The Use of Traditional and Alternative Methods to Study Endocrine Disruption in Model Avian Species

Krittika Mittal

Department of Natural Resource Sciences

McGill University, Montreal

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Abstract

Toxicity testing of chemicals is integral to environmental hazard determination and risk assessment. Traditional whole animal (*in vivo*) testing methods are resource-intensive and raise ethical concerns pertaining to animal use. This has led to a surge in the development of alternative methods (cells and tissues) to screen and prioritize chemicals. Despite the promise shown by such methods, little is known about how they perform against whole animal tests. Overall, this thesis aimed to advance knowledge on A) traditional and alternative toxicity testing methods, and B) the effects of 17β-trenbolone (17βT- an endocrine disrupting chemical used in livestock as a growth promoter) in model avian species. The specific aims were to 1) use *in vivo* exposures and next-generation RNA-Sequencing to examine sex- and developmental stage-related differences in the hepatic transcriptome of Japanese quail (JQ, *Coturnix japonica*) exposed to 17βT; Aim 2) assess molecular and biochemical effects on the quail endocrine system during early stages of *in vivo* exposure to 17βT; Aim 3) compare effects of chemicals on gene expression in three alternative methods: hepatocytes, liver slice culture, and *in ovo* liver of white leghorn chicken (*Gallus gallus domesticus*); and Aim 4) outline key challenges, opportunities, and monetary costs, time and number of animals associated with traditional and alternative testing. In Aim 1, analysis of the quail hepatic transcriptome indicated that early life stages may be more vulnerable to endocrine disruption than adults. Differentially expressed genes were related to processes including cell proliferation, and transport and metabolism of lipids and proteins. In Aim 2, analyses of the JQ endocrine pathway revealed significant differences in plasma hormone levels in exposed males and females that fluctuated over the duration of exposure, but no changes in the expression of associated genes, implying that metabolic

pathways and other receptors may be involved in the mechanisms by which 17βT impairs the endocrine system. In Aim 3, hierarchical clustering analysis of gene expression results across three alternative methods showed similarities between liver slice culture and *in ovo* liver, while hepatocytes were more different. In Aim 4, bibliometric searches of resource-related costs from various sources showed that, realistically, the status quo in toxicity testing will not be able to provide toxicity data for all existing and emerging chemicals. In summary, this work demonstrated the potential of liver slices as alternatives in toxicity testing; further, this work showed that even using traditional methods results may vary depending on factors such as sex, developmental stage, and exposure duration. This thesis advances knowledge on two fronts: A) the potential of alternative testing methods to aid in their future implementation and the reduction of traditional methods in chemical toxicity testing, and B) the importance of including aforementioned factors while examining molecular and biochemical endpoints in toxicity studies.

Résumé

Les essais de toxicité chimique font partie intégrante de la détermination et de l'évaluation des risques. Les tests traditionnels sur les animaux entiers (*in vivo*) consomment beaucoup de ressources et soulèvent des préoccupations éthiques importantes concernant l'utilisation des animaux. Cela a conduit à au développement de méthodes alternatives (sur cellules et tissus) pour cribler et hiérarchiser les produits chimiques. Malgré l'aspect prometteur de ces méthodes, on sait peu de choses sur leur performance comparativement aux tests sur animaux entiers. L'objectif global de cette recherche vise à faire progresser les connaissances sur A) les méthodes traditionnelles et alternatives, et B) les effets toxiques du 17β-trenbolone (17βT- un perturbateur endocrinien (PE) utilisé dans l'élevage comme promoteur de croissance) sur des espèces aviaires modèles. Les objectifs de cette étude étaient : 1) de réaliser des expositions *in vivo* et d'utiliser des méthodes de séquençage ARN nouvelle-génération pour examiner les différences liées au sexe et au stade de développement dans le transcriptome hépatique de la caille japonaise (CJ, *Coturnix japonica*) exposées au 17βT; 2) d'évaluer les effets sur le système endocrinien durant les premiers stades d'exposition au 17βT, 3) de comparer les effets de trois PE sur l'expression génique en utilisant des hépatocytes, des tranches de foie en culture et le foie après exposition *in ovo* chez le poulet blanc Leghorn (*Gallus gallus domesticus*), et 4) de définir les principaux défis et opportunités des tests de toxicité et d'évaluer les coûts (en termes de temps, argent, nombre d'animaux) avec des méthodes traditionnelles et alternatives. Dans l'objectif 1, les analyses transcriptomiques ont révélé que les stades précoces de développement pouvaient être plus vulnérables que les stades adultes aux perturbations endocriniennes. Plusieurs gènes ont été impactés, dont certains impliqués dans la prolifération cellulaire et le transport et le métabolisme

des lipides et protéines. Dans l'objectif 2, les analyses endocriniennes chez la JQ ont révélé des différences significatives dans les taux d'hormones plasmatiques chez les mâles et les femelles qui ont fluctué selon la durée de l'exposition, mais aucun changement dans l'expression des gènes associés, suggérant l'implication possible d'autres voies métaboliques et récepteurs par lesquels le 17βT pourrait altérer le système endocrinien. Dans l'objectif 3, l'analyse par clusters hiérarchiques classifiant l'expression génique à travers trois méthodes alternatives ont montré des réponses très proches entre les tranches de foie en culture et le foie exposé *in ovo*, mais des réponses plus éloignées entre les hépatocytes et le foie exposé *in ovo*. Dans l'objectif 4, les recherches bibliographiques sur les coûts liés aux ressources provenant de diverses bases de données ont montré que, de façon réaliste, le statu quo des essais de toxicité sera probablement incapable de fournir des données de toxicité pour les produits chimiques existants et émergents. En résumé, ce travail a démontré le potentiel des tranches de foie comme méthode alternative pour les tests de toxicité; en outre, ce travail a montré que les résultats issus de méthodes traditionnelles peuvent eux-mêmes varier en fonction de facteurs tels que le sexe, le stade de développement et la durée de l'exposition. Cette thèse fait progresser les connaissances sur deux fronts: A) le potentiel des méthodes alternatives pour faciliter leur mise en œuvre future et la réduction des méthodes traditionnelles dans les essais de toxicité des produits chimiques; et B) l'importance d'inclure les facteurs susmentionnés en examinant les réponses moléculaires et biochimiques dans les études de toxicité.

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Preface and Contribution of the Authors

This thesis fills important knowledge gaps and contributes to the advancement of knowledge as follows:

Chapter 2 was a toxicogenomic study examining sex- and developmental stage-related differences in the hepatic transcriptome in a model avian species – Japanese quail (*Coturnix japonica*) exposed to a model androgenic endocrine disrupting chemical (EDC) – 17β-trenbolone (17βT). This chapter was able to compare results in males vs females, and embryos vs adults and was able to identify biological pathways that could be impaired by differentially expressed genes. Moving beyond the use of traditional quantitative polymerase chain reaction (qPCR) to analyze gene expression, this study used next-generation RNA-sequencing to examine the global transcriptome. This type of transcriptomic information could also be used to predict adverse outcomes in the whole animal.

Chapter 3 investigated the effects of 17βT on several endpoints in the JQ endocrine system at four time-points within the first three weeks of *in vivo* exposure. This study greatly expands upon previous studies on the effects of 17βT in an avian species not only by investigating effects at multiple time-points of exposure but also by investigating effects from a short-term exposure. This could help understand the effects of 17βT on the endocrine system immediately following exposure, which might be precluded in long-term exposure studies. We examined molecular (gene expression) and biochemical (plasma hormone levels) endpoints that could be used to establish links to adverse outcomes. This chapter fills an important knowledge gap by examining changes in the endocrine system from a short-term time-course study on 17βT exposure in an avian species.

Chapter 4 was a comparative study of three alternative toxicity testing models, and examined changes in hepatic gene expression upon exposure to three model chemicals. These methods included primary hepatocyte culture, liver slice culture and the *in ovo* liver from White Leghorn Chicken (*Gallus gallus domesticus*). To the best of our knowledge this is the first study to perform a comparison of these alternative methods in an avian species; additionally, we were able to measure changes in the expression of genes that have not yet been studied in response to EDCs in an avian species. This is also the first study to develop and use an avian liver slice culture for toxicity testing.

Chapter 5 examined the progression in the field of toxicology from the use of traditional whole animal-based toxicity testing methods to the development of alternative approaches such as *in silico*, *in vitro* and early life stage methods. This chapter pulls information from various data-streams to highlight key events, challenges and opportunities in the field of environmental toxicity testing. This chapter also synthesizes information on the monetary costs, number of animals and times associated with traditional and alternative toxicity testing methods and presents select case studies that can demonstrate potential benefits of alternative methods. To the best of our knowledge, an in-depth comparison on the resources needed for toxicity testing does not exist in ecotoxicology.

This thesis consists of 4 chapters authored by the candidate and intended for publication. The initial study design of Chapter 2 was developed by Drs. Natalie K. Karouna-Renier and Paula F.P. Henry at the United States Geological Survey (USGS) and samples were collected by various collaborators from the USGS; Dr. Robert Cornman at the USGS was responsible for processing the raw sequencing counts and goseq analysis. Aside from the initial aspects of the study, the candidate was responsible for performing all differential expression analysis, analysis

and interpretation of the data, discussion of the results, and preparation of the manuscript, and was provided advice by the candidate's supervisor Dr. Niladri Basu. This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu and collaborators Drs. Natalie K. Karouna-Renier and Paula F.P. Henry and Robert Cornman at the USGS. For chapter 3, the candidate, in collaboration with the co-authors, was responsible for the study design and development, and sample collection; the candidate was responsible for sample analysis, data analysis and interpretation, discussion of the results, and preparation of the manuscript, and was provided advice on all aspects of the study by the candidate's supervisor Dr. Niladri Basu. This chapter is co-authored by Dr. Niladri Basu and collaborators Drs. Natalie K. Karouna-Renier and Paula F.P. Henry at the USGS, and Drs. Cheryl A. Murphy and Brandon Armstrong (currently at Department of Environmental Quality, State of Michigan) at Michigan State University. For chapter 4, the candidate was responsible for the study design and development, sample collection and analysis, analysis and interpretation of the data, discussion of the results, and preparation of the manuscript, and was provided advice on all aspects of the study by the candidate's supervisor Dr. Niladri Basu. This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu and Doug Crump at Environment and Climate Change Canada (ECCC). Doug Crump provided guidance on the study design and technical advice on the arrays. For chapter 5, the candidate was responsible for the design of bibliometric searches, compilation and interpretation of information, discussion of results, and preparation of the manuscript and was provided advice on all aspects by the candidate's supervisor Dr. Niladri Basu. This chapter is co-authored by the candidate's supervisor Dr. Niladri Basu, Doug Crump (ECCC) and Dr. Markus Hecker (University of Saskatchewan) who provided advice and guidance on the manuscript preparation.

