting frame (*) are counted; Structure of interest present in both A and B (black arrow) is not counted.

2.3.3. The Fractionator

As it is indicative by the name, the fractionator principle is a method based on fractionating the tissue.[150] The fractionator principle enounces that, when collecting a random sample in a known fraction of a population, the unbiased estimate for the population is the value from the sample divided by the fraction, as represented by the basic mathematical formula in Figure 2.12[150,152]:

$$\hat{X} = \frac{x}{f}$$

Figure 2.12 – Basic Fractionator formula

The symbol X represents the quantity of interest. The X with the caret is the estimate of X. The lowercase x is the value, either measured or estimated, from the sample. f is the fraction sampled.

It is fundamental to refer that estimate of X is only as unbiased as the value x is also unbiased[152].

Applying this concept to biological tissue, the total number of cells in an organ or region can be estimated by multiplying the number of cells counted in the final sample by the inverse of the sampling fraction for each sampling step[147,150].

In practice, the structure in question has to be fractionated into sections representative of the whole organ with equal probability of each section to be selected and evenly distanced. This will occur through systematic random sampling[142,149,150].

The total number of cells is determined using either physical (different sections) or optical (planes within a thick section) disectors and are known respectively as the physical or optical fractionator. This methodology allows for a direct number estimation without knowledge of total volume, regardless of object size and shape[136,147,150].

2.3.4. Minimizing the human-in-the-loop

At the moment, the most commonly used technique to acquire quantitative information from tissue sections is 2D morphometry where manual or automated analysis is performed on single or multiple tissue sections to obtain quantitative information. However, the ensuing data can only be valid in the sections being evaluated. For example, number counts performed on single thin 2D sections have no correlation with what is present in a 3D structure[153].

Unlike histomorphometry, stereology does not make assumptions about the tissue and can supply accurate 3D estimates, but these techniques are still mainly performed by humans with their inherent biases. To minimize this, stereology in its fundamental base already provides strategies and methods to eliminate the “human bias” with systematic random sampling throughout all the steps (sample collection, sectioning, field selection for measurements) instead of allowing this to be chosen[152].

Computerized stereology applications continue to minimize or eliminate the need for user-based data collection. Systems, such as the newCAST Visiopharm stereological software are already available for accurate and highly efficient quantification, with minimal “human-in-the-loop” interaction. Central to these novel approaches is the combination of unbiased stereology with technology for image segmentation and feature recognition based on size, staining, texture, shape, and other characteristics of biological microstructures.

3. Materials and Methods

The research field of endocrine disruption by ubiquitous substances has seen a rise since the 60s, however the impact of the environmental exposure to these substances in animals in research settings is still lacking. Keeping in mind that a properly controlled environment is key for validation of several models of disease, toxicology testing and microbiota assays increasing the information in this area of knowledge is paramount.

To fill the gaps in this area this project started with the elaboration of a comprehensive list of known EDCs present in the production of C57BL/6J mice at a renowned research institute in the Lisbon area (Phase I). Because there was no previous statistical analysis on the reproductive performance of these mice, this was also prepared. Data on reproductive performance from 2012 to 2019 of the production of C57BL/6J mice was compiled and analysed (Phase II).

Amidst the previous objectives this study also aimed to implement a reliable, and relatively inexpensive, method to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute. (Phase III).

The mice were kept in a positive pressure, temperature-controlled room maintained at 20-24ºC on a constant light-dark 12h:12h cycle with humidity in an interval between 30-70%. They had constant free access to food and water and both the diet, and the method of water purification has been the same since 2012. The basic housing and husbandry conditions are transverse to all three phases of the present study.

3.1. Research method and variable classification

Due to the triphasic nature of the present study each Phase has its own methodology separately described. However, research method classification and variable classification is thus presented. Phase I of this project is classified as exploratory and transversal study., Phase II is classified as a retrospective exploratory study and, finally, Phase III is classified as an exploratory study as well [154].

In Phase I, as it consisted in a list elaboration, no variables were assessed. The variables in phases II and III were assessed and classified regarding type and scale and can be consulted on Appendix I [154].

3.2. Hypothesis formulation

As the present study is first and foremost an exploratory one and, generally, exploratory research (often denominated hypothesis-generating research) aims to uncover possible relationships between variables and no prior assumptions or hypotheses are formulated. However, the design of this project relied on the hypothesis that on Phase I the presence of EDCs on the components of material used in the production of C57BL/6J mice would be found. On Phase II, the results were expected to be comparable to those provided by the supplier companies regarding reproductive performance.

Finally, for Phase III no hypothesis was formulated as the objective was to implement a methodology and establish baseline values for future research.

3.3. Phase I

The first part of the present study consisted in the elaboration of a comprehensive list of the known EDCs present in the production of C57BL/6J mice in the institute. This list was obtained with the collaboration of the institute's own research rodent facility.

Through contact with the facility it was possible to obtain the standard conditions that the mice are maintained in the production room.

To ensure the elaboration of a comprehensive list, on September 2019 a Google docs file was created with the title "Production Materials" and shared with the both the production Technical staff (2 members), Rodent facility technician responsible for ordering the supplies (1 member), the Rodent Facility Manager (1 member) and the Head of Unit of the Model Organisms Facility (1 member) at the institute. All elements had permission to edit the file to fill the following categories depicted on Table 3.1:

Table 3.1 – Table of contents available to production Rodent Facility Staff

Categories	Description
Type	Personal Protective Equipment, Diet, Environmental Enrichment and, Housing
Product	Name of the product according to the vendor for easier traceability
Reference	Vendor code of the product
Brand	Identifier the company name that produces/resells the product
Vendor	Company that supplies the product
Composition	Detailed list of chemical composition; Label composition description

The list included only material currently used in the production room for the core breeder's strain of the study, C57BL/6J.

When composition was not detailed enough contact with the providing companies was established to try and add a more thorough description.

Likewise, to access the conditions in which the mice of this strain are produced and maintained by the companies who provide them, contact in person and through e-mail was established.

The process was conducted also trough company specific websites to maximize the detail of the conditions studied.

According to the composition of the diverse instruments, feed and environmental enrichment the data was cross-referenced trough the online website edlists.org[16]. This website, administered, on behalf of all participating national authorities, by The Danish Environmental Protection Agency (Danish EPA), aims primarily to inform stakeholders about the current status of substances identified as endocrine disruptors, or under evaluation for endocrine disrupting properties within the European Union (EU).

In more detail, the substances are separated by the three lists:

- List I - Substances identified as endocrine disruptors at EU level

This list contains substances that have undergone the full evaluation process for endocrine disruption as regulated in the EU under the Plant Protection Products Regulation (PPPR), the Biocidal Products Regulation (BPR) or REACH (the Candidate- and Authorization Lists).

- List II - Substances under evaluation for endocrine disruption under an EU legislation

This list contains substances that are currently under evaluation in an EU legislative process due to explicit concerns for possible endocrine disrupting properties and are supported by National Competent Authorities in Belgium, Denmark, France, the Netherlands and Sweden.

- List III: - Substances considered, by the evaluating National Authority, to have endocrine disrupting properties

This list contains substances for which a participating National Authority has evaluated endocrine disrupting properties based on scientific evidence.

Although there are other lists to cross-reference with none were perfect and this website search method was chosen because it was user friendly (with a search engine to find compounds by name, CAS or EC number), due to its bi annual update system (with the

last update on May 2020) and the reliability of the institutions involved. A comprehensive table was elaborated with the results.

3.4. Phase II

The second stage of the project pertains to the statistical analysis of the Reproductive Performance of the most used *Mus musculus* strain (C57BL/6J) core breeders under the institute production room conditions. The rodent facility has vast records to be analysed, each table (example in Annex I) with detailed identification including Cage number (refers to a mating pair - always a duo for core breeders), Room and Rack encompassing its husbandry process.

Both male and female are identified (ID) and preferably, pending on availability, are brother and sister from the same litter. Date of Birth (DOB) is also always present.

It is also possible to observe the data pertaining the litters produced by the pair adding to the DOB, the date of weaning (DOW) and the proportion of males and females at weaning. There is also a basic statistic portion with totals and the average of pups per litter. To decrease the possible error this data was disregarded while compiling the database and was calculated again within the new database. The data to be analysed started on February 22nd, 2012 (the first new breading pair of the year) up to December 31st, 2019 (counting only the terminated cages). As standard operating procedure the facility usually terminates breeding pairs after a year or, if there are no litters produced, after 3 months. All breeding pairs were counted even if there were no litters produced.

3.4.1. Reproductive Performance of IGC C57BL/6J mice

In this subsection of Phase II, data on C57BL/6J core breeder at IGC was compiled in a GraphPad database[155] and exploratory data analysis (descriptive statistics) was performed. The data collected aimed to be compared with the most recent records published by the company that supplies the specific strain (and substrain) under study to populate the core breeding in the institute. (Figure 3.1).

Strain of JAX® Mice (stock number)	Number of breed-ing pairs	Number of pups weaned per female (mean)	Number of litters per female* (mean)	Maternal age (days)		Litter size (pups)		Percent wean: born‡	Percent females weaned	Maternal age (months) when last litter weaned§ (mean, range)	Dates of data gen-eration (mo/yr)
				At pairing (range)	Birth of first litter (mean)	At birth (mean)	At wean† (mean)				
C57BL/6J (000664)	50	29.5	5.4	24-31	67	5.9	5.5	92	51	8.3 (5.7-11.2)	12/05-7/07

*The number of litters produced during the optimal breeding period (see § below), not the total number of litters a breeding pair *can* produce. (Includes litters for which no pups were weaned.)
†Includes litters from which no pups survived (weaned litter size = 0) and litters with more pups weaned than "born," which occurred occasionally due to undercounting litter size at birth.
‡Excludes litters with more pups weaned than "born" to provide a more accurate estimate of neonatal mortality.
§A breeding pair was retired when the caretaker judged that the period of optimal breeding performance had ended for that breeding pair, based on experience and general guidelines for the strain.

Figure 3.1 – Summarizing table of C57BL/6J reproductive performance according to JAX

Adapted from Currer, J. *The Jackson Laboratory Handbook on Genetically Standardized Mice*.p138

Due to specific standard operating procedures, regarding animal welfare, in the rodent facility there is no data of litter size at birth so the variables “Litter size at birth” and “Percent wean: Born” could not be assessed.
Keeping these results in mind and to facilitate result comparison, in this study the same variables were assessed.

3.4.2. Reproductive Performance - A group comparison

In the data provided by the rodent facility there were also records on the reproductive performance of new mice, bought to repopulate the breeding to ensure minimal genetic drift. These mice were thought to be a possible control group with distinct EDC environmental exposure background. The information on these animals was also compiled in a GraphPad database[155] .
Initially, two distinct groups were created and compared using exploratory data analysis techniques (location & dispersion measurements) and hypothesis testing. The latter varied accordingly with data homoscedasticity and distribution. Variables tested were “Number of Litters *per* Female”, “Litter size (pups) at Wean”, “Male/Female Pup Ratio”, “Number of Pups Weaned *per* Female”, “Difference between 1st Mating and 1st Litter in days” and “Difference between 1st Mating and Last Litter in days”. Variables were assessed and classified regarding type and scale and can be consulted on Appendix I[154]. The Shapiro–Wilk test revealed that in all variables one group did not follow a normal distribution, so a non-parametric approach was implemented. The non-parametric Wilcoxon Test was used to verify if there were significant differences for a significance level of 0,05. Exploratory data analysis and hypothesis testing were performed in R.[156]

3.5. Phase III

For the third and final phase of this study, surplus wild-type male and female animals of *Mus musculus* strain C57BL/6J, kept on a 12-hour light:12-hour darkness regimen under specific pathogen-free (SPF) conditions with mouse feed and water freely available, were provided in set time points by the institute rodent facility.
These mice are collected specifically from the core breeders of this strain to maintain the relation with Phase I and II of the project.

3.5.1. Ethical considerations

In this phase of the present study animal were sacrificed with the purpose of collecting samples for analysis. As in all cases where sentient beings are involved there are ethical and legal considerations to take in account. As such, and even though according to *Decreto Lei* nº113/2013[157] (document that regulates laboratory animal use for research purposes in Portugal and transposes the European Directive nº2010/63/UE to Portuguese juridical order) the aim of this project would not require submission to *Direcção-Geral de Alimentação e Veterinária* (DGAV) or the institute's ethical commissions, a technical/legal opinion was requested to the Model Organisms Facility Head of Unit obtained with a positive ruling. (Annex II)

In short, the use of laboratory animals exclusively to collect organs is not considered a procedure and, as such, does not require approval from the competent authorities.

Even so, the institute and its rodent facility are invested in animal welfare with the support of *Orgão Responsável pelo Bem-Estar Animal* (ORBEA; in english Animal Wellfare Organism – AWO) and in this study, as in all of the institute's procedures, the 3Rs principle is upheld.