List of Abbreviations

17βHSD	17β-hydroxysteroid dehydrogenase
17βT	17β-trenbolone
3βHSD	3β-hydroxysteroid dehydrogenase
3Rs	Reduce, Replace, and Refine
AOP	Adverse Outcome Pathway
Apo	Apolipoprotein
AR	Androgen Receptor
ATP	Adenosine triphosphate
BP	Biological Process
CCAC	Canadian Council on Animal Care
CFIA	Canadian Food Inspection Agency
CMP	Chemicals Management Plan
CV	Coefficient of Variation
CYP1A4	Cytochrome P450 1A4
CYP11A	Cholesterol Side-Chain Cleavage enzyme
CYP17A1	Steroid 17α-monooxygenase
CYP19A1	Aromatase
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
E2	17β-Estradiol
ECM	Extracellular Matrix

EDCs	Endocrine Disrupting Chemicals
EDSP	Endocrine Disruptor Screening Program
ELISA	Enzyme Linked Immunosorbent Assay
ER	Estrogen Receptor
EU	European Union
FC	Fold change
FHM	Fathead minnow
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GO	Gene Ontology
HGP	Human Genome Project
HPGL	Hypothalamic-Pituitary-Gonadal-Liver
HSI	Hepatosomatic Index
IPC	Inter-Plate Calibrator
JQ	Japanese quail (*Coturnix japonica*)
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBFABP	Liver Basic Fatty Acid Binding Protein
LH	Luteinizing Hormone
MF	Molecular Function
NAM	New Approach Method
NCBI	National Center for Biotechnology Information
NIH	National Institute of Health
NTC	No Template Control

NRC	National Research Council
NRT	No Reverse Transcription Control
OECD	Organization for Economic Co-operation and Development
OPFRs	Organophosphate Flame Retardants
PANTHER	Protein Analysis through Evolutionary Relationships
PBDE	Polybrominated Diphenyl Ethers
PCB	Polychlorinated Biphenyls
PCPs	Personal Care Products
PPAR	Peroxisome Proliferator Activated Receptor
PR	Progesterone Receptor
RNA-seq	RNA Sequencing
qPCR	Quantitative Polymerase Chain Reaction
QSAR	Quantitative Structural Activity Relationship
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RNA	Ribonucleic Acid
SCD	Stearoyl CoA Desaturase
SDE	Significantly Differentially Expressed
SE	Standard Error
SHBG	Steroid Hormone Binding Globulin
SRM	Standard Reference Material
SULT1E1	Estrogen Sulfotransferase
T	Testosterone
TbA	Trenbolone Acetate

TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TGF	Transforming Growth Factor
ToxCast	Toxicity Forecaster
TSCA	Toxic Substances Control Act
TT21C	Toxicity Testing in the 21st Century
TXRF	Total Reflection X-Ray Fluorescence
US EPA	United States Environment Protection Agency
USGS	United States Geological Survey
VTG	Vitellogenin
Zn	Zinc

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Chapter 1

1. Introduction and Literature Review

1.1 General Introduction

Over the past few decades there has been a significant increase in societal awareness that widespread environmental contaminants can exert sub-lethal effects on vertebrates and invertebrates (1). Seminal papers in recent years have further emphasized the threat posed by chemical contamination on our planet and have reported that pollution is the largest environmental cause of disease and premature death in the world (2,3). There are in excess of 100,000 chemicals lacking in toxicity information, with hundreds of new chemicals being introduced every year (4). Toxicity testing is an integral aspect of hazard determination and assessing the risk posed by these chemicals. Traditional toxicity testing involves exposing whole animals to chemicals of interest and measuring apical outcomes. However, this is not feasible for every species-chemical scenario (5,6). This realization has resulted in a surge in the development of alternative toxicity testing methods that use components of relevant tissues (such as cells and tissue slices) or early-life stages (embryos) to predict adverse effects, and screen and prioritize chemicals for further animal testing (5). However, we need to understand differences among various testing methods to be able to use alternative methods to predict adverse outcomes in the whole animal. Among the many types of chemical contaminants are endocrine disrupting chemicals (EDCs) which can adversely affect the reproductive pathway. This pathway in vertebrates is mediated by the hypothalamic-pituitary-gonadal-liver (HPGL) axis. The multitude of hormones controlling the HPGL axis and the intricacies of their regulation make the HPGL

axis a prime target of EDCs (7). While evidence of endocrine disruption was initially observed in avian species, much of the ensuing research has shifted focus to mammalian and aquatic species. The overall objective of this research was to use traditional and alternative toxicity testing methods to study the effects of 17β-trenbolone (17βT- an EDC used in livestock as a growth promoter) using Japanese quail and domestic chicken as model avian species.

1.1.1 Specific Aims

Chapter 2: How is the transcriptome of model avian species at multiple stages affected upon exposure to endocrine disrupting chemicals?

- Aim: Examine sex- and developmental stage-related differences in the hepatic transcriptome of Japanese quail (*Coturnix japonica*) exposed to 17βT, using RNA Sequencing. This aim was explored in Chapter 2 where the specific objectives were to: Determine the genes that are significantly differentially expressed due to 17βT exposure and examine the differences in the hepatic transcriptome between
    - males vs females, and
    - embryos vs adults
- Identify biological pathways that are significantly enriched with differentially expressed genes and might be affected by exposure to 17βT

We hypothesize that genes and pathways implicated in steroidogenesis and metabolism will be differentially affected across sex and developmental stage in treated birds compared to controls.

Chapter 3: Are the effects of short-term exposure to 17βT on the avian endocrine system different from prolonged exposure, and do these effects vary over the course of the exposure period?

Aim: Investigate effects on male and female Japanese quail endocrine system during the first three weeks of *in vivo* exposure to 17βT. This aim was explored in Chapter 3 where the specific objectives were to:

- Examine difference in egg production, and morphometric changes in the treated birds.
- Examine changes in the plasma concentrations of key sex hormones and proteins.
- Examine changes in the expression of key genes in the gonad and liver.

We hypothesize that 17βT exposed birds will exhibit differential changes in plasma hormone (including luteinizing hormone, E2, and T) levels and expression of associated genes, and that these changes will vary over the exposure duration.

Chapter 4: How do changes in gene expression differ across various alternative methods?

Aim: Compare three alternative testing methods by studying gene expression in hepatocyte culture, liver slice culture, and *in ovo* liver from a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*) exposed to three model chemicals. This aim was explored in Chapter 4 where the specific objectives were to:

- Examine changes in the expression of chosen genes in the three methods.
- Use hierarchical clustering analyses to determine which of the methods are more similar to each other.

We hypothesize that changes in gene expression will differ across all three methods, however, liver slices will be more similar to the *in ovo* liver than hepatocytes.

Chapter 5: Can alternative toxicity testing methods be more efficient and ethical compared to traditional animal-based methods in terms of resources i.e., money, time, and number of animals used?

Aim: Examine the evolving field of toxicity testing and study whether alternative toxicity testing can be more resource efficient than traditional toxicity testing. This aim was explored in Chapter 5 where the specific objectives were to:

- Highlight key events, opportunities and challenges in the field of environmental toxicity testing.
- Synthesize information from various data-streams on toxicity testing costs associated with money, number of animals needed for testing and testing times
- Present select case studies demonstrating potential benefits.

1.2 Literature Review

1.2.1 Toxicity Testing

1.2.1.1 Environmental Toxicity Testing

Toxicity testing of chemical contaminants forms an integral part of hazard determination and risk assessment. Environmental toxicology is the branch of toxicology that studies the adverse effects of chemicals on living organisms. The field came into prominence with the publication of Silent Spring in 1962, which documented the decline in bald eagle (*Haliaeetus leucocephalus*) populations in North America due to the excessive use of pesticides such as dichlorodiphenyltrichloroethane (DDT) (8). Since then, studies have examined the effects of a

few hundred chemicals in both wild species and model laboratory species. Toxicity testing can involve any number of methods including field and laboratory studies in the whole animal, and alternative methods including *in vitro* assays using isolated biological components such as cells and tissues from a whole animal.

1.2.1.2 Traditional Toxicity Testing

Traditional toxicity testing entails the use of whole animals (*in vivo*) that are directly exposed to the chemical of interest through several routes of exposure including injections, supplemented diet or water, or oral gavage, to directly measure adverse outcomes (including death, disease, reproductive failure, and developmental toxicity). One of the first known instances of toxicity testing was the development of the LD50 study in 1927 (9). There are several advantages to using *in vivo* methods for toxicological evaluation: it is possible to study effects on development, reproduction, cognition, behavior and multiple generations; *in vivo* studies also account for plasticity of biological pathways since internal regulatory feedback mechanisms are intact; cellular interactions in tissues are preserved and organs possess their original architecture; additionally, since all biological components and pathways are intact, metabolism of exogenous chemicals can be accounted for, in these studies (10). The availability of internationally standardized guidelines for several tests make it easy to interpret and accept the data (11). Due to these advantages, since the emergence of environmental toxicity testing in the 1960s and 70s, *in vivo* studies have been considered the gold-standard, and played an important role in addressing the chemical contamination issues of the 20th century (6). However, animal-based studies often may not account for sex, developmental stage or duration of exposure (12–14). Such studies tend to be prohibitively expensive because of high monetary costs, requirement of large numbers of

animals and lengthy duration of the tests (10). Additionally, results from such studies may be difficult to interpret not only because of complexity of biological interactions but also because studies may concentrate on adverse outcomes without looking at the underlying mechanistic effect (10).

1.2.1.3 Need for Alternative Toxicity Testing

There are currently over 100,000 chemicals registered in commerce with 500-1000 new chemicals being introduced annually (4). These numbers are much higher if one considers regulatory testing needs associated with municipal and industrial effluents such as Canada's Environmental Effects Monitoring program (Sect 36, *Fisheries Act*) and the EU's Water Framework Directive (2000/60/EC). Seminal papers in recent years have further emphasized the threat posed by chemical contamination on our planet and have reported that pollution is the largest environmental cause of disease and premature death in the world (2,3). Despite the hundreds of chemicals that are purported to have the potential to cause sub-lethal effects, there exists data only for a fraction of these chemicals (15,16).

The logistical constraints and resource-intensive nature of *in vivo* studies render them infeasible to be applied to every chemical-species scenario. In addition to the ethical concerns associated with whole animal testing, these approaches rely on extrapolating from laboratory model species to native species of ecological relevance thus potentially leading to inaccurate estimations of risks. In 2007, the National Research Council (NRC) report released the landmark report titled Toxicity Testing in the 21st century: A Vision and a Strategy (5), which essentially pointed out that the status quo in toxicity testing is not equipped to tackle the diverse chemical contamination issues of the 21st century. It emphasized the need for a change from a toxicity

testing framework based primarily on whole animal methods to one based primarily on screening chemicals through suites of mechanistic assays using alternative approaches (5).