3.5.2. Sample collection and preparation

Histological analysis was performed using unbiased stereology methods for both ovaries and testis in two different time points: neonates (at most in day 1 with no direct exposure to EDCs) and at seven weeks (average age the mating pairs are set in the institute).

Sample collection methods were age specific as both the recommended euthanasia and dissection methods differ.

3.5.2.1. C57BL/6J mice: Neonates

Pregnant females are routinely monitored, and two surplus litters were selected for this study in a total of 14 pups (males, n=8 and females, n=6). The full litter was collected as soon as the birth was detected and, in this specific time point, animals were sacrificed by decapitation with sharp scissors. This method of euthanasia was selected based on DGAV recommendations. Injection of chemical anaesthetics (e.g., pentobarbital), inhalation of carbon dioxide (CO_2) or isoflurane from a vaporizer (used with appropriate safety considerations) are appropriate but were not considered as all require a

secondary physical method of euthanasia to ensure death (for example decapitation) and all, excluding CO_2, have higher associated costs[158].

Recommendations state that decapitation should be used as a last resort method[157], however neonates' resistance to hypoxia at this age may result in a prolonged time to unconsciousness, with exposure times coming up to 50 minutes[159]. In addition, decapitation was the recommended method of euthanasia by the experienced personnel in the rodent facility.

Freezing in liquid nitrogen wasn't considered due to the ice damage it would induce in the samples for histopathology analysis[160].

To facilitate the specific approach to dissection, pups were checked to determine gender using the method described by Wolterink-Donselaar *et al,* 2009. In male neonates and one day old neonates a pigment spot on the anogenital region is visible to the naked eye[161]. After gender determination the pup was euthanized, the head discarded, and the torso was placed upside down on tissue paper to remove the blood (to facilitate dissection). Pups were then placed on a cork base, attached with pins on both front and hind limbs and place under a Nikon® Stereo Microscope SMZ800N. Both testis (from males) and ovaries (from females) were collected to separate containers (Left – L and Right – R), fixed in *Bouin's* fixative (Sigma – Aldrich® HT10132-1L) for 24h at room temperature, routinely processed for paraffin embedding on Leica Biosystems® HistoCore PEARL Tissue Processor (Protocol in Annex III) and embedded with a random orientation. The workflow can be observed on Figure 3.2.

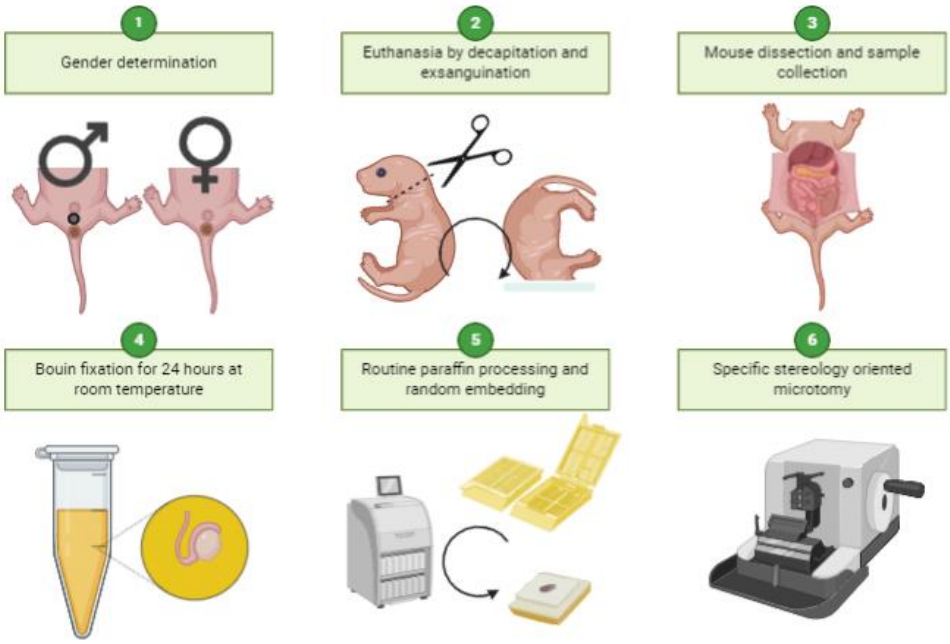


Figure 3.2 – Workflow schematics for neonate sample collection and preparation

Figure originally created with BioRender.com

3.5.2.2. C57BL/6J mice: Seven weeks

For the collection of testis and ovaries in seven-week mice, surplus animals with the appropriate age were selected in a total of 10 animal (5 males n=5; 5 females n=5) and euthanized by CO_2 inhalation.

Due to the size of the animals at this age both the dissection and sample collection were less complex.

Testis samples are very easy to remove without damaging however, ovaries are very small organs and to guaranty that there was no damage produced by the tweezers or scissors during collection the terminal portion of the uterine horn and oviduct were also collected. In most cases the ovary fat pad was also collected.

Both testis (from males) and ovaries (from females) were collected to separate containers (left – L and night – R), fixed in *Bouin's* fixative (Sigma – Aldrich® HT10132-1L) for 48h at room temperature and routinely processed for paraffin embedding on Leica Biosystems® HistoCore PEARL Tissue Processor (Protocol in Annex III). The workflow can be observed on Figure 3.3.

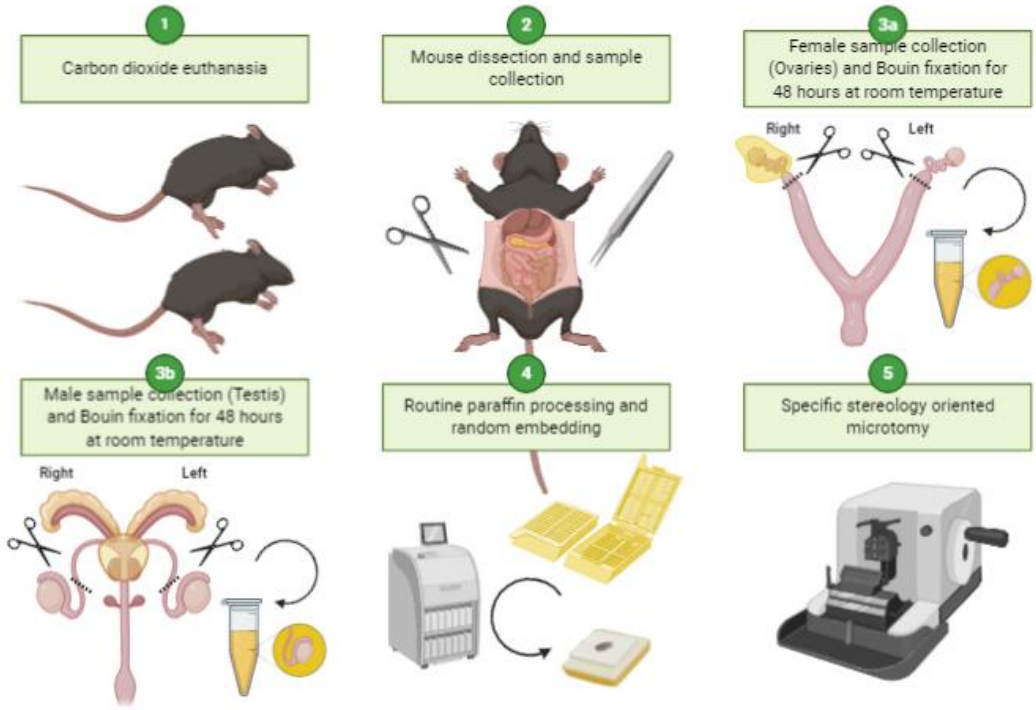


Figure 3.3 – Workflow schematics for seven weeks sample collection and preparation

Figure originally created with BioRender.com

As described for neonates, samples were embedded with a random orientation. However, unlike neonate samples the orientation was purposely randomized.

During the embedding process samples were placed in the bottom of the embedding mould taking special care to orientate the sample randomly. Both testis and ovaries (with the collected annex structures) are ellipsoid and both tend to rest with the bigger axis parallel to the bottom of the mould, restricting the orientation of sectioning.

To counteract this effect, samples were attached to the bottom of the embedding mould rotating the position of sample and assuring that the next one would be different. (Figure 3.4).

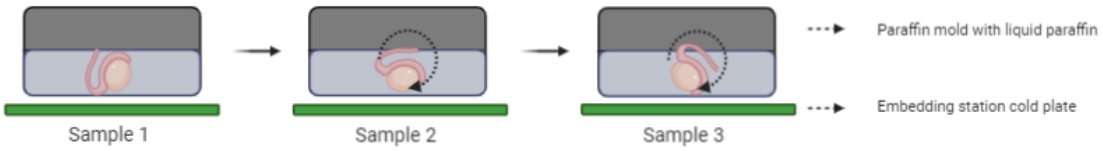


Figure 3.4 – Schematics for seven weeks testis sample embedding with random orientation

Figure originally created with BioRender.com

3.5.3. Sectioning, staining and stereology methods

Samples were exhaustively sectioned on a Leica Biosystems® HistoCore AUTOCUT microtome. A sampling strategy was defined for each specimen with the goal to collect 8 to 10 pairs of sections, as recommended[162]and, at every defined sampling interval (x µm), two sequential sections were collected onto glass slides (as described below). The first section was de nominated the 'reference section' and the second corresponded to the 'look-up section'. Sections were then routinely stained with Haematoxylin & Eosin stain (HE) (Protocol in Annex IV) and scanned on Hamamatsu® NanoZoomer-SQ Digital slide scanner with a 40x objective. Low magnification examples for each sample and time point can be seen on Figure 3.5. Measurements were made using the newCAST stereological software (Visiopharm, Hørsholm, Denmark).

The measures were considered acceptable with a coefficient of error between 2% and 5% [152]for volume and below 10% for number estimation[163]. The event count was

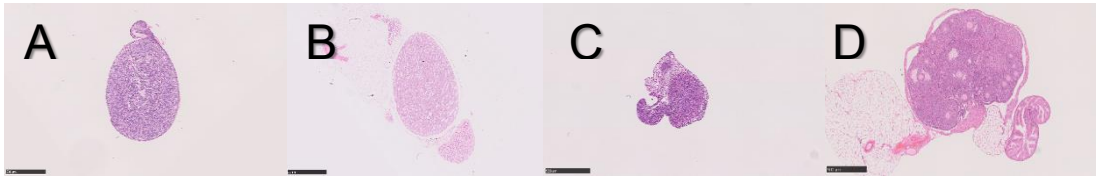


Figure 3.5 – Low magnification microphotographs representative for each group stained with HE

A: Neonate testis at 20x magnification; B: Seven weeks testis at 2x magnification; C: Neonate ovary at 20x magnification; D: Seven-week ovary at 20x magnification.

expected to be in the 100-200 interval[152]. Visiopharm® software was used to estimate both sample volume and object number.

3.5.3.1. C57BL/6J mice: Neonates - Testes

On neonate testes samples, one sample (exploratory test) was exhaustively sectioned at 3 µm to assess sample size and decide the sampling strategy. After obtaining 168 sections (totalling 504µm), and to respect the set goal, every 16th and 17th section was selected systematically from a random uniform starting point for volume and number estimation. (Random number tables in Appendix II). A fractionator/physical disector design[150] was used to sample tissue in conjunction with an unbiased counting frame[149] as described by Myers[136]. The area of the counting frame and the step length was optimized to give the most efficient counting protocol for both volume and number estimation.

Following the validation of the sampling method, samples from 8 animals (n=8, including both left and right testes) were sectioned following the established protocol. The remaining tissue between selected sections was discarded.

Neonates – Testes: Volume estimation

Volume estimations (n= 7) were performed according to the *Cavalieri* principle, using only the first section from each pair of sections (reference section). These measurements were performed with a 20x magnification with a point grid composed of 9 points (3x3), each one associated with an area of 28613,44 µm² (area per point: a/p). The percentage counted for this level of sampling was 50%. The shape factor used for testicular sections was 3, which represents cut-sections of approximately circular, ellipsoid, profiles.

The automatic output of the estimated volume was calculated with the formula in Figure 3.6:

$$V = T\ a/p \sum P$$

Figure 3.6 – Calculating formula for volume estimation using the *Cavalieri* method in Visiopharm

Mathematical formula where V (Estimated volume); T (section distance); a/p (area per point) P (number of events counted)

Neonates – Testes: Gonocyte number estimation

To assess reproductive potential, gonocytes were counted through a combination of fractionator and physical dissector methods. (n= 5).

On these test samples, every 16th section was nominated the “reference section” and the 17th the “look up” section, resulting in a sampling interval of 48 µm. The fields were automatically generated with a 40x magnification and a sampling grid composed of squares and a known sampling fraction within the section, in this case 10%. These fields were generated following a systematic uniform random fashion, with a constant distance (step length) in the X- and Y-axis of 641,90 µm. A counting frame with an area of 46514,71 µm² (241,13 µm x 192,90 µm) was used. Number estimations are performed by counting the number of cells that are present in the reference section, but not on the look-up section. To double the sampling fraction at this level of sampling, the analysis was performed in both directions (reference->look-up, as well as look-up->reference).