In the years following the NRC report, seminal publications further emphasized the significance of this paradigm shift, and the need to exploit the revolution in biology and biotechnology to improve the toxicity testing framework (6,17). This realization and concomitant advances in toxicogenomics, bioinformatics, and computational toxicology, has provided the impetus needed to transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods (5). As part of this effort, the EPA launched the Toxicity Forecaster (ToxCast) program in 2007, a multi-year effort to develop high-throughput screening platforms to expose living cells or cellular components to chemicals (18). Set up as a tiered system, chemicals consistently showing potential toxic effects for certain sensitive endpoints are identified and then tested more intensively in dose-response studies. The overarching vision of the program is to develop a suite of innovative methods that are mechanistically based and evaluate biologically significant perturbations. In 2011 the National Institute for Health (NIH) commenced an initiative - Tissue Chip for Drug Screening, which aims to develop 3-D human tissue chips that model the structure and function of human organs such as the lung, liver and heart and further combine these into an integrated system that can mimic complex functions of the human body (19).

Thus, in the decade following the 2007 NRC report, there has been an upsurge in the research related to alternative testing methods in toxicity testing. This interest in alternative methods can be seen in the regulatory and private sector where regulators, and companies such as Shell and Unilever are not only seeking improved methods that would be capable of screening the thousands of existing and emerging chemicals but also for compliance testing of effluents

(20–22). Much of these recent advances have been focused on human health using biomedical models. There are fewer options of alternative tools for wildlife and these are mainly focused on mammalian and aquatic species; there are fewer options still for avian species.

1.2.1.4 Alternative Toxicity Testing Methods

Alternative toxicity testing methods are those that incorporate the 3Rs principle by reducing the number of animals used in a study, replacing the use of animals in a study, or refining the study by making it less stressful or painful for the test animal (23,24). These methods can be *in silico* or computational, i.e., the use of computer modelling approaches to predict the toxic potential of a chemical; *in vitro* i.e., performing tests on a biological component of an organism such as cells, tissues, or organs; or early-life stages, i.e., performing toxicity tests on embryos. Of the several methods that have been developed, three methods that show great promise in toxicity testing are primary cell culture, tissue slice culture and tissues from *in ovo* exposed embryonic organisms. Since the liver is the main source of xenobiotic metabolism and biotransformation, many of these alternative methods are based on the liver as the target tissue.

1.2.1.4.1 Primary Hepatocyte Culture

Liver cell or hepatocyte culture has been the gold standard in *in vitro* toxicity assays since the cells can maintain function for up to 96 hours, allowing for short-term, medium throughput studies with measurable biochemical and molecular endpoints (25). Hepatocyte cultures have been widely used in avian toxicology to study mechanistic effects of various contaminants (26–28). This method allows us to treat hepatocytes with chemicals of interest and measure gene expression and enzyme activity. However, hepatocytes are a rather simplistic method and far removed from the intact organism. Liver tissue from several birds need to be pooled before

hepatocytes can be isolated, thus results are not obtained from an individual bird, rather as an average. Primary cell cultures are also not representative of the intact organism because single hepatocytes are isolated from the liver, and hence they do not retain all the characteristics of an intact liver. Thus, the assumption that the hepatocytes will be affected by the chemical or affect the chemical the same way as an intact liver may not hold true (29).

1.2.1.4.2 Tissue Slice Culture

An approach that has shown fidelity to the intact tissue is tissue slice culture which involves extracting fresh tissues that are sliced and cultured under appropriate conditions to maintain function and measure biochemical and molecular endpoints (25,29). Tissue slices have the advantages of being more similar to freshly isolated tissue, retaining cell-cell interactions and better retention of architecture than regular 2-D cell culture (30–32). However, they are not without their disadvantages: it is difficult to obtain slices reproducibly, the exposure and activity of cells in slices may vary, and they have a limited *in* vitro lifespan (up to 5 days). The number of slices obtained depends on the size of the organ; for example, numerous slices may be obtained from the embryonic liver, however only a few may be obtained from the gonads in an embryonic chicken. Nevertheless, slices can maintain some of their natural functions outside the organism (e.g., the body of the bird or fish) because all necessary machinery required for cell- or tissue-specific functioning is present (29,33,34). Additionally, slices obtained from one individual can be used for several exposure experiments thus at least partially contributing to a reduction in animal use (25,35).

As such, precision cut liver slices from rats have been successfully cultured for biomedical research and assessing mammalian toxicity (25,29,36–39). In a few cases, human liver slices have also been used to assess hepatotoxicity of chemicals (34,40–42). Recently, a few

studies were conducted using hypothalamic, pituitary, gonadal and liver slice cultures from fathead minnow (FHM; *Pimephales promelas*) and other North American fish species to investigate the effects of chemicals including forskolin, prochloraz and 17α-ethinylestradiol (43–45). Despite the success of tissue slice cultures, this method has not yet been exploited for avian toxicology.

1.2.1.4.3 Early-life Stage Methods

Another method that is widely used in toxicity studies is the use of early-life stages, i.e., embryos that have been exposed to the chemical of interest *in ovo*. Since embryos are nonself-feeding organisms, they are considered to be an alternative method (25,46). This approach allows us to study the effects in a whole organism without the use of adult animals. Additionally, it allows us to examine the effects of chemicals at a developmental stage where an organism might be more vulnerable to exogenous stress. Several studies have used the embryo as a model to examine the effects of chemicals including organophosphate flame retardants (OPFRs), perfluoroalkyl compounds, and metals and metalloids such as mercury (Hg) and arsenic (As) on the developing embryo (47–50).

1.2.1.5 Alternative to Traditional Methods Comparison

Alternative and traditional methods are inherently different from each other as one uses isolated biological components while the other generally examines effects in a whole animal. Thus, it is not clear how results from an alternative method such as cells or slices perform against whole animal tests. A few studies have attempted to determine how accurate different alternative methods are in comparison to *in vivo* methods. These studies have predominantly been performed using liver slices from rats and compared endpoints related to xenobiotic metabolism.

Elferink et al. (36) examined gene expression in rat liver slices and determined that slices were able to accurately predict hepatotoxicity in the *in vivo* liver. Ghantous et al. (51) examined the biotransformation and cytotoxicity of sevoflurane, an anesthetic, in rat liver slices and observed no signs of hepatotoxicity similar to results in the *in vivo* liver. Boess et al. (52) compared a number of *in vitro* methods including primary cell culture and liver slice culture from rats to *in vivo* rat liver dosed with chemicals. They used hierarchical clustering analysis of microarray data and concluded that while the liver slices were more similar to the *in vivo* liver, neither of the methods examined were directly comparable to the *in vivo* method, not on a gene-by-gene basis at least (52). This emphasizes that it is unlikely for any single alternative method to be able to replace whole animal testing. Rather, a suite of the best alternative approaches is likely to be employed. Thus, further research on how various alternative approaches differ from each other is necessary.

1.2.2 Endocrine System

1.2.2.1 Hypothalamic-Pituitary-Gonadal Axis

The reproductive pathway in vertebrates is regulated via the Hypothalamic-Pituitary-Gonadal (HPG) axis. It ultimately controls reproductive physiology, behavior and is responsible for proper development and function of reproductive organs (53). Internal cues such as neurotransmitters and hormones regulate the release of gonadotropin releasing hormones (GnRHs) from the hypothalamus (53,54). Reproductive activities of vertebrates are primarily regulated by these neurohormones which bind to receptors in the pituitary, inducing gonadotropin production and release into circulation. The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, bind to receptors in the gonads

(female – ovary; male – testes) to regulate normal reproductive function through the release of sex steroid hormones– androgens (primarily testosterone - T) and estrogens (primarily 17β-Estradiol – E2) (55). These sex steroids are transported through the circulatory system, and as part of their functions, feed back onto the hypothalamus and pituitary, inhibiting the release of GnRHs and gonadotropins (54,55). Thus, these sex steroid hormones as part of their functions play a part in regulating the HPG axis by completing a negative feedback circuit.

Several enzymes play critical roles in the biosynthesis and metabolism of sex steroid hormones. Some of these key enzymes are the P450 family of enzymes including CYP11A – cholesterol side chain cleavage enzyme and CYP17A1 – steroid 17α-monooxygenase, and 17βHSD - 17β hydroxysteroid dehydrogenase and 3βHSD - 3β hydroxysteroid dehydrogenase, which are involved in the synthesis of T from its precursor cholesterol. Testosterone is further converted to E2 through an aromatization process by the rate limiting enzyme CYP19A1 aromatase (56). In addition to these hormones there are others including androgens (such as dihydrotestosterone - DHT) and estrogens (such as estrone) and progesterone present in vertebrates that are also involved in processes surrounding reproductive function (55).

1.2.2.2 Liver

In egg-laying or oviparous species, the liver has a crucial role in the reproductive pathway. 17β-Estradiol from the ovaries binds to estrogen receptors (ER) in the female liver to induce production of vitellogenin (VTG) and apolipoprotein (Apo). Apolipoprotein is involved in the transport of lipid molecules, while, VTG is a phosphoprotein which is a precursor to egg yolk proteins that are transported through the blood to the ovary and are deposited in the developing oocyte (57). Egg yolk phosphoproteins are not typically synthesized by roosters and immature

chickens, but synthesis of these proteins can be induced in their liver by administering E2 (58). Thus, in oviparous species, due to its role in egg production, the hypothalamic-pituitary-gonadal-liver (HPGL) axis forms an essential part of the endocrine system, and an impairment at any of these components could have deleterious effects on the reproductive pathway (7).

1.2.2.3 The Endocrine System and other Biological Processes

While the endocrine system is mainly known to be involved in the reproductive pathway, it also interacts and influences numerous other biological processes. The neurohormones (such as GnRHs) are a part of the neuroendocrine system that play an important role in controlling functions such as growth, stress, metabolism, energy balance and other processes involved in maintaining homeostasis (59). Androgens and estrogens have been shown to be involved in processes including lipid and carbohydrate metabolism and bone mineralization (60). Previous research has provided evidence on the existence of interactions between the androgen receptor and growth factor mediated signaling pathways (61). An *in vitro* study demonstrated that androgens such as DHT and T were able to stimulate the proliferation of bone cells (62). In addition to these process, sex steroids have also been implicated in the regulation of the immune system (63). Thus, the impact of interferences in the endocrine system can extend beyond reproductive processes to those surrounding homeostasis, metabolism and growth.