The automatic output of the estimated cell number was calculated with the formula in Figure 3.7:

$$N = \frac{1}{bsf}\frac{1}{ssf}\frac{1}{asf}\sum Q^{-}$$

Figure 3.7 – Calculating formula for number estimation using the physical fractionator method in Visiopharm

Mathematical formula where N (Estimated cell number), bsf (block sampling fraction), ssf (section sampling fraction), asf (area sampling fraction), Q^{-} (counted objects)

The gonocytes counted were classified according to the following morphological characteristics (Figure 3.8):

- Nucleus was equated to cell, meaning that cells with only the cytoplasm visible were not counted;

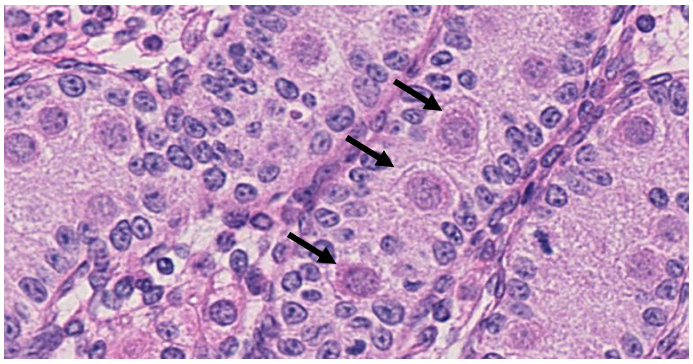


Figure 3.8 – Gonocytes (Black arrow)

Neonate mouse testis histology section stained with HE at 40x magnification

- Located within the centre of seminiferous cords migrating to the periphery as they develop [164];
- Large cells with a spherical euchromatic nucleus, two nucleoli and a surrounding, ring-like cytosol; [127,128]
- Eosinophilic and vast cytoplasm [128].

3.5.3.2. C57BL/6J mice: Neonates - Ovaries

On Neonate ovary samples one sample (exploratory test) was sequentially sectioned at 3µm in full to assess sample size and decide the sampling strategy. After obtaining 221 sections (totalling 363µm), sections were stained with HE and scanned. Every 12th and 13th section was selected systematically from a random uniform starting point for volume and number estimation. (Random number tables in Appendix II)
Following the validation of the sampling method, samples from 6 animals (n=6, including both left and right testes) were sectioned and both validated methods (for volume and number estimation) were applied.

Neonates– Ovaries volume estimation

Ovary Volume measurements (n= 6). were performed with a 60x magnification and a point grid composed of 9 points (3x3), each one associated with an area of 3179,27 µm² (area per point: a/p). The percentage counted for this level of sampling was 50%. The shape factor used for ovary sections was 4, which represents cut-sections of approximately ellipsoid, bean-like, profiles.
The automatic output of the estimated volume was calculated with the same formula previously described.

Neonate – Ovaries primordial follicle number estimation

To assess reproductive potential, primordial follicles were counted through a combination of fractionator and physical dissector methods (n= 5).
On these test samples, every 12th section was nominated the “reference section” and the 13th the “look up” section, resulting in a sampling interval of 36 µm. The fields were automatically generated with a 100x magnification and a sampling grid composed of squares and a known sampling fraction within the section, in this case 10%. These fields were generated following a systematic uniform random fashion, with a step length of 160,47 µm. A counting frame with an area of 2575,21 µm² (56,74 µm x 45,39 µm) was

used. Number estimations were performed in both directions and trough the disector method as previously referenced.

The primordial follicles counted were classified according to the following morphological characteristics, the same used for the seven-week time point.

- Oocyte nucleus was equated to primordial follicle, meaning that follicles with only the oocyte cytoplasm visible or only squamous granulosa cells with no oocyte nucleus were not counted;
- Oocyte surrounded by a partial or complete layer of squamous granulosa cells [117];
- Oocytes not yet enclosed in primordial follicles or oocytes in or external to the surface epithelium were also counted [163];
- Oocytes with predominantly cuboidal granulosa cells are classified as primary and thus excluded from counting[136]. (Figure 3.9):

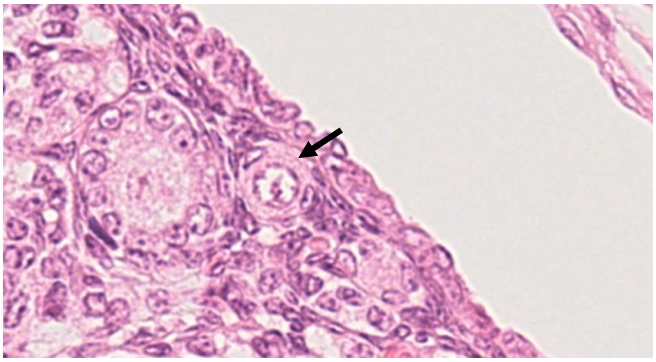


Figure 3.9 – Primordial Follicles (Black arrow)
Seven weeks mouse testis histology section stained with HE at 100x magnification

3.5.3.3. C57BL/6J mice: Seven weeks - Testes

On 7 weeks testes samples, two samples (exploratory test) were sectioned at 2 µm to assess sample size and decide the sampling strategy. Initial sampling methodology of P.J Baker e P.J. O'Shaughnessy [165] was applied to paraffin sections. Due to the high number of slides obtained, (35 and 32 for each sample respectively) an extra sampling was performed. Slides were selected with sampling fraction of 1/6 (with a random starting point between 1 and 6), resulting in 13 and 12 slides analysed per sample.

This extra sampling allowed for counting on 8 to 10 pairs of sections, as recommended [162].

Following the validation of the sampling method, samples from 5 animals (n=5, including both left and right testes) were sectioned from a random uniform starting point, collecting

every 150th and 151st section. The remaining tissue between selected sections was discarded.

Seven weeks – Testes volume estimation

Volume estimations (n= 5) were performed with a 5x magnification with a point grid composed of 6 points (3x2), each one associated with an area of 686 722,42 µm². The percentage counted for this level of sampling was 50% and the measures were considered acceptable for the same ranges mentioned for volume before. The shape factor used for testicular sections was 3, which represents cut-sections of approximately circular, ellipsoid, profiles.

The same *Cavalieri* formula previously mentioned was used to calculate the automatic output of the estimated volume.

Seven weeks – Testes Leydig cells number estimation

To assess cell number, Leydig cells were counted through a combination of fractionator and physical dissector methods. (n= 5).

On these test samples, every 150th section was nominated the "reference section" and the 151st the "look up" section, resulting in a sampling interval of 300µm. The fields were automatically generated with a magnification of 100x and a sampling grid composed of squares and a known sampling fraction within the section, in this case 1%. These fields were produced following a systematic uniform random fashion, with a constant step length of 2870,76 µm. A counting frame with an area of 6592,54 µm² (90,78 µm x 72,62 µm) was used.

The automatic output of the estimated cell number was calculated with the same formula previously mentioned.

The Leydig cells counted were classified according to the following morphological characteristics:

- Nucleus was equated to cell, meaning that cells with only the cytoplasm visible were not counted;
- Round nucleus with dispersed chromatin, with one or two nucleoli at the periphery;
- Extensive eosinophilic cytoplasm [117];
- Leydig cells are in the interstissium, outside of the protective blood–testis barrier [119];

- Occurring singly or in clusters [117]. (Figure 3.10)

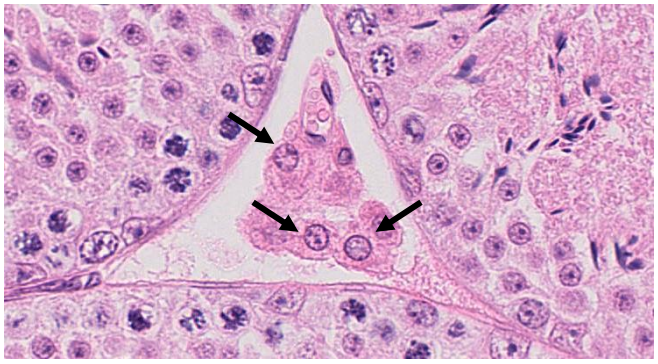


Figure 3.10 – Leydig Cells (Black arrow)

Seven weeks mouse testis histology section stained with HE at 40x magnification

3.5.3.4. C57BL/6J mice: Seven weeks - Ovaries

On 7 weeks ovary samples, two samples (exploratory test) were sectioned at 3 µm to assess sample size and decide the sampling strategy. Initial sampling methodology of Myers[136]was applied to paraffin sections. Due to the high number of slides obtained (52 and 68 for each sample respectively) an extra sampling was performed with the same approach used in *Seven weeks – Testes* section. The sampling fraction used was 1/6 (with a random starting point between 1 and 6), resulting in 14 and 13 slides analysed.

Following the validation of the sampling method, samples from 5 animals (n=5, including both left and right ovaries) were sectioned from a random uniform starting point, collecting every 30th and 31st section. The remaining tissue between selected sections was discarded.

<u>Seven weeks – Ovaries volume estimation</u>

Volume estimations (n= 5) were performed twice for these samples with a magnification of 20x. In the first trial a point grid composed of 6 points (3x2), each one associated with an area of 42 920,15 µm² (area per point: a/p) and a second one with 4 points (2x2) each one associated with an area of 64 380,22 µm² .The percentage counted for this level of sampling was 50% and the measures were considered acceptable if a coefficient of error between 2% and 5% was achieved and an event count in the 100-200 interval. The shape factor used for ovarian sections was 4, which represents cut-sections of approximately ellipsoid, bean-shaped, profiles.

The same *Cavalieri* formula previously mentioned was used to calculate the automatic output of the estimated volume.

Seven weeks – Ovaries primordial follicle number estimation

To assess reproductive potential, primordial follicles were using the same methodology, although with a different sampling strategy as neonate ovaries (n= 5).
On these test samples, every 30th section was nominated the “reference section” and the 31st the “look up” section, resulting in a sampling interval of 90 µm. The fields were automatically generated with a magnification of 60x and a sampling grid composed of squares and a known sampling fraction within the section, in this case 50%. These fields were generated following a systematic uniform random fashion, with a step length of 191,38 µm. A counting frame with an area of 18312,60 µm² (151,30 µm x 121,04 µm) was used. Number estimations were performed in both directions and trough the disector method as previously referenced.
The cells (primordial follicles) counted were classified according to the same morphological characteristics as used for neonate ovaries with the added location reference that the primordial follicles, in the adult mouse are located in the periphery of the ovary. [117,136,163]

3.5.3.5. Statistics

Phase III, as an exploratory study, also required descriptive statistics and results were presented as Mean ± Standard Error of the Mean for volume and number estimation. Possible differences between Left and Right organs were also assessed. Two groups were created (Left and right) and compared using exploratory data analysis techniques (location & dispersion measurements) and hypothesis testing. The latter varied accordingly with data homoscedasticity and distribution. For hypothesis testing, considering the objective of comparing two organs from the same animal the measurements were considered to be paired. To assess differences between the left and right organs data distribution was assessed and Shapiro–Wilk test revealed that variables did not follow a normal distribution, so a non-parametric approach was implemented. The non-parametric Wilcoxon Test was used to verify if there were significant differences for a significance level of 0,05. Variables were assessed and classified regarding type and scale and can be consulted on Appendix I[154]. Exploratory data analysis and hypothesis testing were performed in R.[156]

3.5.4. Cost assessment

To assess the cost effectiveness of the techniques applied the current price list at the Histopathology Facility (Annex V) was used and average cost per sample per object of study (Volume/Number) was calculated. Technician time is reflected in the items price, however, time taken to perform the specific stereological counts was measured in hours and charged as “Pathology assessment” time as this specific step is routinely performed by the resident pathologist. Results were presented as Mean ± Standard Deviation. Variables was assessed and classified regarding type and scale and can be consulted on Appendix I[154].

4. Results

As defined in the 3. Materials and Methods section, the present study was divided in three main phases (I, II, and III) and, as so, the results are presented accordingly.

4.1 Phase I

The first part of the present study consisted in the elaboration of a comprehensive list of the known EDCs present in the production of C57BL/6J mice in the institute with submission to the list of choice[16] to ascertain the EDC status of each component.

After submission, no component matched with the list used indicating that no component was considered an EDC in any of the three lists (I, II, or III). A comprehensive table with all the results was placed in Appendix III.

To try and circumvent this pitfall all components were searched in the available literature trough PubMed with the search terms “endocrine disruptor” + “component” and a table with the relevant results was elaborated (Table 4.1). The products are colour coded for Personal Protective Equipment (Blue), Diet (Yellow), Environmental Enrichment (Red) and Housing (Grey):

Table 4.1 – Possible EDCs in C57BL/6J production

Composition	Products	Observations	Literature Review
2-Phenylphenol	Tunel carton 100mm	o-Phenylphenol (synonym)	[166]
Aflatoxin B1	SizzleNest (Enviro-dri) Cocoons Mouse Igloo Red + Mouse Wedge Tunel carton 100mm		[167,168]
Aldrin	Mouse Igloo Red + Mouse Wedge		[169,170]
Arsenic (As)	SizzleNest (Enviro-dri) Cocoons Mouse Igloo Red + Mouse Wedge Tunel carton 100mm		[171–173]
Cadmium (Cd)	Cocoons Mouse Igloo Red + Mouse Wedge Tunel carton 100mm		[174–176]

Table 4.1 (cont.)– Possible EDCs in C57BL/6J production

Composition	Products	Observations	Literature Review
Cis-chlordane/ Trans-chlordane	Mouse Igloo Red + Mouse Wedge		[177] (1)
Cypermethrin	SizzleNest (Enviro-dri Tunel carton 100mm)		[178–180]
De-hulled Extracted Toasted Soya	Rat and Mouse No.3 Breeding Autoclavable	Phytoestrogens: Genistein (List II)	(2)
Diazinon	Mouse Igloo Red + Mouse Wedge		[39,181]
Dieldrin	Mouse Igloo Red + Mouse Wedge		[169,182]
Dithiocarbamates	Glove Semperguard® Nitrile Xpert powder-free		[183,184]
Full Fat Soya	Rat and Mouse No.3 Breeding Autoclavable	Phytoestrogens: Genistein (List II)	(2)
Gamma-hexachlorocyclohexane (lindane)	Mouse Igloo Red + Mouse Wedge		[182,185,186]
Heptachlor	Mouse Igloo Red + Mouse Wedge		[187,188]
Heptachlor epoxide	Mouse Igloo Red + Mouse Wedge		[188]
Hexachlorobenzene (HCB)	Mouse Igloo Red + Mouse Wedge		[189–192]
Lead (Pb)	SizzleNest (Enviro-dri) Cocoons Mouse Igloo Red + Mouse Wedge Tunel carton 100mm		[174,175,193–195]
Malathion	Mouse Igloo Red + Mouse Wedge		[196,197]
Mercury (Hg)	SizzleNest (Enviro-dri) Cocoons Mouse Igloo Red + Mouse Wedge Tunel carton 100mm		[174,175,193–195]
Mirex	Cocoons		[198]
Nitrate/nitrite	SizzleNest (Enviro-dri) Cocoons Tunel carton 100mm		[199,200]
o,p'-DDD(o,p'-TDE);o,p'-DDE;o,p'-DDT;p,p'-DDD (p,p'-TDE);p,p'-DDE;p,p'-DDT	Mouse Igloo Red + Mouse Wedge		[94,201–203]

(1) Study of possible connection to Autism;(2) Known subcomponent/derivate submitted to the list;

Table 4.1 (cont.)– Possible EDCs in C57BL/6J production

Composition	Products	Observations	Literature Review
Parathion-methyl	Cocoons		[182]
Pendimethalin	SizzleNest (Enviro-dri)		[204]
Permethrin	Tunel carton 100mm		[205]
Phorate	Mouse Igloo Red + Mouse Wedge		[206,207]
Piperonyl butoxide	Tunel carton 100mm		[208,209]
Policarbonate	Mouse Igloo Red + Mouse Wedge	BPA (List I)	(2)
Polychlorinated biphenyls (PCBs)	SizzleNest (Enviro-dri) Cocoons Mouse Igloo Red + Mouse Wedge Tunel carton 100mm	References for: PCB 28, PCB 52, PCB 101, PCB 138, PCB 153, PCB 180	[13,210–214]
Polypropylene	PPE: 3-Ply Mask, Blue Clip Cap		[215,216]
Polysulfone	Type III-D cage bottom in polysulfone Type II-C cage bottom in polysulfone Type III-D Lid in polysulfone Type II-C Lid in polysulfone	Old cages in use: Policarbonate - BPA(List I)	(2,3) BPS [217,218]
Selenium (Se)	Mouse Igloo Red + Mouse Wedge		[219,220]
Soya Oil	Rat and Mouse No.3 Breeding Autoclavable	Phytoestrogens: Genistein (List II)	(2)
Zinc (Zn)	Tunel carton 100mm		[175,221,222]
Zinc mercaptobenzothiazole	Glove Semperguard® Nitrile Xpert powder-free		[223,224]

(1) Study of possible connection to Autism, no more references found;(2) Known subcomponent/derivate submitted to the list;(3) Known subcomponent/derivate not listed but referenced in literature;

Even though housing and diet were already expected to figure in this list, as described in the literature, most of the results indicating a possible EDC are not from components but from contaminants detected in environmental enrichment.

4.2 Phase II

In the second stage of the present study statistical analysis of the Reproductive Performance of the C57BL/6J core breeders under the institute production room conditions was assessed. A total of 134 breeding pairs were used for this reproductive performance analysis. In this group 33 breeding pairs (n=33) were composed by animals externally purchased to populate the breeding's and 101 were in-house breeding pairs (n=101):

4.2.1 Reproductive Performance of IGC C57BL/6J mice

As it was not possible to guaranty that the externally purchased animals had the same environmental exposure to EDCs as the in-house ones, for the descriptive statistical analysis and comparison with the supplier's data, only the 101 in-house breeding pairs were utilized (n=101). Results can be observed in Table 4.2.

Table 4.2 – Descriptive statistical analysis and comparison of C57BL/6J reproductive performance

Data Source	Number of breeding pairs	Number of pups weaned per female (mean)	Number of litters per female (mean)	Maternal age (days)	
				At pairing (range)	Birth of the first litter (mean)
JAX[85]	50	29,5	5,5	24-31	67
IGC	101	23,4	5,9	38-219	96
Data Source	Litter size (pups)		Percent wean born	Percent females weaned	Maternal age (months) when last litter weaned (mean, range)
	At birth (mean)	At wean (mean)			
JAX[85]	5,9	5,5	92	51	8,3 (5,7-11,2)
IGC	-	4,9	-	50	13,2 (3,4-16,2)

4.2.2 Reproductive Performance - A group comparison

While assessing differences between the IGC mice (n=101) and the external animals control group (n=33) the non-parametric Wilcoxon Test (α=0,05) found no significant differences between the two sources of animals in “Number of Litters *per* Female” (*p*=0,77), “Litter size (pups) at Wean” (*p*=0,93), “Male/Female Pup Ratio” (*p*=0,94), “Number of Pups Weaned *per* Female” (*p*=0,33) and “Difference between 1st Mating and 1st Litter in days” (*p*=0,50). However, for “Difference between 1st Mating and Last Litter in days” there were enough evidences to conclude that there are significant differences regarding the time-span between the mating date and the last litter weaned between IGCs C57BL7/6J mice and the ones bought from the supplier (*p*=0,03).

4.3 Phase III

In line with the previous two phases, this study also aimed to implement a reliable, and relatively inexpensive, method to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute. A full table with the individual results per sample can be consulted in Appendix II.

Relevant results for the two time-points (neonates and seven weeks) and both female (ovaries) and male (testes) gonads are presented below:

4.3.1 C57BL/6J mice: Neonates - Testes

Neonate testes histology showed *tunica albuginea* surrounding the testicular parenchyma composed of seminiferous cords containing germ cells (gonocytes) (Figure 4.1). These gonocytes were located, mainly, in the centre of the seminiferous cords. However, it was possible to observe gonocytes with locations closer towards the basal membrane. Sertoli cells, line the interior testis cords and in the interstitium, Leydig cells, peritubular myoid cells, macrophages, vasculature, and other cell types such as fibroblasts and vascular-associated cells can be observed.

Volume results are expressed in mm^3 in the format of Mean ± Standard Error of the Mean (S.E.M.).

Mean of estimated sample volume (n=7), with event counts between 89 and 136 was 0,3188±0,0111mm^3. For gonocytes estimated number(n=5), with event counts between 118 and 178, was 11 576±556.

Regarding the paired sample hypothesis test, there was no evidence to say there were any significant differences between left and right neonate testis (p=1,00) Absolute values can be consulted in Appendix II.

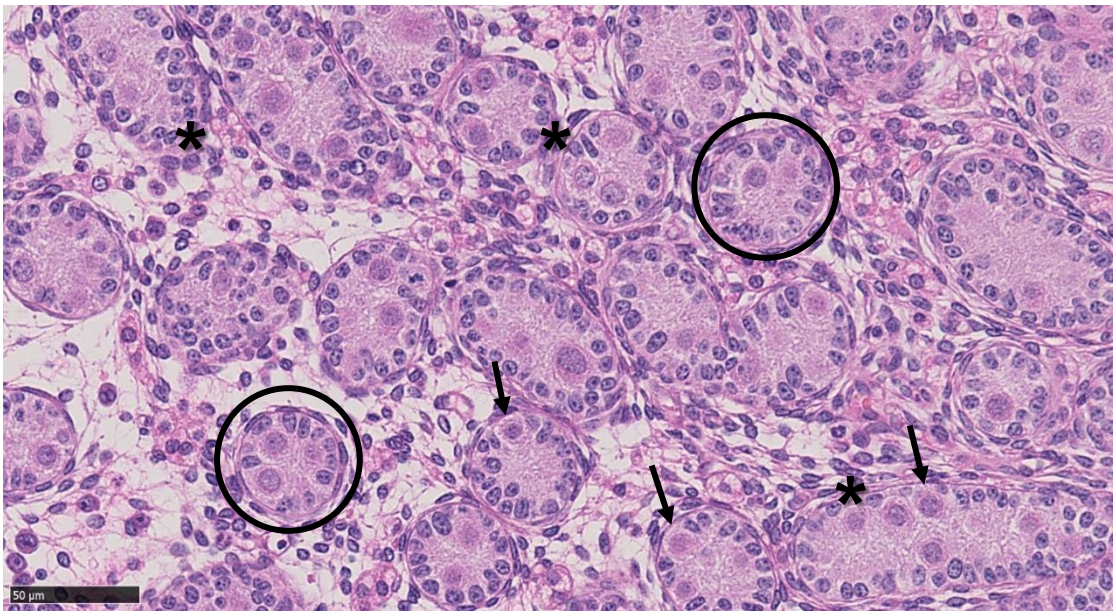


Figure 4.1 – Neonate testis

Neonate mouse testis histology section stained with HE at 40x magnification; Gonocyte in the center of the seminiferous cords (black circles); Gonocytes with locations closer towards the basal membrane (black arrows); Sertoli cells lining seminiferous cords interior (black asterisks)

4.3.2 C57BL/6J mice: Neonate – Ovaries

In neonate ovary samples, oocytes were present in both medullary and cortical region occasionally forming nests with no granulosa cells associated. However, the majority of oocytes could be identified as a primordial follicle based on their association with flat granulosa cells. The stroma was composed by capillaries, mast cells and pyknotic bodies (compacted, densely staining structures lacking cellular detail). The epithelium

in the ovary periphery was composed by a single layer of squamous or cuboidal cells. Occasionally oocytes were identified lying close to the surface of the ovary (Figure 4.2).

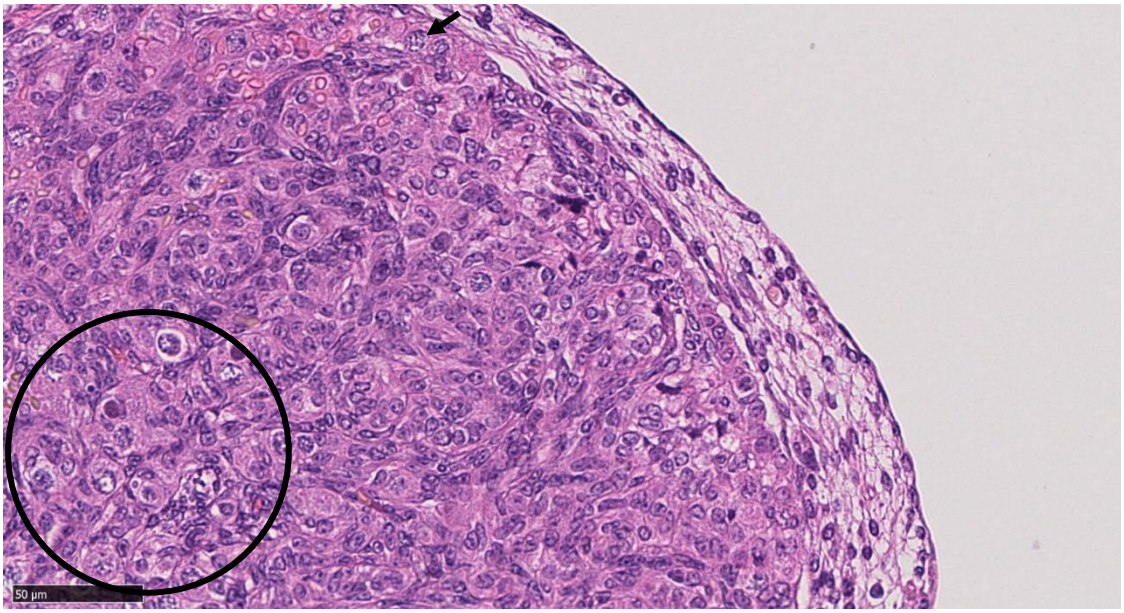


Figure 4.2 – New born ovary

Neonate mouse ovary histology section stained with HE at 20x magnification; Oocytes identified lying close to the surface of the ovary (Black arrows); Oocytes forming nests with no granulosa cells associated. (Black circle)

Mean of estimated sample volume (n=6), with event counts between 100 and 162 was 0,0301±0,0013mm^3. For oocytes and primordial follicle estimated number(n=5), with event counts between 75 and 117, was 5786±265.