1.2.3 Endocrine Disruption

1.2.3.1 Endocrine Disrupting Chemicals

Endocrine disrupting chemicals (EDCs) are defined as chemicals that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones

that are present in the body, and can thus cause deleterious effects on reproductive organs, function and behavior (64). The multitude of hormones, proteins, enzymes, and receptors that form a part of the HPGL axis and the complexity of their regulation make the HPGL axis a major target of EDCs (7). Endocrine disrupting chemicals can be naturally occurring products such as metals and metalloids – Hg and As, phytochemicals such as isoflavones – genistein, or synthetic chemicals such as pesticides, organophosphate flame retardants (OPFRs) and personal care products (PCPs). The negative effects of EDCs can also be observed in processes such as growth and metabolism due to the interactions of the endocrine system with these processes. Among the many classes of EDCs are those that mimic estrogens and androgens, thus affecting estrogen and androgen synthesis and metabolism (64). While these EDCs were generally known to act via nuclear estrogen and androgen receptors, more evidence has been generated indicating that there are numerous other pathway involving non-nuclear receptors, enzyme and non-genomic pathways through which EDCs may exert deleterious effects on the endocrine system (59,65,66).

1.2.3.2 Avian Endocrine Disruption

As mentioned earlier, one of the first cases of environmental contamination – the adverse effects of DDT on bald eagles and herring gulls, was also the first evidence of the endocrine disrupting potential of chemicals (8,67). Other cases of chemicals such as DDE, and polychlorinated biphenyls (PCBs) in the Great Lakes region and Green Bay, Wisconsin have also been implicated in similar adverse effects such as egg mortality in avian species including double-crested cormorants (68,69). A number of laboratory-based studies have examined the negative effects of chemicals on model avian species including: PCBs (70), BPA (71,72), atrazine (73), methoxychlor (MXC) (74,75), nonylphenol (76,77), vinclozolin (78) and PCPs such as EE2 and

diethylstilbesterol (79). Collectively, these studies and many more have demonstrated that exposure to environmental contaminants, whether naturally occurring or manmade, can cause reproductive impairment, decreased survival of offspring, and neurobehavioral effects in avian species including wildlife, ultimately impacting entire populations (80,81). However, despite birds being the earliest indicators of endocrine disruption in the wild, much of the ensuing research has been focused on mammalian and aquatic species. Additionally, while there have been numerous studies examining the impacts of estrogenic chemicals such as BPA, EE2, nonylphenol, MXC, and polychlorinated biphenyls (PCBs), the effects of androgen mimicking chemicals have not been explored in depth.

1.2.3.3 17β-trenbolone: A Model Androgenic Chemical

Trenbolone, commercially sold as trenbolone-acetate (TbA), is a synthetic androgenic steroid used as a livestock growth promoter. While concrete numbers on trenbolone production are not available, it is estimated that several tons of trenbolone are manufactured and implanted annually (82). Upon ingestion, TbA is metabolized to 17α-Trenbolone (17αT) and 17β-trenbolone (17βT) (82,83). 17β-trenbolone is the more active metabolite with a greater affinity for androgen receptors (AR) than endogenous T (84). There is no information available on the environmental body burden of 17βT in birds; however, studies looking at environmental levels have reported 17βT concentrations up to 0.0043 ppm in solid dung collected from a livestock farm containing cattle implanted with TbA (82). Fish and wildlife can be exposed to 17βT in waterways, livestock fields, or feedlots that have been fertilized with manure from livestock.

Previous research has shown that 17βT disrupts key physiological processes related to the endocrine system in various fish species. In FHM, a 21-day exposure to 17βT altered plasma

hormone levels such as E2 and T (85). While plasma E2 levels were increased in males, in females, E2 and T were decreased. A time-course 17βT study in FHM also showed a decrease in T synthesis from gonads (86). This decrease was suggested to be due to the internal feedback mechanism of the endocrine system compensating for the higher perceived total androgens, where total androgen is seen as the sum of exogenous 17βT and endogenous T (85,86). Concomitant decreases in plasma E2 levels and changes in associated genes were also observed along with downstream effects such as a decrease in VTG production (86). Additional studies in fish species including Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), sheepshead minnow (*Cyprinodon variegatus*) and guppy (*Poecilia reticulata*) have shown similar effects at molecular and biochemical levels, and demonstrated impairment in reproductive organs, function and behavior (87–92)

In comparison, there have been three studies examining effects of 17βT on the endocrine system in birds - Japanese quail (JQ; *Coturnix japonica*). Quinn et al. (93) studied the effects of a one time *in ovo* injection of 17βT in the yolk of JQ eggs, on reproductive behavior. Characteristics of reproductive behavior including the number of mount attempts and successful cloacal contacts, and egg production, were assessed at various points following the onset of puberty till 18 weeks of age. The authors found that onset of puberty was delayed in males. The adult cloacal gland area was also decreased in the males compared to the controls. The different characteristics of copulatory behavior such as attempts to mount and successful copulations were significantly lower compared to the controls. Henry et al. (94) investigated the effects of dietary exposure to 5 or 20 ppm 17βT in 12.5-week old JQ. They found decreased female plasma testosterone and a decrease in the number of maturing yellow follicles and overall egg production in the 20 ppm group. A further study looking at multi-generational exposure

investigated effects of *in ovo* and dietary 17βT exposure across F0, F1 and F2 generations in JQ (95). The authors found that plasma estradiol levels were elevated while plasma testosterone levels were decreased in the females. In the males, plasma estradiol levels were elevated. Additionally, an increase in CYP19A1 mRNA expression was observed in the F1 females and males. While the concentrations used in these studies were higher than what might be encountered in the environment, using concentrations that demonstrate measurable results can allow us to better understand mechanistic effects (95).

1.2.3.4 Model avian species

For decades the Japanese quail and white leghorn chicken (*Gallus gallus domesticus*), have consistently been used as model species in various areas of research such as basic reproductive biology, and several disciplines of toxicology (96,97). For example, the Organization for Economic Cooperation and Development (OECD) test guidelines 206 and 223 concerns reproductive and acute toxic effects. With the chicken genome sequence being completed in 2004, the development of high throughput screening platforms and bioinformatics tools, and the physiological similarity of JQ to humans, there are many attributes that have advanced the JQ and chicken to model organism status (98). Additionally, fertilized chicken eggs are easily obtained commercially, and a JQ colony was made available at the USGS. Eggs from these birds can be successfully incubated and hatched under artificial conditions. The resources required to incubate quail and chicken eggs are minimal and the incubation period is a short 18-21 days after which tissue samples are easily obtainable. Collectively, these allow the JQ and chicken to serve as optimal model avian species.

1.2.4 Conclusion

We live in a polluted world; animal-based methods that have long been considered the gold-standard in toxicity testing are also flawed. This has generated great interest in the development of alternative testing approaches. However, there is uncertainty in how such methods compared with whole animal tests. Thus, more research is warranted on increasing understanding of traditional whole animal as well as various alternative approaches. Overall, this thesis aimed to use traditional and alternative methods and examine the impacts of chemicals that affect the endocrine system in model avian species.

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Preface to Chapter 2

Chapter 2 describes the study of global hepatic gene expression in Japanese quail exposed to 17β-trenbolone (17βT), an androgenic endocrine disrupting chemical (EDC). This study attempts to look at transcriptomic differences related to sex (male vs female) and developmental stage (embryo vs adult) upon exposure to a model EDC. Birds were exposed to 17βT *in ovo* (embryos) or *in vivo* via diet for 17 weeks (adults). Additionally, moving beyond the traditional method of using qPCR to analyze expression of a few genes, this study examined changes in gene expression in the entire transcriptome using RNA Sequencing. Performing a transcriptome wide analysis enabled us to look at pathway-based effects of 17βT exposure. To the best of our knowledge, this is the first study to investigate changes in the hepatic transcriptome of an avian species upon exposure to an EDC.

This chapter is authored by the candidate and coauthored by the candidate's supervisor Dr. Niladri Basu, Drs. Natalie K. Karouna-Renier, Paula F.P. Henry and Robert Cornman. The initial study design was developed by Drs. Karouna-Renier and Henry, and they along with collaborators at the USGS performed sample collection; Dr. Cornman was responsible for processing the raw sequencing counts and goseq analysis. The candidate was responsible for performing all differential expression analysis, analysis and interpretation of the data, discussion of the results, and preparation of the manuscript, and was provided advice by the candidate's supervisor Dr. Niladri Basu. It is planned for submission to Environmental Toxicology and Chemistry.

Chapter 2

2 Sex- and Developmental Stage-related Differences in the Hepatic Transcriptome of Japanese quail (*Coturnix japonica*) Exposed to 17β-trenbolone

2.1 Abstract

Endocrine disrupting chemicals (EDCs) have long been known to cause reproductive dysfunction in various avian and aquatic species. Molecular and biochemical interactions between the endocrine system and biological processes including metabolism, transport, and cell growth extend the scope for observable effects of EDCs beyond reproduction. Analyzing changes in the transcriptome of target tissues (e.g., liver, gonads) in response to EDC exposure is an integral part of understanding the mechanisms of action. We investigated sex- and developmental stage-related transcriptomic differences in F1 generation Japanese quail liver (*Coturnix japonica*) from *in ovo* and dietary exposure to 17β-trenbolone (17βT, a model EDC) at 0, 1 or 10 ppm. Our objectives were: A) to identify differentially expressed hepatic genes and assess perturbations of biological pathways by performing gene set enrichment analysis in all treatment groups, and B) to examine the differences in response in embryos vs adults and males vs females. Sequencing data were processed using Bowtie2, and NetworkAnalyst was used for differential expression analyses. Gene Ontology and pathway enrichment were analyzed using goseq and NetworkAnalyst. In male embryos, 546 genes and 178 genes were up- and down-regulated (>2-fold), respectively, in response to 17βT (10ppm). Similarly, 240 and 133 genes in

female embryos, 55 and 30 genes in adult males, and 68 and 30 genes in adult females were up- and down-regulated, respectively. Vitellogenin and apolipoprotein were upregulated in male adults exposed to 17βT (10ppm). The molecular functions and biological processes that were highly represented included metabolism and transport of lipids and proteins, enzyme activity and extracellular matrix (ECM) interactions. Several pathways were found to be enriched e.g., female embryos (peroxisome proliferator-activated receptor, cardiac muscle contraction, gluconeogenesis); male embryos (growth factor signaling, focal adhesion, Hedgehog signaling, adipocytokine signaling); female adults (focal adhesion, ECM receptor interaction); male adults (bile acid biosynthesis, pyruvate and pyrimidine metabolism). Since one of the main uses of 17βT is as a livestock growth promoter, these results suggest potential effects on biological functions related to cell proliferation, differentiation, growth, and metabolism and transport of biological molecules.