Regarding the paired sample hypothesis test, there was no evidence to say there were any significant differences between left and right neonate ovaries (p=0,69). Absolute values can be consulted in Appendix II.

4.3.3 C57BL/6J mice: Seven Weeks – Testes

In seven week, mice testes, *tunica albuginea* surrounded the testicular tissue composed by seminiferous tubules visible in different orientations. Seminiferous tubules were encased in a layered wall of collagen fibres and myofibroblast cells (*lamina propria*) with one layer of peritubular cells surrounding the tubular walls. The seminiferous epithelium contained germ cell during the different steps of differentiation encased, near the basal membrane, by Sertoli cells. In the interstitium small groups of Leydig cells and blood vessels could be observed.

Mean of estimated sample volume (n=5), with event counts between 113 and 154 was 53,8116±1,7634mm^3. For Leydig cells estimated number(n=5), with event counts between 169 and 279, was 1 520 149± 72 080.

Regarding the paired sample hypothesis test, there was no evidence to say there were any significant differences between left and right seven-week testis (p=0,81) (Figure 4.3). Absolute values can be consulted in Appendix II.

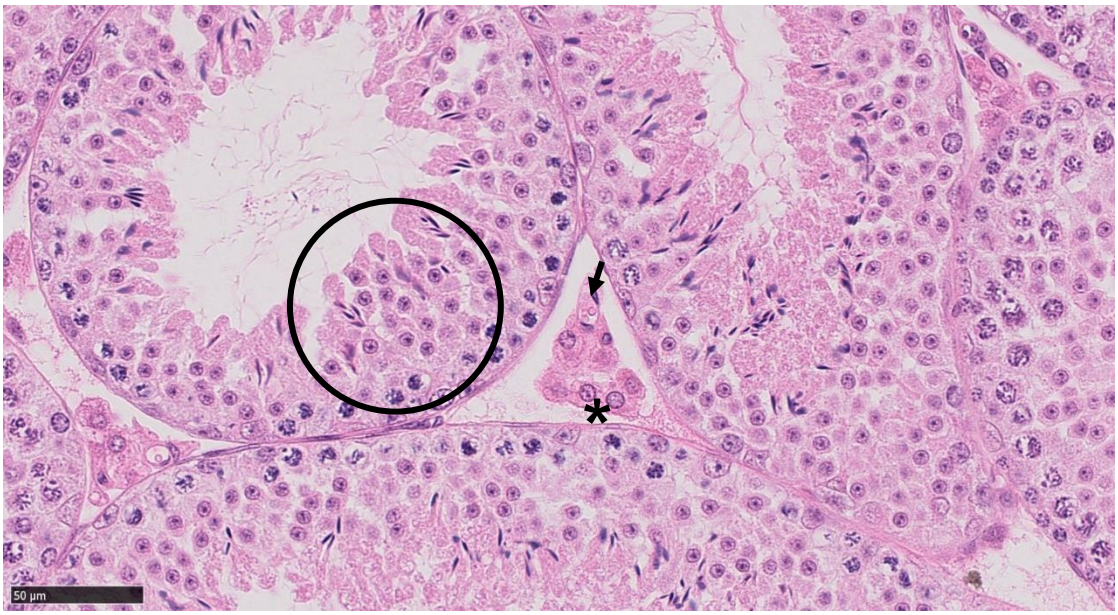


Figure 4.3 – Seven weeks testis

Seven weeks mouse testis histology section stained with HE at 40x magnification; Seminiferous epithelium containing germ cell during the different steps of differentiation (Black Circle); Leydig cells (Black Asterisk), Blood vessels (Black arrows).

4.3.4 C57BL/6J mice: Seven Weeks – Ovaries

At the seven weeks' time point, primordial follicles were normally present on the periphery of the ovary, with rare twin oocytes or oocytes closely apposed lacking intervening granulosa cells. Ovaries appeared normal, containing numerous *corpora lutea* as well as follicles in various stages of development or atresia. The cortical stroma was composed of strands of loose connective tissue supporting scattered interstitial cells and blood vessels. (Figure 4.4)

Mean of estimated sample volume (n=6), with event counts between 113 and 224 were 1,3690±0,0924mm^3. For primordial follicle estimated number(n=5), with event counts between 29 and 81, was 1706±168.

Regarding the paired sample hypothesis test, there was no evidence to say there were any significant differences between left and right seven-week ovaries (p=0,63). Absolute values can be consulted in Appendix II.

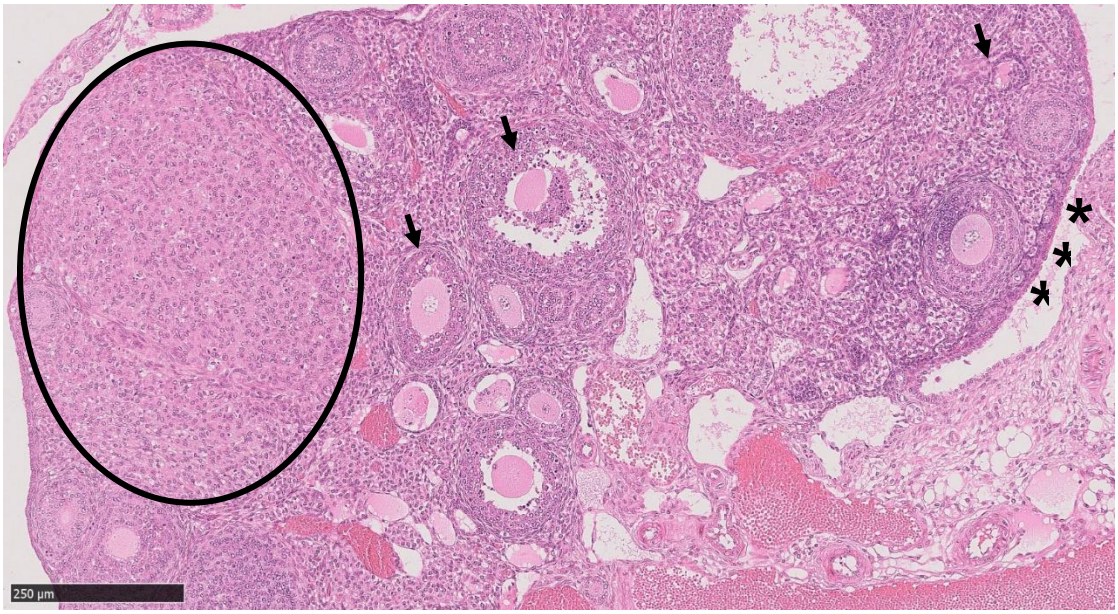


Figure 4.4 – Seven weeks ovary

Seven weeks mouse ovary histology section stained with HE at 10x magnification; Primordial follicles present on the periphery of the ovary (Black asterisks); *corpora lutea (B*lack circle); follicles in various stages of development or atresia (black arrows).

4.3.5 Cost Assessment

As a final objective of the present study, it was planned to implement a reliable, and relatively inexpensive, method to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute. The calculated average cost (with standard deviation represented as ± SD) associated with each sample was 106,18€±39,33€ for volume estimation, 136,36€±45,86€ for number estimation and 245,23€±81,95€ for both.

5. Discussion

As it was previously mentioned, exposure to EDCs has been associated with reproductive effects, neurobehavioral and neurodevelopmental changes, metabolic syndromes, bone and immune disorders, and cancer[4] and it is known that these anthropogenic ubiquitous substances can be found in every individual and ecosystem tested so far [11,12].

Even though a properly controlled environment is key for validation of several models of disease, toxicology testing and microbiota assays, the impact these ubiquitous substances have on the animal production in research settings is still lacking.

The present study aimed to be the starting point in the methods applied to research animal production and maintenance regarding EDCs environmental exposure.

In Phase I, the main hurdle to overcome became one of the most interesting results. The selected list for cross-reference, apparently user friendly, has no use for the facility staff in the rodent facility. None of the 133 components identified in the products labels could be directly identified as an EDC. This list, as many, does not cross-reference the component with possible byproducts. For example, polycarbonate will return a “No Match” result but BPA (a known biproduct of polycarbonate[225]) will be categorized as a List I substance (Substances identified as endocrine disruptors at EU level). This pitfall reveals a need to implement better suited EDC lists for cross-reference but, most importantly, for facility staff, researchers and companies to be more aware of possible EDC content of the products in use in the production and experimentation of research animals. For this all stakeholders need to keep informed on the latest research on this topic.

While compiling the list of components from all the products used in IGC’s C57BL/6J production (the most controlled environment in the rodent facility) the preliminary results were as expected. Already reported sources of EDCs environmental exposure in these facilities such as type of diet, bedding, caging, and water bottles were also present in the production of C57BL/6J mice[226].

The maintenance diet used across the rodent facility is a closed-formula diet were components are listed but not their detailed percentage. In laboratory animals this is considered the primary source of exogenous estrogenic substances[227] and natural dietary ingredients such as soybeans (and derivates) are major dietary ingredients reported to contain phytoestrogens namely daidzein, genistein, and glycitein directly correlated with the percentage of soybean present in the diet[228] that will be converted

to their bioactive forms of daidzein, genistein, and glycitein by intestinal bacteria[229]. Eliminating soy or other natural phytoestrogen rich components from the feed is not feasible, however the use of open-formula feed could be a strategy to better characterize the conditions in which the mice are produced and experimented on.

Caging and water bottles are also a known source of EDCs in the services under study[226].

Particularly, in the production of C57BL/6J mice where conventional polycarbonate cages are still in use the potential environmental exposure to EDCs, specifically BPA, is well documented[225,230]. Recently the conventional polycarbonate cages started to be replaced by polysulfone cages presented as low-BPA. While this is a great effort to try and minimize BPA exposure low-BPA can still mean enough BPA to cause endocrine disruption[231,232]. BPA, in line with other EDCs, follows a non-traditional dose-response dynamic, specifically presenting a non-monotonic u-shaped curve[59,62,65] where negligible levels of exposure (as expected in polysulfone cages) can in fact have higher deleterious effects than the higher levels present in polycarbonate cages. Moreover, BPA tends to be substituted by other, less studied, bisphenols, particularly BPS. Reports already show that BPS can cause similar effects as BPA[233].

Another particular aspect to keep in mind is related to the longevity of these cages. To guaranty a clean environment in the production the cages are washed and autoclaved periodically. With time, wear and high temperatures these plastics tend to deteriorate, increasing the levels of BPA (and/ or BPS) released into the environment [225,226]. To try and circumvent this issue cages should be kept as new as possible, renewed frequently and the amount of BPA (or/and BPS) released should be assessed. There is published evidence that even new cages release bisphenols[225].

Water bottle composition could not be confirmed in time for this study, but often these products are composed by polycarbonate plastics so it is safe to infer that the recommendations for cages should also be applied. In the institute rodent facility, the water purification method in use is reverse osmosis so the water bottles are autoclaved empty and are left to cool down before being filled reducing the leaching of BPA to the water to be consumed by the mice[215,230].

The bedding was not assessed in this project because it has recently been changed to corncob. Regarding this bedding type research shows that, when contaminated by zearalenone (a mycotoxin produced by a variety of fungi belonging to the genus *Fusarium*) estrogenic effects have been detected such as higher aggression when

compared to cardboard-based bedding, fewer estrogen receptor-α-positive cells and suppressed expression of phosphorylated ERK in several brain regions [234].

By themselves, the type of diet, bedding, caging, and water bottles are important sources of environmental exposure to mixtures of EDCs (phytoestrogens, phenols, mycotoxins) however, this study unveiled a possible new source for concern. Environmental enrichment provided to the animals, apparently composed by relatively innocuous material such as cotton and paper, presents a vast list of contaminants present (detected and the list provided by the companies that sell these items). Every contaminant listed was reviewed in available publications trough PubMed and the majority was linked at least once to endocrine disruption and is represented on Table 4.1. The list includes pesticides, herbicides, fungicides and others. It was not the aim of this study to evaluate if the doses and/ or mixtures present would be enough to case endocrine disruption or other physiological, epigenetic or morphologic alterations. However, it is important to keep in mind that all of the above-mentioned EDCs are simultaneously, and trough different exposure routs, contacting with the mice under study. Traditionally, independent action of each EDC has been considered the main principle in EDCs mixture toxicity. However, recent studies have demonstrated co-exposure to these agents, particularly in critical windows, may induce hazardous effects [68]. Alternatively, these mixtures may be having synergistic, additive and/or antagonistic effects between themselves in a multitude of combinations and it is a possibility that these interactions are masking the more extreme effects obtained in toxicology studies of single, isolated EDCs.

As was previously mentioned, exposure to EDCs in a research animal facility context is expected to be in low enough doses that fertility or fecundity are not greatly affected. This seems to be the case while comparing the reproductive data on IGC with the values provided by the supplier of the mice to populate the breeding's as the conditions in which the animals are kept at the institute are not expected to be drastically different from the ones in which the mice commercially available are kept. The "Litter size at Wean" and the "Number of Pups Weaned *per* Female" is slightly lower in IGC mice however IGC mice tend to have a higher "Number of Litters *per* female". These slight discrepancies might be explained by standard operating procedures and condition differences between the IGC and the supplier. In IGC, lower numbers in litter size and "Pups Weaned *per* Female" could be due to less a controlled environment (temperature, humidity, noise) in a building with more than 50 years. On the other side, even though C57BL/6J females are paired later at IGC, these dams produce more litters throughout

their lives. The purpose of the mice produced in a research setting is to be employed by researchers in controlled testing. In a supplier company, animals are produced to supply a vast array of institutes, academia and pharmacology companies. It is possible that animal facility staff allows for longer lifespans at IGC then the supplier companies permit.

Nevertheless, none of these possibilities are confirmed, EDC action cannot be excluded, and further studies are required.

With the comparison between the control group (mice bought from the supplier to populate the breeding's) and IGCs own production most of the results were confirmed. There was no evidence to determine a significant difference between the variables compared. The only case where the variables were found to have significant differences was when comparing the timespan that the females were productive. In the previous portion of this study this apparent difference was credited to technical staff *modus operandi* being different between a research institute and a commercial facility. However, in this control group, the results might indicate a lower reproductive potential, for example, fewer primordial follicles associated with EDC effects[107,235]. To evaluate these findings, it would be relevant to repeat the methods applied in Phase III for primordial follicle count this time from mice directly bought from the supplier at the same timepoints used in this study.

Ultimately, regarding the results obtained in Phase II of this study, it is important to acknowledge that the environmental exposure to EDCs in the supplier's mice conditions are unknown and as so, it was not possible to establish any cause-effect relation between the findings. Likewise, for IGC animals, with no experimental groups isolating the sources of EDC exposure, no comparison can be made.

Moreover, Phase III of this study produced both a reliable and relatively inexpensive technique to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute.

In neonate testes samples general morphology appeared normal and for both the estimated volume and gonocyte number estimation Coefficient of Error was below the established values of 2-5% and 10%, respectively [136,163].

For estimated volume of one neonate testis the results showed 0,3188±0,0111mm^3 and it was in accordance with the available reference[165]. However, this study was not performed in C57BL/6J mice.

For gonocyte number estimation the counts were 11 576±556 concurring with the available reference[236]. However, in this study the C57BL/6 substrain "J" is not referred

so comparison should be made with caution. The viability of the cells was also not an aim of this study and to fully assess fertility potential a starting point could be using the same stereology techniques defined in this project associated with a Ki67, caspase-3 immunohistochemical assay to distinguish proliferating gonocytes (that will give rise to Type A spermatogonia and are Ki67 positive) from apoptotic gonocytes (caspase-3 positive). Quiescent gonocytes should also be counted (no staining with the previous antibodies)[237].

Regarding neonate ovary samples, it was also not possible to find in the literature references for comparison for volume estimation within the same strain, time point and stereology techniques. Therefore, the value obtained for this present study (0,0301±0,0013mm^3) can be used as a reference for future studies.

For primordial follicle number estimation event counts were below the 100-200 interval with a coefficient of error averaging 10% and, primordial follicle counts are slightly lower than the available literature[163]. In the present study results have shown 5786±265 primordial follicles while in the literature the estimate is 7924±1564. This is expectedly due to the difficulty to correctly identify primordial follicles in day 1 neonates, reducing the event count and, consequentially the number estimation. An immunohistochemistry assay with CYP1B1 antibody has proven to facilitate follicle identification[238] but its relevance in neonate ovaries is yet to be determined. Validation of the technique and *a posteriori* association with stereology techniques could improve primordial follicle counts in this time point.

Protocols for seven weeks testes were easily implemented and the results for volume estimation did not find a comparable reference to compare values. Leydig cell number estimation was in concordance with the available literature although the tests were not performed in C57BL/6J mice [165] That being said, the values reported in this study should be better controls for the C57BL/6J strain under IGC conditions.

Finally, for ovary results regarding volume were slightly lower than what was previously reported for C57BL/6N mice with ages between 8-10 weeks[239]. Again, the same issue arises as different sub-strain and different time points require very cautious comparison. Regarding primordial follicle counts the results were also lower than previously reported [163]. In this project the sampling was reduced to roughly one third of what was reported by Myers *et al* (2002) to reduce the amount of work and the costs associated with the stereology assays. In this case event count was under the 100-200 established as a goal and coefficient of error was above 10% [136].

Nevertheless, the results are similar to the ones previously reported (1706±168 in this study and 1976±306 in the literature for C57BL/6J mice at this timepoint[163]).To increase event counts and decrease the coefficient of error two strategies can be approached. For one, decreasing the sampling fraction to obtain more sections per organ (increasing the events counted, reducing the coefficient of error but also the amount of work and the cost per sample) or changing the strategy by previously defining the region of interest as only the periphery of the ovary. This strategy has been previously described [240] and could be one way to get results within the ranges of event count and coefficient of error recommended while maintaining the stereology view of “Do more, Less well”.

While deciding to implement new techniques and strategies to quantify certain parameters it is important to evaluate if the cost-effectiveness is worth it in the user’s perspective. To do so the present study also has calculated the average cost *per* sample. As expected, stereology was able to provide precise, unbiased data, and it is considered the gold standard for quantitative microscopy. This quantitative unbiased data can be obtained at a relatively low price. To evaluate the possibility to further reduce the costs the paired sample hypothesis test was performed, and it concluded that there were no evidences of significant differences between left and right gonads in any of the samples under this study. Thus, it is possible to select only one of the bilateral organs, applying the other for other purposes and still obtaining precise, unbiased quantitative data.

Furthermore, regarding volume results, it is important to refer that due to sample shortage, it was not possible to asses tissue shrinkage, due to fixation and processing, to correct the measurements. This limitation, the relatively low number of samples and the need to add more observers points to the prerequisite to obtain more samples to strengthen the results.

On the other hand, data from cell numbers *per* organ was obtained using unbiased stereological methods that have been validated in mouse[136,163,165]. Results are quantitative estimates that are number-weighted rather than volume-weighted, and are independent of sample volumes, cell size, shape and distribution, and do not rely upon correction factors based on assumptions associated with these parameters or section thickness.

Although stereology is the method of choice to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute it proves to be unpractical for routine EDC exposure assessment as it is too time consuming and expensive for this purpose.

One possible venue to explore was proposed by Kolla *et al* (2017) referring the mouse mammary gland has the potential to be used as a sentinel organ to evaluate and distinguish animal colonies raised in different environmental conditions including distinct potential EDC exposures [241].

Overall, while using a whole mount stain of the mammary gland with carmine alum and quantifying the results it could be possible to perform *a posteriori* evaluation to screen for possible unintentional contamination with EDCs in animal colonies, especially after new materials (feed, caging, water bottles) have been introduced or establish a sentinel like protocol for this type of chemical interference.

As an exploratory study, this project sets a baseline for endocrine disruption assessment in research animals at IGC and implements tools to access the potential effect of this disruption.

6. Concluding Remarks

Exposure to EDCs, as it was aforementioned, has been linked to several effects such as reproductive alterations, neurobehavioral and neurodevelopmental deviations, metabolic syndromes, bone and immune disorders, and cancer[4] and the presence of these, mostly human-made, and pervasive substances can be found in every individual and ecosystem tested so far[11,12]. The deduction that the same facts can be transposed to a research environment is easily obtained.

This project listed the known EDCs present in the production of C57BL/6J mice at IGC, analyzed the data on reproductive performance from 2012 to 2019 and implemented a reliable, and relatively reasonably priced, method to obtain quantitative parameters to act as a baseline for future endocrine disruptor studies and their impact on the research developed in the institute.

However, during this process and as it often happens in research, many questions were answered, and an equal number of new ones were raised.

To the authors' best knowledge this is the first EDC comprehensive list elaborated for these purposes. Nevertheless, the exact composition of each item was difficult to obtain due, in many cases, to the confidentiality of the components of several of the resources used in the facility, limiting the details of the final list.

In Phase I, besides improving the details of the composition to include quantities of each deleterious component, the next step should be determinate the amounts of EDCs present in each resource in use, ascertain if, at these concentrations, any effects are reported and investigate the impact of mixtures with special focus on possible antagonistic mechanisms masking the expected effects for each individual compound.

While analyzing the data on reproductive performance of the C57BL/6J mice in the production of the institute the results, as expected, were in accordance to the ones reported by the supplier reports and recommendations. However, the conditions in which the mice are maintained, although now characterized in the IGC mouse production, are unknown in the supplier and intermediary companies. Further research needs to be done on this portion of the study. In parallel, exploring the differences in the timespan that the females were productive can bring new and relevant data regarding possible alteration in reproductive potential.

Finally, with Phase III of this study the knowledge of stereological techniques to assess gonocyte, Leydig cell and primordial follicular number and, simultaneously, ovary and testicular volume of C57BL/6J mice in both timepoints selected for this study, has

substantially improved. However, there is still a need to increase the number of samples to strengthen the results obtained.

Even though this is a great step to bridge the gap in the knowledge available regarding environmental exposure to EDCs in a research setting, more should be done.

The key to a more controlled environment in a research setting comes from the knowledge of all the possible variables at play.

Overall, data obtained in this study should be used as a tool to further evaluate the potential impact of EDCs on the different studied models of disease, on toxicology testing and microbiota assays. The possible impact of this study, and the subsequent ones might be vast, changing the way biomedical animal research is performed, how the animals are maintained and bred and, possibly, unveiling new endocrine disruption pathways and interactions.

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Annexes

Annex Index

Annex I – Example table from the rodent facility records encompassing the C57BL/6J production core breeders husbandry process of one breeding pair

Table An. I – Table from the rodent facility records

Status	Core Breeding		
Cage	105		
Room	C		
Rack	1-A		
Male			
ID	DOB	Obs.	
1-B997	10/04/12	CX82CB	
Female #1			
1-B999	10/04/12	CX82CB	
Female #2			
ID	DOB	Obs.	
M+F	20/06/12		
Litters			
DOB	**DOW**	**# M**	**# F**
12/07/12	27/08/12	2	3
04/09/12	08/10/12	2	2
30/10/12	04/12/12	3	3
01-01-13	06/02/13	0	5
	FIM (10-04-13)		
TOTAL		7	13
TOTAL pups		20	
Litters		4	
Pups per litter		5,0	
Obs.			
Founder colony. Mice were purchased to Charles River. Pups from these mice will populate the breeding.			

Annex II - Technical review from the current Model Organisms Facility Head of Unit regarding the present project

INSTITUTO GULBENKIAN DE CIÊNCIA

Parecer

Na condição de Coordenador do Biotério do Instituto de Ciência, como membro da Comissão de Ética, e também do Órgão Responsável pelo Bem-Estar Animal (ORBEA) do Instituto Gulbenkian de Ciência (IGC), venho por este meio esclarecer que o projeto a realizar no âmbito do Mestrado em Tecnologias clínico-laboratoriais, na Escola Superior de Tecnologoa da Saúde de Lisboa – Instituto Politécnico de Lisboa, de Joana Carina Ferreira Rodrigues Lóios, Nº de Aluno – 2018643, com o título da Tese: "Endocrine Disrupting Chemicals: Exposure impact in C57BL/6J mice reproductive performance and morphology" não necessita de revisão e aprovação ética por parte nem da Comissão de Ética, nem do ORBEA do IGC, apesar de usar tecidos de murganhos com origem neste Instituto. A base legal para o exposto neste Parecer encontra-se fundamentada no Decreto-Lei n.º 113/2013 (documento que em Portugal regula o uso de animais de laboratório na investigação científica e que transpõe a Directiva Europeia n.º 2010/63/UE para a ordem jurídica portuguesa), mais precisamente no artigo 3, pontos 1 e 2. Neste artigo é referido que:

«Procedimento», qualquer utilização, invasiva ou não, de um animal para fins experimentais ou outros fins científicos, com resultados conhecidos ou não, ou para fins educativos, suscetível de lhe causar um nível de dor, sofrimento, angústia ou dano duradouro equivalente ou superior ao provocado pela introdução de uma agulha em conformidade com as boas práticas veterinárias, incluindo qualquer ação destinada ou suscetível de conduzir ao nascimento ou à eclosão de um animal, ou à criação e manutenção de uma linhagem geneticamente modificada, excluindo o abate de animais unicamente para utilização dos seus órgãos. ou tecidos;

«Projeto», um programa de trabalho com um objetivo científico definido e que envolva um ou mais procedimentos.