Keywords: Sex, developmental stage, 17β-trenbolone; Japanese quail; liver; RNA-Seq;

2.2 Introduction

Toxicity studies have long since demonstrated that exposure to endocrine disrupting chemicals (EDCs) can have adverse effects in wildlife such as eggshell thinning and decreased survival of offspring, ultimately, leading to population-level impacts (1–4). EDCs act by directly stimulating or inhibiting the production of hormones or by mimicking or blocking the effects of hormones at target receptors or tissues (5). Adverse effects of EDCs may be observed beyond impairment of reproductive pathways, such as growth, metabolism and stress (6-8). Thus, several biological molecules such as proteins, enzymes, receptors and their ligands involved in these processes and associated genes are also likely affected (7,9). Much of the research on transcriptomic effects of

EDCs has been focused on mammalian and aquatic species, and not much is known about transcriptomic effects in birds (Figure 2.1).

Variations in the abundance of key biological molecules in males and females may result in differential responses upon exposure to EDCs (10,11). Studying sex- and developmental stage-related differences in animals in response to exogenous stress is a key aspect in clinical research. However, often, toxicological studies may examine one gender and stage, and generalize or ignore them as factors (10,12,13). Sexual dimorphism and differences in individual and social behavior are important in the wild and maybe overlooked in laboratory studies (12,14). Additionally, embryos may be more vulnerable to exogenous stress than adults since they lack fully developed detoxifying and metabolizing processes (15-17). Reviews of avian toxicology have examined numerous studies and, in many cases, exposures were performed only in males or females, and embryos or later in development (18-20). These and other publications have further emphasized the need to include sex and developmental stages in endocrine disruption studies (21,22).

Trenbolone, commercially available as trenbolone acetate (TbA), an EDC, is an anabolic steroid used in the livestock industry to increase muscle mass. Upon consumption, TbA is hydrolyzed to 17β-trenbolone (17βT) (23,24). 17β-trenbolone has a long half-life, and thus higher potential to accumulate in higher trophic levels and soils treated with manure from animals having TbA implants (24). Wildlife can be exposed to 17βT in waterways, livestock fields, or feedlots that have been fertilized with manure from livestock. While there are no reports on body burden of 17βT in birds in the environment, previous studies have considered the Japanese quail (JQ; *Coturnix japonica*) as the model avian species to investigate effects of EDCs (30). While, adverse effects of 17βT have been well characterized in fish (25-27), in

comparison, there are fewer avian studies. These have demonstrated deleterious effects such as changes in plasma hormone levels, reproductive behavior and decreased egg production (28-30).

Understanding the effects of EDCs at the molecular level (such as effects on gene expression) is an integral aspect of investigating the mechanism of action. A powerful and sensitive method for transcriptomic analyses is RNA sequencing (RNA-seq), which can generate gene and pathway level information (31-33). The objective of this study was to examine differences related to sex and developmental stage in the hepatic transcriptome of JQ exposed to *in ovo* and dietary 17βT. Additionally, by performing pathway enrichment analyses on the significantly differentially expressed (SDE) genes, we aim to identify perturbed biological pathways containing higher numbers of SDE genes. We hypothesize that hepatic genes related to endocrine processes such as steroidogenesis, and metabolism will be differentially affected upon exposure to 17βT across sex and developmental stages. This information could help deepen our understanding of the broader effects of androgens in the avian liver, and by further characterizing the effects of 17βT, advance our knowledge of the mechanisms by which it can disrupt biological pathways in Japanese quail.

2.3 Methods

2.3.1 17β-trenbolone

Wildlife International Gamebird Ration (Cargill # 108564-WI; Wildlife International Ltd. Easton, MD, USA) containing 0, 1, or 10 ppm (mg/kg) feed of 17βT (CAS 10161-33-8; Sigma, St. Louis, MO, USA) was used. The Gamebird Ration contained ≥ 27% protein, ≥ 2.5% crude fat, ≤ 3.8% crude fiber, and approximately 1.0% calcium. Calcium was supplemented to 3% in the diet to meet the minimum required for breeding quail. Levels of 17βT in feed (recoveries 1

ppm= 105.1%, 10 ppm = 102.8%) were verified by the US Army Public Health Command, Directorate of Laboratory Sciences (Aberdeen, MD, USA) using HPLC-UV.

2.3.2 Animal Husbandry

All procedures involving the handling of animals were reviewed and approved by the USGS Patuxent Wildlife Research Center's (PWRC) Animal Care and Use Committee, Laurel, MD and the Institutional Animal Care and Use Committee at Virginia Tech, Blacksburg, VA. The methods for animal husbandry and 17βT exposure for the samples obtained are described in detail in Karouna-Renier et al. (28). Briefly, a total of 96 healthy, compatible, unrelated, and actively breeding pairs were randomly selected for the F0 generation. The F0 pairs were randomly assigned to a treatment group (n=16 pairs/group; 0, 1, or 10 ppm feed of 17βT) and were started on treatment upon onset of sexual maturity at 6.1 weeks of age. These concentrations were selected from the range of concentrations used in the multi-generational study (29) to represent a low and high dose.

Eggs collected from the F0 pairs (7-8 weeks after initiation of treatment) were incubated to provide adults for the F1 generation. Eggs collected over weeks 9-10 were also incubated and the resulting embryos (males and females) were euthanized at embryonic day 12 by decapitation providing the embryo samples for this study. The F1 generation adult quails (n = 11-13 per treatment group) received feed containing the same level of 17βT as their parents, from day 1 post-hatch, for 17 weeks. Male and female F1 birds were paired when 90% of control birds exhibited foam production or egg laying (9.3 weeks) and remained paired until the end of the exposure period. The F1 quails were euthanized by decapitation at 17 weeks of age providing the

adult samples for this study. A diagrammatic representation of the Japanese quail exposure is provided in Figure 2.2. No mortality was observed in the treatment groups used for this study.

2.3.3 Sample Collection and RNA Extraction

The methods for sample collection and RNA extraction are explained in detail in Karouna-Renier et al. (28). Briefly, during necropsy, liver from F1 embryos (embryonic day 12) and F1 adults (17-week old) were removed and a piece was immediately placed in RNALater® (Life Technologies, Grand Island, NY, USA) solution, refrigerated overnight at 4 °C, and then frozen at –20 °C until analysis. Total RNA was isolated from liver (n = 3 per treatment group for each sex and developmental stage) using RNAzol (Molecular Research Center, Inc., Cincinnati, OH, USA) in combination with the RNeasy Mini Kit (Qiagen, Gaithersburg, MD, USA). Total RNA concentration and A260/A280 were determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA), and RNA quality was assessed visually by gel electrophoresis using the Northern-Max-Gly Sample Loading Dye protocol (Life Technologies). RNA was stored at -80 °C until analysis.

2.3.4 RNA Sequencing

2.3.4.1 Transcriptome sequence data processing

Raw fastq and biosample information was submitted to GenBank under BioProjects PRJNA313918 and PRJNA313931. Reads were trimmed in CLC Genomics Workbench v7 (Qiagen). Adapter sequences introduced during library preparation were trimmed using the default adaptor trimming algorithm. Base calls with an error probability greater than 0.01 were trimmed. Retained reads had a minimum length of 40 and at most 2 ambiguous characters. Reads

were mapped to predicted transcripts of *Coturnix japonica* assembly 2.0 (GenBank accession GCF_001577835.1)) using Bowtie2 (34) with --very-sensitive and --end-to-end parameter presets. As transcript models include alternative isoforms of the same genetic locus, locus identifiers for each transcript were parsed from NCBI's official mapping of accessions to entries in the Gene database. Mappings were then filtered on a Phred-scaled map quality of 20 and tabulated with the idxstats function of SAMtools and summed by locus identifier (35).

2.3.4.2 Quantification of differential mRNA expression levels

Filtering and normalization of the raw count numbers and statistical assessment of differential gene expression were conducted using the EdgeR statistical package in NetworkAnalyst (36,37). The interquartile range (IQR) used was 15 and the baseline count was set at 4 to remove genes with low variance or near constant gene expression values. Normalization was performed based on the trimmed mean of M values (TMM). Pairwise differential analyses were conducted for each age, sex, and dose class vs the respective control using a negative binomial model. Genes were considered to be differentially expressed when the adjusted p-value as determined by the likelihood ratio test was <0.05. Genes for which the $\log_2$ fold change exceeded 1 or -1 and were considered for pathway enrichment analysis. Descriptive statistics were performed for each class vs control to determine differences in numbers of genes that were SDE. Visualization of gene expression classifications among all differentially expressed genes was performed with bar graphs and Venn diagrams.

2.3.4.3 Gene ontology and pathway enrichment analyses

Identification of ontology terms from the Generic GO Slim developed by the GO consortium (38,39) enriched among differentially expressed genes was investigated with goseq using the default approach to length bias correction (40). As no ontology was available for *C. japonica* at the time of this analysis, annotation information was derived from homologous genes in the chicken (*Gallus gallus*) genome. Protein models for *G.gallus* were downloaded from Ensembl (access date 5/19/16), and the best BLASTX match for each *C. japonica* mRNA with a minimum bit score of 100 was assigned as a putative functional homolog. Gene ontology for *G.gallus* were downloaded from Ensembl Biomart (access date 5/20/16). As multiple *C. japonica* mRNA can have the same *G. gallus* homolog, the *G. gallus* homolog was considered to be in the differentially expressed category if any of those *C. japonica* mRNAs were differentially expressed. The European Bioinformatics Institute (EBI) part of the European Molecular Biology Laboratory (EMBL) QuickGO database was used to study gene ontology (GO) results within the GO hierarchy – biological process (BP), cellular component (CC) and molecular function (MF). The PANTHER (Protein Analysis Through Evolutionary Relationships) biological database of gene and protein families and their functionally related subfamilies was used to identify functional groups of the genes (41,42). Pathway enrichment analysis was performed using NetworkAnalyst for the significantly differentially expressed genes in each individual class (female adult, female embryo, etc.) on pathways described in the KEGG database (33,36).

2.4 Results and Discussion

In this study, our objectives were to examine how changes in the JQ hepatic transcriptome exposed to 17βT differed across sex and developmental stage. To this end, we looked at

differentially expressed genes, and also biological pathways that were significantly enriched by these genes.

2.4.1 Overall significant differential expression

Hereafter, female embryo and male embryo at 10 ppm dose will be referred to as FE10 and ME10, respectively. Male and female adults at 10 ppm dose will be referred to as FA10 and MA10, respectively. Similarly, female and male - embryo and adults at 1 ppm dose will be referred to as FE1, ME1, FA1, and MA1, respectively. There were 98 genes in FA10, 85 in MA10, 373 in FE10, and 724 in ME10 that were significantly up- or down-regulated by at least 2-fold (i.e. log2 fold = 1). There were 3 genes in FA1, 20 in MA1, 94 in FE1, and 633 in ME1. In each group the number of genes that were upregulated were higher than the number of genes downregulated (Supplementary Table 2.1).

2.4.2 Descriptive Statistics

Descriptive statistics were performed to compare differences between developmental stages (embryo vs adult) and sex (male vs female):

1) Embryo vs adult: The number of genes differentially expressed by at least 2-fold in FE10 (373) was about four times the number of genes differentially expressed in FA10 (98). Similarly, the number of genes differentially expressed in ME10 (724) was about nine times the number in MA10 (85). Among all genes that were SDE in ME10 (724) and MA10 (85), only 11 genes were common (Figure 2.3A). Similarly, among all SDE genes in the FE10 (373) and FA10 (98), only

9 genes were common (Figure 2.3B). A list of common genes in the ME10-MA10 and FE10-FA10 pairs is provided in Supplementary Table 2.2.

Thus, it appears that more genes are differentially expressed in the developing embryos. Research in mammalian models has shown that the embryo and fetus are more sensitive to exogenous stress since they not only lack a blood-brain barrier but also possess suboptimal hepatic detoxifying and metabolizing capabilities (16,43). Additionally, due to mechanisms that compensate for exogenous stress, genes that are perturbed in embryos could be restored to normal function in the developed adults (44). These factors could contribute to fewer differentially expressed genes in adults compared to the developing embryo. Genes that were common among the male embryo and adults included osteoglycin, olfactomedin, phosphoribosylformylglycinamidine synthase, rhophilin 1, and TNF receptor associated factor. Genes common among the female embryo and adults included phosphoenolpyruvate carboxykinase (PCK1), heat shock protein 9, MK167, and orosomucoid 2. The reasons for these genes to be differentially expressed across life stages is not yet clear. However, several of these genes are found to be involved in steroid transport, gluconeogenesis, bone formation, purine biosynthesis, and regulation of cell proliferation. This indicates that processes related to transport, metabolism and growth might be affected upon exposure to 17βT.

2) Male vs female: It seems that while in the embryonic stage, number of genes that are affected in the males (724) is about twice that of in the females (373). However, in adults, this difference evens out in general and there are a few more genes SDE in females (98) than in males (85). Among all the genes that were SDE in the ME10 (724) and FE10 (373), there were 175 genes that were common (Figure 2.4A). Similarly, among all genes in MA10 (85) and FA10 (98), only

7 genes were common (Figure 2.4B). A list of common genes in the ME10-FE10 and MA10-FA10 pairs is provided in Supplementary Table 2.2. Among the genes that were common between each set of pairs, there were no dimorphic genes (i.e. upregulated in males but downregulated in the females or vice versa) in the 1 ppm dose male-female pairs. There were eight dimorphic genes in the FE10-ME10 pair: dynein assembly factor 3, troponin I3 cardiac type, PEPCK and RAD52 motif containing 1 were downregulated in males but upregulated in females; protocadherin 20, SUN domain containing protein 3-like, catenin delta 2 and carboxymethylenebutenolidase (CBML) homolog were upregulated in males but downregulated in females. There were three dimorphic genes in the FA10-MA10 pair: hemopexin, orosomucoid 2, and matrilin 4 (MATN4); all three genes were upregulated in the males but downregulated in the females.

While the reasons for the sex-based dimorphism observed in these specific genes are unclear, the three dimorphic genes in the MA10-FA10 pair are known to be involved in inflammatory response, and Miller et al. (45) observed sex-based differences in other genes also involved in inflammatory response. A deficiency in MATN4 was shown to be associated with increased cell proliferation of hematopoietic stem cells which is linked to a disturbance in homeostasis (46). Among the other genes, PEPCK is known to be a rate-limiting step in gluconeogenesis, and a folic acid supplemented diet in rats resulted in sex-based differences in PEPCK expression (47). These further indicate the involvement of processes indirectly related to the endocrine system. A study with zebrafish (*Danio rerio*) exposed to four different EDCs also observed sexual dimorphism in hepatic genes involved in metabolic pathways (48). Collectively, these results suggest that one of the mechanisms by which EDCs function is by affecting metabolism.

Differences in response to EDCs between males and females can occur since the plasma concentrations and functions of sex hormones, can substantially differ between males and females (11). Depending on the exogenous stress due to EDCs, exposure, life-stage, and endpoints being measured, either male or female may be more vulnerable than the other (10,49). Males may not have the same ability to reduce their contaminant burden as females through maternal deposition of chemicals into their eggs. Previous studies with American kestrels (*Falco sparveritus*) and glaucous gulls (*Larus hyperboreus*) have shown that in certain cases males may be more vulnerable than females (11,50). The sex-based transcriptomic differences in response to exogenous stress are still relatively unknown in avian species. The absence of consistent patterns in the current study and observations from previous studies further demonstrates the importance of including both males and females while examining endocrine disruption.

2.4.3 Gene Annotation and Gene Ontology

The major endocrine pathway genes that were SDE were vitellogenin (VTG) and apolipoprotein (Apo) in MA10 (>5 log2 fold). Another study looking at 17βT exposure in male fathead minnow (*Pimephales promelas*) (FHM) also found an increase in plasma VTG levels (25). However, while studies have observed a decrease in VTG expression and protein levels in female fathead minnow exposed to 17βT (25,51), we did not find any changes in VTG expression in the female quails. VTG and Apo are both estrogen responsive genes that are typically expressed upon binding of estradiol to estrogen receptors (ER). However, we did not observe any changes in ER expression. Separate analyses of ER expression using qPCR also showed no changes in mRNA levels (unpublished data). It is possible that changes in ER mRNA expression alone may not be

sufficient to explain changes in VTG expression, rather investigating changes in binding of estradiol to ER might be a better indicator.

Among other SDE genes, gene annotation revealed that many belonged to categories including protein binding (e.g., troponin, plastin-1, transgelin, myosin binding factor), nucleic acid binding (e.g., DNA primase, histone acyltransferase), cell communication (such as tetraspanin, calsequestrin), enzyme activity (such as iodothyronine deiodinase, uridine phosphorylase, Rho associated protein kinase), transport (such as phospholipid transporting ATPase, cationic amino acid transporter, anion exchange protein), and protein, lipid, and carbohydrate metabolism (such as phosphoenolpyruvate carboxykinase, prostaglandin E synthase, hexosyltransferase). To summarize the data, plots of the % of SDE genes corresponding to their function (determined using corresponding GO terms) are presented for each class (Figure 2.5 and Figure 2.6). Interestingly, even though only a few genes were commonly SDE across sex and developmental stage, similar groups of functions and processes seem to be represented. Metabolic and cellular processes were the most highly represented biological processes. Other categories of processes represented were response to stimuli, developmental process, and biological regulation. Catalytic activity and binding were the most highly represented molecular functions. Other molecular functions represented were transporter and receptor activity.

Gene ontology identified certain categories in the different classes which were significantly expressed and are provided in Table 2.. For example, GO ID:0005856 which is involved in cell division, cellular movement, movement of organelles was identified in ME1, ME10, and FE10. Similarly, GO ID:0008092 which is involved in cytoskeletal protein binding was found enriched in ME1, ME10, and FE10. GO ID:0007010 which is involved in the function

of extracellular matrix organization and providing structural support to cells, was enriched in ME1 and ME10. Some other GO categories found to be enriched such as GO ID: 0007049, 0051301 and 0007067 are involved in processes such as cell division and cell cycle. The ECM consists of complex mixtures of molecules that play an important role in maintaining the structure and functions of cells and tissues. Studies have shown that interactions between cells and the ECM can be involved in the regulation of various cellular activities such as adhesion, and cell proliferation and migration (52,53).

There is a lack of information on transcriptomic changes due to EDC exposure in JQ. However, previous research looking at endocrine effects of androgenic chemicals such as trenbolone, testosterone, and methyldihydrotestosterone, in various fish species such as zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), found SDE genes belonging to many of the same functional groups such as binding, enzyme activity, and transport (54-56). Another study looking at the effect of androgenic chemicals on the proteomic profiles in FHM also found cellular and metabolic processes to be among the most represented biological processes, and binding and catalytic activity to be among the most represented molecular functions (57). Thus, it appears that the toxicogenomic effects of the androgenic chemical 17βT may be observed in genes involved in biological functions that are not directly related to the androgen receptor pathway. Previous research in FHM has shown that estrogenic compounds can affect processes such as metabolism, development, and response to stimulus (58). Previous studies on 17βT in JQ and FHM have shown a temporally dependent increase or decrease in plasma estradiol levels (28,44). It is possible that the effects observed in genes in these biological processes are due to changes in the estrogenic metabolites in response to 17βT exposure (54). Note that while most

studies did specify the form of trenbolone used for the exposure as 17β-trenbolone or trenbolone acetate, in some cases it was simply mentioned as 'trenbolone or TRB'.

2.4.4 Pathway Enrichment Analysis

A list of the KEGG pathways that were found to be enriched in the different classes is provided in Table 2.. No pathways were enriched in the FE1 class. In FE10 the main pathway to be enriched was the peroxisome proliferator associated receptor (PPAR) pathway. A complex pathway, it is described in the KEGG database and has three subtypes: PPARα plays a role in the clearance of circulating or cellular lipids by regulating expression of genes involved in lipid metabolism in liver and skeletal muscle; PPARβ/δ is involved in lipid oxidation and cell proliferation; PPARγ promotes adipocyte differentiation to enhance blood glucose uptake (59,60). Within the PPAR pathway, 9 genes (such as PPARγ, fatty acid binding protein 1, acyl CoA synthetase, phosphoenolpyruvate carboxykinase) were found to be differentially expressed under the three subtypes. These genes are known to be involved in the transport and metabolism of both fatty acid and lipids. While there are no avian studies that have examined the effects of EDCs on the PPAR pathway, a previous study on effects of 17βT exposure in Japanese medaka (*Oryzias latipes*) also found the PPAR pathway to be enriched and observed that genes related to lipid metabolism and cholesterol synthesis were significantly upregulated (26). Lopes et al. (61) studied PPAR expression in response to estrogenic and androgenic chemicals in brown trout (*Salmo trutta*). They found that brown trout treated with 10 and 50μM testosterone had significantly reduced PPARγ expression compared to control individuals. Since 17βT is commonly used as a growth promoter, some information is available on transcriptomic changes in livestock. Chung et al. (62) administered estradiol, 17βT, or a combination of 17βT /estradiol

to yearling steers and measured gene expression of select genes including PPARγ in bovine skeletal muscle. They noticed that PPARγ expression was decreased in the 17βT implanted individuals. Other studies in humans, mice, and rats have also reported that steroids alter PPARγ expression (63,64). Since the PPAR pathway is involved in lipid metabolism, it is possible that there is also a link with cholesterol synthesis. Cholesterol is a known precursor to androgen biosynthesis, and hence this could be one of the pathways through which 17βT affects gene expression.