Sendo assim, à luz do DL n.º 113/2013, um animal que não tenha sido utilizado em procedimentos e que foi occisado somente com o intuito de recolha de órgãos, não está abrangido pela Diretiva respeitante à proteção dos animais utilizados para fins experimentais e outros fins científicos. Sendo que o projeto da aluna Joana Rodrigues apenas pretende usar tecidos de animais eutanasiados (e que não tenham sido sujeitos a qualquer procedimento de acordo com a lei nacional e europeia), este trabalho não necessita de revisão ética por parte das comissões éticas institucionais, nem por parte da Direção-Geral de Alimentação e Veterinária, órgão que regula a utilização destes animais em Portugal.

Estou disponível para prestar mais esclarecimentos se tal fôr necessário.

Com os meus melhores cumprimentos,

Manuel Rebelo, PhD
Coordenador do Biotério do IGC
Membro da Comissão de Ética do IGC
Membro do Órgão Responsável pelo Bem-Estar Animal (ORBEA) do IGC

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Annex III - Leica Biosystems® HistoCore PEARL Tissue Processor: Processing protocol for regular sized samples

Table An. III – Processing protocol for regular sized samples

Station	Step	Time	Vacuum	Temperature
1	Formalin	01:00h	x	37ºC
2	Processing water	00:02h	-	-
3	Ethanol 70%	02:00h	x	-
4	Ethanol 96%	01:00h	-	-
5	Ethanol 100% I	01:00h	x	-
6	Ethanol 100% II	01:00h	-	-
7	Ethanol 100% III	01:00h	-	-
8	Ethanol 100% IV	01:00h	x	-
9	Xylene I	00:30h	-	-
10	Xylene II	01:00h	-	-
11	Xylene III	01:00h	x	-
12	Paraffin I	00:30h	x	62ºC
13	Paraffin II	01:00h	x	62ºC
14	Paraffin III	01:00h	x	62ºC
Duration: ≃13:30h				

Annex IV – Hematoxylin & Eosin manual staining protocol

Table An. IV – Hematoxylin & Eosin staining protocol

Step	Reagent	Time	Considerations
1.	Xylene I (Dewax)	10’	
2.	Xylene II (Dewax)	10’	
3.	99,9% Ethanol	2’	
4.	99,9% Ethanol	2’	
5.	96% Ethanol	2’	
6.	70% Ethanol	2’	
7.	Tap Water	2’	
8.	Hematoxylin	8’	Remove “mirrored surface”
9.	Running tap water	Wash	
10.	0,5% Chloridric acid alcohol	4 dips	Differentiation step
11.	Warm running tap water	8’	
12.	96% Ethanol	2’	Same as 5
13.	Eosin	2’	
14.	99,9% Ethanol	2’	
15.	99,9% Ethanol	2’	
16.	Xylene I (Mount)	2’	
17.	Xylene II (Mount)	2’	

Reagents:

- Xylene

Xileno (mistura de isómeros), técnico

VWR International - Material de Laboratório, Lda

Reference 28973363

- Ethanol

Ethanol absolute, p.a. Normapur

VWR International - Material de Laboratório, Lda

Reference VWRC20821.330

- Hematoxylin

Hematoxylin Solution, Harris Modified

Sigma-Aldrich Corporation

Reference HHS128-4L

- Eosin

Eosin Y solution, alcoholic

Sigma-Aldrich Corporation

Reference HT1101128-4L

Annex V – Histopathology Facility at *Instituto Gulbenkian de Ciência* price catalog (only relevant prices)

Table An. V – IGC Price catalog

Description	Prices
Technique	IGC, Academic and Collaborators
Processing and paraffin embedding per block (PPE)	0,64€
Sectioning in standard slides per slide (SS)	0,60€
Dewaxing and hydration per slide (DWH)	0,50€
Hematoxylin &Eosin per slide (HE)	1,09€
Pathology consultation (PC)/hour	20,00€

Note: Prices are cumulative.

Appendixes

Appendix Index

Appendix I – Variable classification according to type and scale

Table Ap. I – Variable classification distributed by study Phase

Phase II (3.2.1)			
Variable	**Definition**	**Type**	**Scale**
Number of breeding pairs	Total number of breeding pairs in the study.	Quantitative	Discrete
Number of pups weaned per female	Total number of pups weaned per female/breeding pair until retirement	Quantitative	Discrete
Number of litters per female	Total number of litters produced per female/breeding pair until retirement	Quantitative	Discrete
Maternal Age at pairing	Maternal age, in days, at pairing	Quantitative	Discrete
Maternal age at birth of first litter	Maternal age, in days, at birth of the first litter	Quantitative	Discrete
Litter size at wean	Total number of pups weaned per litter	Quantitative	Discrete
Percent females weaned	Total percentage of female pups weaned	Quantitative	Continuous
Maternal age (months) when last litter weaned	Maternal age, in months, at wean of the last litter produced	Quantitative	Discrete
Phase II (3.2.2)			
Variable	**Definition**	**Type**	**Scale**
Number of litters per female	Total number of litters produced per female/breeding pair until retirement	Quantitative	Discrete
Litter size at wean	Total number of pups weaned per litter	Quantitative	Discrete
Male /female Pup Ratio	Racio between the number of male pups and female pups at wean	Quantitative	Continuous
Number of pups weaned per female	Total number of pups weaned per female/breeding pair until retirement	Quantitative	Discrete
Difference between 1st Mating and 1st litter in days	Difference between the date of the first mating and the date of birth of the first litter	Quantitative	Discrete
Difference between 1st Mating and Last litter in days	Difference between the date of the first mating and the date of birth of the last litter	Quantitative	Discrete

Table Ap. I (Cont.) – Variable classification distributed by study Phase

Phase III			
Variable	**Definition**	**Type**	**Scale**
Volume	Estimated total volume of the sample	Quantitative	Continuous
Number	Estimated total number of objects counted	Quantitative	Discrete
Cost	Average cost per animal per object of study (Volume/Number) in Euros	Quantitative	Continuous

Appendix II – Resumed result tables for Phase III

Table Ap. II.i– Resumed result table for Neonate testes regarding volume and number estimation

Neonate Testes											
Sample Code	Description	Random Number	# Slides	Volume			Number			Cost	
				Estimation	Event Counts	CE	Estimation	Event Counts	CE	Volume	Number
JRL 21/19	M1a, Testis, Left (Neonate)	8	13	0,373577022	136	0,022510984	11280	141	0,085078059	110,55 €	120,55 €
JRL 22/19	M1a, Testis, Right (Neonate)	4	17	0,362589443	132	0,024310797	12960	162	0,07883371	178,35 €	188,35 €
JRL 23/19	M2a, Testis, Left (Neonate)	12	14	0,362589408	132	0,023436363	14240	178	0,075278803	125,86 €	135,86 €
JRL 24/19	M2a, Testis, Right (Neonate)	14	15	0,329626779	120	0,025520953	10720	134	0,087067786	142,27 €	152,27 €
JRL 25/19	M3a, Testis, Left (Neonate)	1	17	0,36258943	132	0,024574948	13440	168	0,077377137	178,35 €	188,35 €
JRL 26/19	M3a, Testis, Right (Neonate)	5	13	0,321386117	117	0,025602889	10000	125	0,089442719	110,55 €	120,55 €
JRL 35/19	M1b, Testis, Left (Neonate)	3	11	0,315892337	115	0,024715475	9440	118	0,093056482	83,19 €	93,19 €
JRL 36/19	M1b, Testis, Right (Neonate)	5	13	0,304904755	111	0,026409523	13200	165	0,079702346	110,55 €	120,55 €
JRL 37/19	M2b, Testis, Left (Neonate)	4	10	0,315892264	115	0,025590996				71,14 €	
JRL 38/19	M2b, Testis, Right (Neonate)	13	16	0,324133007	118	0,026403595				159,76 €	
JRL 41/19	M3b, Testis, Left (Neonate)	4	12	0,258207645	94	0,028962685	11040	138	0,086311263	96,32 €	106,32 €
JRL 42/19	M3b, Testis, Right (Neonate)	15	14	0,255460716	93	0,030981578	9440	118	0,092367374	125,86 €	135,86 €
JRL 43/19	M4b, Testis, Left (Neonate)	3	14								
JRL 44/19	M4b, Testis, Right (Neonate)	7	11								
JRL 47/19	M5b, Testis, Left (Neonate)	16	7	0,244473182	89	0,032034373				41,55 €	
JRL 48/19	M5b, Testis, Right (Neonate)	11	14	0,332373635	121	0,02491749				125,86	

Note: Grey cells represent samples excluded due to sample/tissue loss

Table Ap. II.ii– Resumed result table for Neonate ovaries regarding volume and number estimation

Neonate Ovaries												
Sample Code	Description	Random Number	# Slides	Volume			Number			Cost	
				Estimation	Event Counts	CE	Estimation	Event Counts	CE	Volume	Number
JRL 27/19	F1a, Ovary, Left (Neonate)	5	**10**	0,027011	118	0,029896				71,14 €	
JRL 28/19	F1a, Ovary, Right (Neonate)	12	**9**	0,030903	135	0,024256				60,19 €	
JRL 29/19	F2a, Ovary, Left (Neonate)	6	**11**	0,032505	142	0,024625	6843	114	0,093659	83,19 €	113,19 €
JRL 30/19	F2a, Ovary, Right (Neonate)	4	**7**	0,027469	120	0,025579	4502	75	0,115918	41,55 €	71,55 €
JRL 31/19	F3a, Ovary, Left (Neonate)	8	**11**	0,034336	150	0,023333	5402	90	0,106364	83,19 €	113,19 €
JRL 32/19	F3a, Ovary, Right (Neonate)	4	**11**	0,022891	100	0,031645	4862	81	0,111111	83,19 €	113,19 €
JRL 33/19	F1b, Ovary, Left (Neonate)	12	**7**	0,028156	123	0,026412	6603	110	0,095987	41,55 €	71,55 €
JRL 34/19	F1b, Ovary, Right (Neonate)	2	**7**	0,029529	129	0,024291	5522	92	0,104742	41,55 €	71,55 €
JRL 39/19	F2b, Ovary, Left (Neonate)	9	**8**	0,027469	120	0,027193	7023	117	0,09245	50,32 €	80,32 €
JRL 40/19	F2b, Ovary, Right (Neonate)	1	**9**	0,026553	116	0,027168	5522	92	0,104257	60,19 €	90,19 €
JRL 45/19	F3b, Ovary, Left (Neonate)	11	**8**	0,036854	161	0,021367	6122	102	0,100153	50,32 €	80,32 €
JRL 46/19	F3b, Ovary, Right (Neonate)	4	**9**	0,037083	162	0,021663	5462	91	0,105214	60,19 €	90,19 €

Note: Grey cells represent samples excluded due to sample/tissue loss

Table Ap. II.iii– Resumed result table for Seven weeks testes regarding volume and number estimation

Seven weeks Testes											
Sample Code	Description	Random Number	# Slides	Volume			Number			Cost	
				Estimation	Event Counts	CE	Estimation	Event Counts	CE	Volume	Number
JRL 1/19	M1, Testis, Left (7 weeks)	15	13	63,45315	154	0,020419	1365672	183	0,073923	110,55 €	120,55 €
JRL 2/19	M1, Testis, Right (7 weeks)	23	12	52,74028	128	0,022991	1261194	169	0,078309	96,32 €	106,32 €
JRL 3/19	M2, Testis, Left (7 weeks)	56	12	58,09672	141	0,021475	1447761	194	0,073271	96,32 €	106,32 €
JRL 4/19	M2, Testis, Right (7 weeks)	25	14	51,91622	126	0,024526	1320896	177	0,07569	125,86 €	135,86 €
JRL 5/19	M3, Testis, Left (7 weeks)	108	11	46,55978	113	0,025185	1559701	209	0,069897	83,19 €	93,19 €
JRL 6/19	M3, Testis, Right (7 weeks)	86	13	46,97181	114	0,025896	1626866	218	0,069527	110,55 €	120,55 €
JRL 7/19	M4, Testis, Left (7 weeks)	100	16	54,80045	133	0,023908	2082090	279	0,06018	159,76 €	169,76 €
JRL 8/19	M4, Testis, Right (7 weeks)	85	13	60,15688	146	0,021716	1552239	208	0,071331	110,55 €	120,55 €
JRL 9/19	M5, Testis, Left (7 weeks)	61	12	49,03198	119	0,024806	1477612	198	0,071914	96,32 €	106,32 €
JRL 10/19	M5, Testis, Right (7 weeks)	12	15	54,38842	132	0,023633	1507463	202	0,070615	142,27 €	152,27 €

Note: Grey cells represent samples excluded due to sample/tissue loss

Table Ap. II.iv– Resumed result table for Seven weeks ovaries regarding volume and number estimation