Other pathways in FE10 were enriched by fewer genes - cardiac muscle contraction (4 genes), pyruvate metabolism (5 genes), and gluconeogenesis (4 genes). These pathways are involved in functions related to the production of precursor carbon metabolites in the central pathway of converting glucose to generate molecules of ATP, carbohydrate metabolism, and contraction of heart muscle (65). A study using growth promoting implants in beef cattle also found that the gluconeogenesis pathway was enriched (66). This further implies that the underlying mechanisms behind endocrine disruption may be through metabolic pathways.

Certain pathways were enriched in both ME1 and ME10, such as transforming growth factor (TGF) signaling (7 genes in ME1; 10 genes in ME10), cardiac muscle contraction (10 genes in ME1; 8 in ME10) and vascular smooth muscle contraction (10 genes in ME1; 11 genes in ME10). Some other pathways enriched were focal adhesion (15 genes), Hedgehog signaling (7 genes), tight junction (13 genes), and calcium signaling (14 genes). These pathways are generally known to be involved in cell proliferation, regulation of proliferation, motility and differentiation of cells, muscle contraction and signal transduction. It is known that there is crosstalk between adhesion and growth factor-mediated signaling (67,68); TGFs include types of proteins called bone morphogenetic proteins that are involved in the induction of bone and cartilage formation

in the body (69). A previous study using androgenic, growth-promoting implants in beef cattle also found TGF signaling to be enriched (66). Studies have shown that TGF signaling could be related to the AR, which could explain the effect of an androgenic chemical in these biological processes (70). Thus, the aforementioned studies show similar biological pathways being affected upon exposure to androgenic chemicals. Other studies have looked at effects of androgens further downstream such as on cell proliferation. For example, an *in vitro* study showed that androgens such as dihydrotestosterone and testosterone can stimulate proliferation of bone cells (71). Treatment of bovine satellite cells with TbA resulted in an increase in cell proliferation rates (72). TbA implants in yearling steers resulted in an increase in the number of actively proliferating satellite cells (73). Since AR activity is more predominant in the gonads and brain than in the liver, transcriptomic analyses in these additional target tissues could provide more insight into the reason for such signaling pathways to be affected by 17βT exposure (74,75).

No enrichment was possible for FA1, and the only pathway enriched in MA1 was starch sucrose metabolism, however only by 2 genes. In FA10 and MA10, few pathways were enriched since fewer genes were differentially expressed. Most of these pathways were enriched only by 2 or 3 genes. Some pathways such as PPAR, pyruvate metabolism, calcium signaling, and focal adhesion were in common in the embryos as well as adults. Other pathways found exclusively in the adults were pyrimidine metabolism, p53 signaling, bile acid biosynthesis, starch and sucrose metabolism, oocyte meiosis, and extra-cellular matrix (ECM) receptor interaction. These are found to be involved in the synthesis of primary bile acids from cholesterol in the liver and carbohydrate metabolism (76,77). Previous studies looking at transcriptomic profiles have shown links between metabolic and steroidogenic pathways in mice and fish (78,79). This crosstalk

between metabolic and steroidogenic pathways could explain the observed effects resulting from exposure to an androgenic chemical.

2.5 Conclusion

Through this study we increased our understanding of the adverse effects of 17βT on JQ hepatic gene expression across sex and developmental stages, using RNA sequencing. Embryos exposed to 17βT showed more genes that were SDE compared to the adults. Effects also differed between males and females as evidenced by the varying numbers of differentially expressed, and sexually dimorphic genes. The main genes affected in the endocrine pathway were VTG and Apo, only in the adult males. Many SDE genes were found to be involved in cell proliferation, differentiation, protein and nucleic acid binding, lipid and fatty acid metabolism, muscle contraction, etc. Pathway enrichment analyses showed that many of the pathways in female embryos were related to lipid and carbohydrate metabolism. In the male embryos, many of the pathways seemed to be related to cell proliferation, cell differentiation and signal transduction. However, process such as muscle contraction were common in both male and female embryos. Certain pathways such as metabolism and focal adhesion were common between embryos and adults, however, others such as bile-acid biosynthesis and ECM interactions were only enriched in adults. These differing results between males and females, and across embryos and adults highlights the importance to include these factors in endocrine studies. Overall, the gene and pathway findings are in accordance with other studies studying 17βT exposure in fish and cattle that also found similar biological processes to be affected. We know that 17βT is a synthetic androgenic steroid mainly used as a growth promoter in the livestock industry to increase tissue mass. These results suggest that 17βT may exert its effects through indirect processes including metabolism, cell

proliferation and transport of biological molecules. Future studies that look at transcriptomic profiles in JQ during the early stages of exposure and in other target tissues such as ovaries and brain could help increase our understanding of the mechanism of action of androgenic chemicals.

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2.7 Tables and Figures

Supplementary Table 2.1: Significantly differentially expressed (SDE) genes (> 2-fold) in Japanese quail (male and female, embryos and adults) exposed to 0, 1, and 10 ppm 17β-trenbolone. Up represents the number of genes that were significantly up-regulated, down represents the number of genes that were significantly down-regulated.

Up/down regulated	1 ppm				10 ppm			
	Embryo		Adult		Embryo		Adult	
	Male	Female	Male	Female	Male	Female	Male	Female
Up	472	73	15	3	546	240	55	68
Down	161	21	5	0	178	133	30	30

Supplementary Table 2.2: List of common differentially expressed genes across male and female, embryo and adult pairs in Japanese quail exposed to 10 ppm 17β-trenbolone. ME = male embryo; FE = female embryo; MA = male adult; FA = female adult.

ME10MA10	FE10FA10	FE10ME10	FE10ME10	FE10ME10	FE10ME10	FA10MA10
FGD4	ETNPPL	ABCD3	DNAAF3	LOC107309497	RAI14	AKAP12
HHATL	HSPB9	ACTC1	DST	LOC107309544	RASSF9	AMIGO3
LOC107307604	LOC107309544	ACVR2B	EGFR	LOC107310310	RB1CC1	CHRM2
LOC107307605	LOC107315599	ADAM10	ELN	LOC107311487	RBBP6	FGD4
LOC107319873	LTBP2	AHI1	ERG	LOC107311777	ROCK1	HPX
MATN4	MKI67	AKAP9	ESF1	LOC107311808	ROCK2	MATN4
OGN	ORM2	ANGPT1	ESR1	LOC107312463	RPAP3	ORM2
OLFML2B	PCK1	ANKRD12	ESRRG	LOC107314382	RPS24	
PFAS	SMC2	APC	FER	LOC107316692	SCTR	
RHPN1		APOB	FGL2	LOC107318723	SEC62	
TRAF1		ARHGAP5	GCC2	LOC107319047	SEMA3A	
		ARID4A	GCNT7	LOC107320555	SERPINA10	
		ARID4B	GLO1	LOC107325141	SETBP1	
		ASPM	GOLGA4	LRRC2	SLC12A1	
		ATRX	GPR34	LRRCC1	SLC26A1	
		B3GALT1	HELZ	LTBP2	SMC2	
		BAZ2B	HMMR	LYAR	SMC3	
		BDH2	HOOK1	LYVE1	SMC4	
		BOD1L1	HSPB8	MKI67	SMC5	
		BPTF	INHBA	MKL2	SMC6	
		CARS	IQGAP2	MTHFD1L	SMCHD1	
		CASP8AP2	ITGA1	MYOZ2	SNX29	
		CASQ2	IYD	N4BP2	SOX10	
		CCDC171	JPH2	NEBL	SRFBP1	
		CCDC18	KIAA1551	NES	SRP54	
		CCDC91	KIF16B	NIPBL	SSB	
		CDK5RAP2	KIF20B	NKTR	ST3GAL1	
		CENPE	KIF21A	NMRK1	ST8SIA4	
		CENPF	KNOP1	NOL8	SUB1	
		CEP135	LCA5	NRXN1	SYNM	
		CEP152	LIPC	NSRP1	TDO2	
		CEP162	LOC107305888	PCK1	TMEM135	
		CEP290	LOC107305997	PCNT	TMTC3	
		CGNL1	LOC107306196	PDS5B	TMX3	
		CHD1	LOC107306221	PFAS	TNNC2	
		CLSTN2	LOC107306231	PFDN4	TNNI3	
		COBLL1	LOC107306279	PHF3	TPR	
		COLEC11	LOC107306690	PLCB1	TRANK1	
		COPS2	LOC107306788	PNPLA8	TRIP11	
		CTAGE5	LOC107307457	PPIG	TTC6	
		CTNND2	LOC107307559	PRUNE2	UACA	
		CTXN1	LOC107307604	RAD50	ZBED4	
		DEK	LOC107307605	RAD51AP1	ZBTB38	
		ZNF644	ZNF518A	ZNF292		

Table 2.1: Gene Ontology (GO) categories that were significantly enriched in Japanese quail exposed to 0, 1, and 10 ppm 17β-trenbolone and the number of significantly differentially expressed (SDE) genes in each category. CC = Cellular Component; BP = Biological Process; MF = Molecular Function; FE = Female embryo; ME = Male embryo; FA = Female adult; MA = Male adult.

Class	Category	Ontology	Term	# of SDE genes	p-value
FE1	GO:0005694	CC	chromosome	13	0.005
	GO:0007059	BP	chromosome segregation	7	0.020
FE10	GO:0005615	CC	extracellular space	31	0.004
	GO:0008092	MF	cytoskeletal protein binding	31	0.006
	GO:0005856	CC	cytoskeleton	52	0.012
ME1	GO:0005856	CC	cytoskeleton	102	< 0.001
	GO:0008092	MF	cytoskeletal protein binding	55	< 0.001
	GO:0007010	BP	cytoskeleton organization	55	0.023
	GO:0007059	BP	chromosome segregation	19	0.026
	GO:0043226	CC	organelle	363	0.036
	GO:0005815	CC	microtubule organizing center	34	0.035
	GO:0005694	CC	chromosome	37	0.048
ME10	GO:0005856	CC	cytoskeleton	110	< 0.001
	GO:0008092	MF	cytoskeletal protein binding	55	0.001
	GO:0007010	BP	cytoskeleton organization	64	0.005
FA1	NA	NA	NA	NA	NA
FA10	GO:0030198	BP	extracellular matrix organization	7	0.004
	GO:0005694	CC	chromosome	12	0.004
	GO:0005578	CC	proteinaceous extracellular matrix	8	0.004
	GO:0007067	BP	mitotic nuclear division	7	0.006
	GO:0007049	BP	cell cycle	15	0.006
	GO:0000228	CC	nuclear chromosome	8	0.008
	GO:0005615	CC	extracellular space	11	0.024
	GO:0051301	BP	cell division	7	0.048
MA1	NA	NA	NA	NA	NA
MA10	GO:0005615	CC	extracellular space	11	0.046

NA = gene ontology was not possible for FA1 and MA1 due to low number of differentially expressed genes

Table 2.2: List of significantly enriched KEGG pathways obtained from NetworkAnalyst in Japanese quail exposed to 0, 1, and 10 ppm 17β-trenbolone. PPAR = Peroxisome Proliferator Activated Receptor; ECM = Extracellular Matrix; TGF = Transforming Growth Factor; FE = Female embryo; ME = Male embryo; FA = Female adult; MA = Male adult.