Seven weeks Ovaries											
Sample Code	Description	Random Number	# Slides	Volume			Number			Cost	
				Estimation	Event Counts	CE	Estimation	Event Counts	CE	Volume	Number
JRL 11/19	F1, Ovary, Left (7 weeks)	5	14	1,73054	224	0,018204	2455	81	0,111701	125,86 €	185,86 €
JRL 12/19	F1, Ovary, Right (7 weeks)	2	13	1,390613	180	0,021158	2273	75	0,11547	110,55 €	170,55 €
JRL 13/19	F2, Ovary, Left (7 weeks)	22	17	1,699638	220	0,019317	1970	65	0,124894	178,35 €	238,35 €
JRL 14/19	F2, Ovary, Right (7 weeks)	6	15	1,568302	203	0,019922	1848	61	0,128382	142,27 €	202,27 €
JRL 15/19	F3, Ovary, Left (7 weeks)	26	16	1,04296	135	0,027388	879	29	0,185695	159,76 €	219,76 €
JRL 16/19	F3, Ovary, Right (7 weeks)	7	13	1,321082	171	0,022034	1242	41	0,156538	110,55 €	170,55 €
JRL 17/19	F4, Ovary, Left (7 weeks)	18	16	1,645559	213	0,019807	1970	65	0,124697	159,76 €	219,76 €
JRL 18/19	F4, Ovary, Right (7 weeks)	2	14	1,267003	164	0,02273	1939	64	0,126601	125,86 €	185,86 €
JRL 19/19	F5, Ovary, Left (7 weeks)	18	13	0,872996	113	0,029636	1000	33	0,174078	110,55 €	170,55 €
JRL 20/19	F5, Ovary, Right (7 weeks)	10	15	1,151118	149	0,024995	1485	49	0,142857	142,27 €	202,27 €

Note: Grey cells represent samples excluded due to sample/tissue loss

Appendix III – Result tables for Phase II

Table Ap. III– Components and contaminants present in materials used in mice production

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC classification
Personal protective equipment (PPE)	Long sleeve tunic - 100% algodão	N.A.	N.A.	Ultragene	Cotton	No more info	No match
	Pants with elastic waistband and ankles - 100% cotton	N.A.	N.A.	Ultragene	Cotton	No more info	No match
	3-ply mask	Rma.007.A1	Celulosas vascas	Ultragene	Polypropylene		No match
					Fiber glass		No match
					Latex		No match
	1-ply paper mask	Rma.008.Bi	Celulosas vascas	Ultragene	Paper	No more info	No match
	Molded face masks	101-6829	Henry schein INC.	Cuvetrus	No info	Latex and fibreglas free	No match
	Cover-shoes PE 40u blue	O-0003-b	N.A.	Ultragene	Polyethylene		No match
	Blue clip cap	Rgo.018.Bi	N.A	Ultragene	Polypropylene		No match
	Glove semperguard® nitrile xpert powder-free	11807202 + 11897192	Semperit	Fisher scientific	Nitrile (acrylonitrile butadiene rubber)	More components not described	No match
					Dithiocarbamates		No match
					Zinc mercaptobenzothiazole		No match
	BLOC shoes (autoclavable)	BLOC open 03	Wock	Vimatechlab	No info	Covered by shoe covers, shouldn't be an issue	
Cleaning and disinfection	Ethanol (CH3 CH2OH) 5L alcohol pure 96,3% V/V	Etanol.5l.963	N.A.	Manuel vieira & cª (irmão)	Ethanol		No match
	Shower gel - savon perle	3048	Elis	N.A.	Water		No match
					Sodium laureth sulfate		No match
					Cocamidopropyl betaine		No match
					Decyl glucoside		No match
					Glyceryl oleate		No match
					Coco glucoside		No match
					Sodium chloride		No match
					Sodium benzoate		No match
					Citric acid		No match
					Guar hydroxypropyltrimonium chloride		No match
					Perfume		No match
					Sodium sulfate		No match
					Hydrolized wheat protein		No match
					Tetrasodiumglutamate diacetate		No match
					CI 16035		No match

Table Ap. III (Cont.)– Components and contaminants present in materials used in mice production

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC Classification
Cleaning and disinfection	Virkon (S Tablets (100 x 50g) - 5Kg and Virkon S Tablets (50 x 5g))		LanXess	BRADAN LIMITED	Pentapotassium bis(peroxymonosulphate bis (sulfate)		No match
					Malic acid		No match
					Dipotassium peroxodisulphate		No match
	Neodisher Polyklar, 10l	1DW401930	Dr. Weigert	A.Mendes e Silva (Veisil)	Policarboxylates (<5%)	No label extra info	No match
					chloromethylisothiazolinone	Synonims	No match
					Methylisothiazolinone	Synonims	No match
					citric acid		No match
					acrylates		No match
	Neodisher N	DW420126	Dr. Weigert	A.Mendes e Silva (Veisil)	Phosphates (>30%)	No label extra info	No match
					Phosphoric acid		No match
					Citric acid		No match
	Neodisher TP Acid	408326	Dr. Weigert	Ultragene	Inorganic acids	No label extra info	No match
	Neodisher TP rinse	408426	Dr. Weigert	Ultragene	Policarboxylates (<5%)	No label extra info	No match
					chloromethylisothiazolinone		No match
					Methylisothiazolinone		No match
Consumables	LUCART Prosessional Strong C	paper-cellul	Lucart	Blue Sigma	Celulose paper	No label extra info	No match
	Bucket for Diet	n.a.	n.a.	Fragoso e Higino	-	Old plastic with no labels or signs	No match
	Cirurgical Instruments	n.a.	Fine Science Tools (F.S.T.)	Ultragene	Stainless Steel		No match
Conventional Housing	Type III-D cage bottom in polysulfone	1290D00SU	Techniplast	Ultragene	polysulfone	No label extra info	No match
	Type II-C cage bottom in polysulfone	1264C00SU	Techniplast	Ultragene	polysulfone	No label extra info	No match
	Type III-D Grid in stainless steel wire	1290D116	Techniplast	Ultragene	Stainless Steel		No match
	Type II-C Grid in stainless steel wire	1264C116	Techniplast	Ultragene	Stainless Steel		No match
	Type III-D Lid in polysulfone	1290D400SU	Techniplast	Ultragene	polysulfone	No label extra info	No match
	Type II-C Lid in polysulfone	1264C400SU	Techniplast	Ultragene	Polysulfone	No label extra info	No match
	Replacement filter sheets (Type III-D)	1290D420R	Techniplast	Ultragene	-		No match
	Replacement filter sheets (Type II-C)	1264C420R	Techniplast	Ultragene	-		No match
	Water Bottle Graduated up to: 600 ml	ACBT0702SU	Techniplast	Ultragene	Polysulfone (?)		No match
	Water Bottle Graduated up to: 400 ml	ACBT0402SU	Techniplast	Ultragene	Polysulfone (?)		No match
	Standard Bottle Cap	ACCP2521	Techniplast	Ultragene	Stainless Steel		No match
	Special Bottle Cap (long)	ACCP6521	Techniplast	Ultragene	Stainless Steel		No match

Table Ap. III (Cont.)– Components and contaminants present in materials used in mice production

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC Classification
Food	Rat and Mouse No.3 Breeding Autoclavable	1011039	SDS Special Diets Service	LILLICO SERVING BIOTECHNOLOGY	Wheat		No match
					Wheatfeed		No match
					De-hulled Extracted Toasted Soya		No match
					Barley		No match
					Macro Minerals		No match
					Yeast		No match
					Dextrose Monohydrate		No match
					Potato Protein		No match
					Hydrolised Wheat Gluten		No match
					Full Fat Soya	Genistein (?)	No match
					Soya Oil	Genistein (?)	No match
					Maize Gluten Meal		No match
					Amino Acids		No match
					Vitamins		No match
					Micro Minerals		No match
					Analytical Constituents		
					Crude Fat		No match
					Crude Protein		No match
					Crude fibre		No match
					Crude ash		No match
					Lysine		No match
					Methionine		No match
					Calcium		No match
					Phosphorus		No match
					Sodium		No match
					Magnesium		No match
					Copper		No match

Table Ap. III (Cont.)– Components and contaminants present in materials used in mice production

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC Classification
Enviromental Enrichment	SizzleNest (Enviro-dri)	CS1A09	Datesand	Sodispan	Paper	No label extra info	No match
					Contaminants:		
					Cypermethrin		No match
					Pendimethalin		No match
					Piperonylbutoxide		No match
					2-Phenylphenol		No match
					Nitrate Nitrite		No match
					Boron Fluorine, detected as Fluoride		No match
					Copper (Cu)		No match
					Zinc (Zn)		No match
					Cadmium (Cd)		No match
					Lead (Pb)		No match
					Mercury (Hg)		No match
					Arsenic (As)		No match
					Aflatoxine B1		No match
					Aflatoxine B2		No match
					Aflatoxine G1		No match
					Aflatoxine G2		No match
					PCB 28		No match
					PCB 52		No match
					PCB 101		No match
					PCB 138		No match
					PCB 153		No match
					PCB 180		No match

Table Ap. III (Cont.) – Components and contaminants present in materials used in mice production

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC Classification
Enviromental Enrichment	Cocoons	COCOON	Datesand	Sodispan	Cotton		No match
					Contaminants:		
					Nitrate Nitrite		No match
					sodiumnitrite (calculated from nitrite)		No match
					Boron Fluorine, detected as Fluoride		No match
					Copper (Cu)		No match
					Zinc (Zn)		No match
					Cadmium (Cd)		No match
					Lead (Pb)		No match
					Mercury (Hg)		No match
					Arsenic (As)		No match
					Aflatoxine B1		No match
					Aflatoxine B2		No match
					Aflatoxine G1		No match
					Aflatoxine G2		No match
					PCB 28		No match
					PCB 52		No match
					PCB 101		No match
					PCB 138		No match
					PCB 153		No match
					PCB 180		No match
	Nestlets	CS3A01	Datesand	Sodispan	Fibers (?)	No more info	No match

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC classification
Enviromental enrichment	Mouse igloo red + mouse wedge	Ref: K3327 + ref: SZWEDGE	Datesand	Sodispan	Policarbonate		No match
					Contaminants:		
					Arsenic		No match
					Cadmium		No match
					Mercury		No match
					Lead		No match
					Selenium		No match
					Aflatoxin B1		No match
					Aflatoxin B2		No match
					Aflatoxin G1		No match
					Aflatoxin G2 7		No match
					Aldrin		No match
					Carbofenothion		No match
					Cis-chlordane		No match
					Trans-chlordane		No match
					Diazinon		No match
					Dieldrin		No match
					Disulfoton		No match
					Endrin		No match
					Endosulfan A		No match
					Endosulfan B		No match
					Ethion		No match
					Hexachlorobenzene (HCB)		No match
					Heptachlor epoxide		No match
					Heptachlor		No match
					Isodrin		No match
					Malathion		No match
					Mirex		No match
					Methoxychlor		No match
					2,2',4,5,5'-pentachlorobiphenyl (PCB congener 101)		No match
					2,3',4,4',5-pentachlorobiphenyl (PCB congener 118)		No match
					2,2',3,4,4',5-hexachlorobiphenyl (PCB 138)		No match
					2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153)		No match
					2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180)		No match
					2,4,4'-trichlorobiphenyl (PCB congener 28)		No match
					2,2',5,5'-tetrachlorobiphenyl (PCB congener 52)		No match
					Parathion-ethyl		No match
					Parathion-methyl		No match
					Phorate		No match
					Toxaphene (camphechlor)		No match
					Trifluralin		No match
					Alpha-hexachlorocyclohexane (alpha-hch)		No match
					Beta-hexachlorocyclohexane (beta-hch, beta-bhc)		No match
					Delta-hexachlorocyclohexane (delta-hch)		No match
					Gamma-hexachlorocyclohexane (lindane)		No match
					O,p'-ddd (o,p'-tde)		No match
					O,p'-dde		No match
					O,p'-ddt		No match
					P,p'-ddd (p,p'-tde)		No match
					P,p'-dde		No match
					P,p'-ddt		No match

Type	Product	Reference	Brand	Vendor	Composition	Observations	EDC classification
Enviromental enrichment	Tunel carton 100mm	Cs3b02a	Datesand	Sodispan	Paper		No match
					Contaminants:		
					2-phenylphenol		No match
					Anthraquinone		No match
					Cypermethrin		No match
					Diphenylamine		No match
					Permethrin		No match
					Piperonyl butoxide		No match
					Propiconazole		No match
					Nitrate nitrite		No match
					Boron fluorine, detected as fluoride		No match
					Copper (Cu)		No match
					Zinc (Zn)		No match
					Cadmium (Cd)		No match
					Lead (Pb)		No match
					Mercury (Hg)		No match
					Arsenic (As)		No match
					Aflatoxine B1		No match
					Aflatoxine B2		No match
					Aflatoxine G1		No match
					Aflatoxine G2		No match
					Pcb 28		No match
					Pcb 52		No match
					Pcb 101		No match
					Pcb 138		No match
					Pcb 153		No match
					Pcb 180		No match