Class	Pathway	Total genes	Randomly Expected	# of SDE genes	P-Value
FE1	PPAR	61	0.357	2	0.049
FE10	PPAR	61	1.52	9	< 0.001
	cardiac muscle contraction	35	0.87	4	0.010
	pyruvate metabolism	64	1.59	5	0.021
	glycolysis/gluconeogenesis	53	1.32	4	0.042
ME1	cardiac muscle contraction	64	2.54	10	< 0.001
	focal adhesion	192	7.63	15	0.009
	vascular smooth muscle contraction	109	4.33	10	0.011
	tight junction	162	6.43	13	0.011
	regulation of actin cytoskeleton	188	7.47	14	0.016
	calcium signaling	172	6.83	13	0.018
	ECM-receptor interaction	81	3.22	7	0.041
	TGF-beta signaling pathway	83	3.3	7	0.045
ME10	TGF beta signaling pathway	83	3.5	10	0.002
	cardiac muscle contraction	64	2.7	8	0.005
	vascular smooth muscle contraction	109	4.59	11	0.006
	hedgehog signaling	42	1.77	6	0.008
	adipocytokine signaling	63	2.66	7	0.016
FA1	NA	NA	NA	NA	NA
FA10	p53 signaling	63	0.507	4	0.002
	ECM receptor interaction	81	0.651	4	0.004
	calcium signaling	172	1.38	5	0.011
	focal adhesion	192	1.54	5	0.017
	oocyte meiosis	97	0.78	3	0.042
MA1	starch sucrose metabolism	32	0.0468	2	0.001
MA10	bile acid biosynthesis	15	0.0768	2	0.003
	pyruvate metabolism	35	0.179	2	0.013
	pyrimidine metabolism	104	0.532	3	0.015
	herpes simplex infection	141	0.721	3	0.034
	PPAR	61	0.312	2	0.038
	adipocytokine signaling	63	0.322	2	0.040

NA = pathway enrichment was not possible for FA1 and MA1 due to low number of differentially expressed genes

Figure 2.1: Number of publications looking at transcriptomic analyses and endocrine disruption from Web of Science (1997 – 2017). Search terms were: (toxic* AND ("endocrine disruption" OR "endocrine disruptor" OR "endocrine disruptors" OR EDC* OR reproduct* OR androgen* OR estrogen*) AND ("RNA Sequencing" OR "RNA-Seq" OR "RNA Seq" OR "RNA seq" OR "RNA-seq" OR microarray* OR transcriptom*)). Search results were refined for species, human/mammalian: (human* OR rat* OR mice OR mammal*); fish studies: (fish* OR minnow* OR medaka* OR trout* OR perch* OR walleye* OR sturgeon*); avian studies: (bird* OR avian* OR chick* OR quail* OR finch* OR duck* OR mallard*). Black circles = RNA-Sequencing and endocrine disruption studies; Open diamond = human/mammalian studies; Grey triangle = fish studies; Black square = bird studies. (Accessed February 28th, 2018)

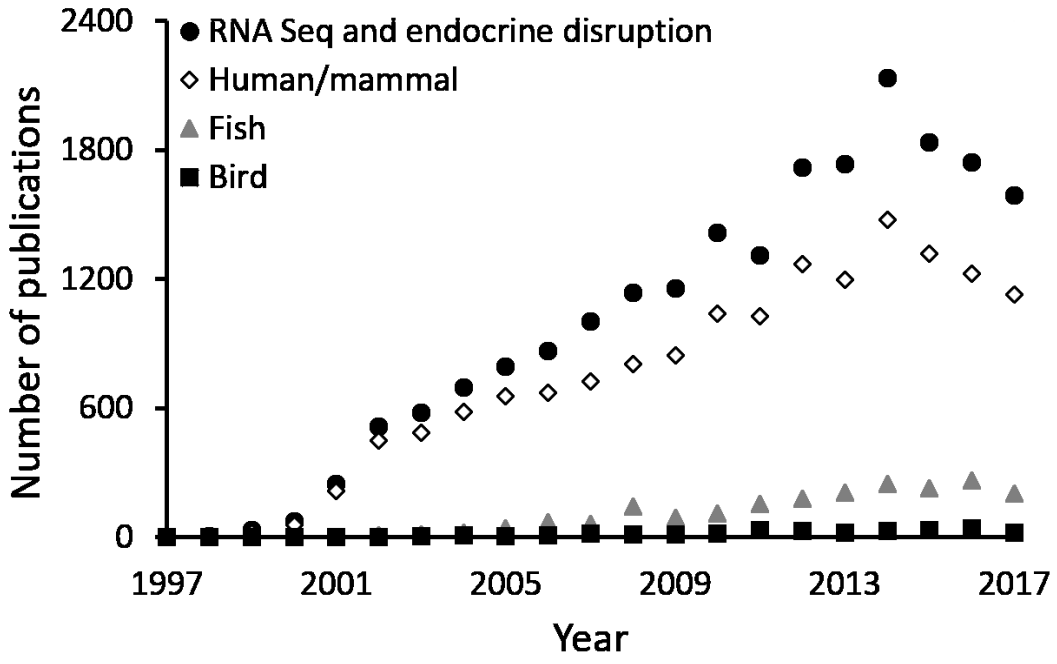


Figure 2.2: Diagrammatic representation of Japanese quail (JQ) 17β-trenbolone (17βT) exposure (0, 1, 10 ppm treatment groups). n = 3 per treatment group for each embryo (male and female) and adult (male and female).

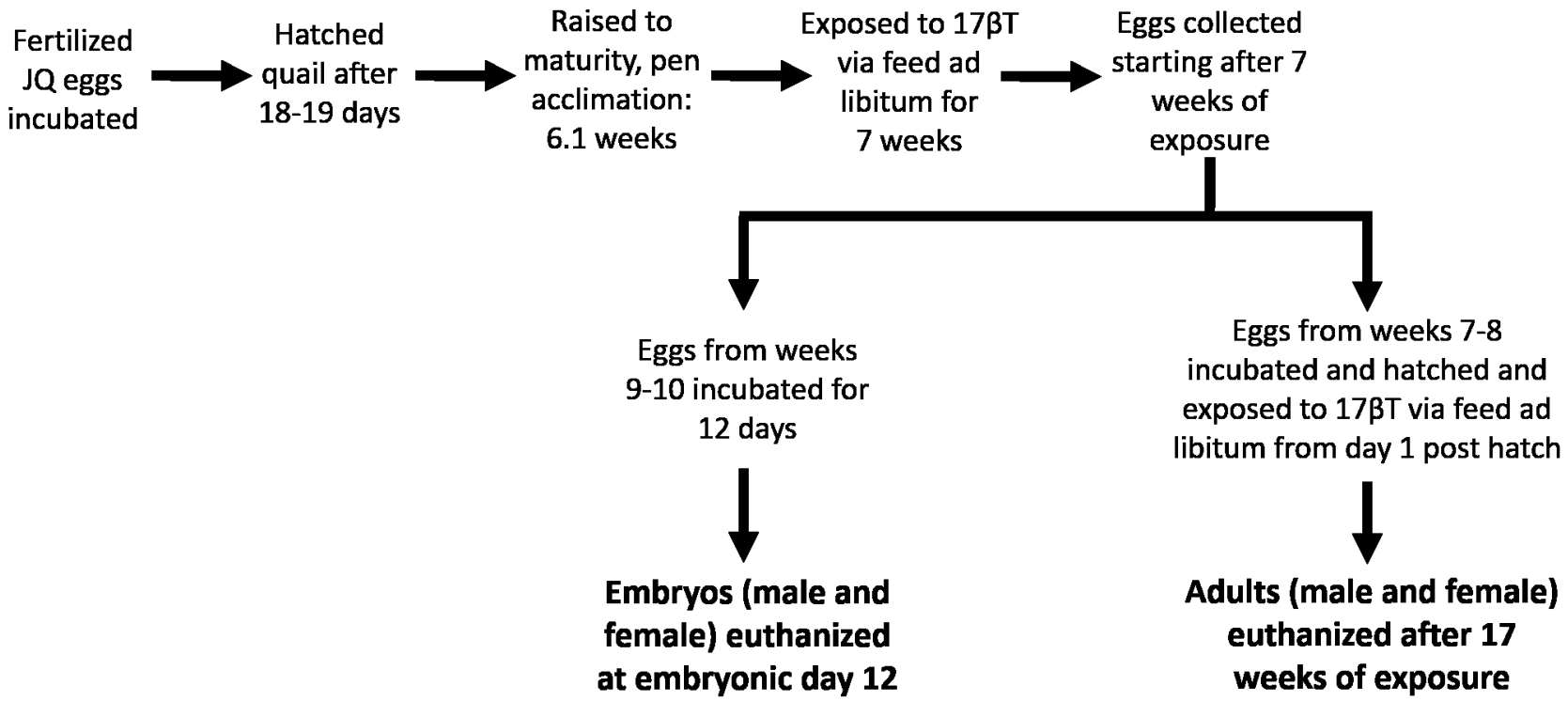
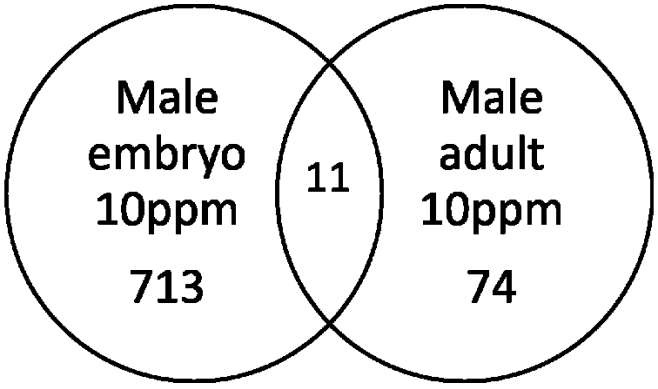


Figure 2.3: Number of significantly differentially expressed genes across embryonic, and adult Japanese quail exposed to 10 ppm 17β-trenbolone. (A) Males; (B) Females.

(A)



B)

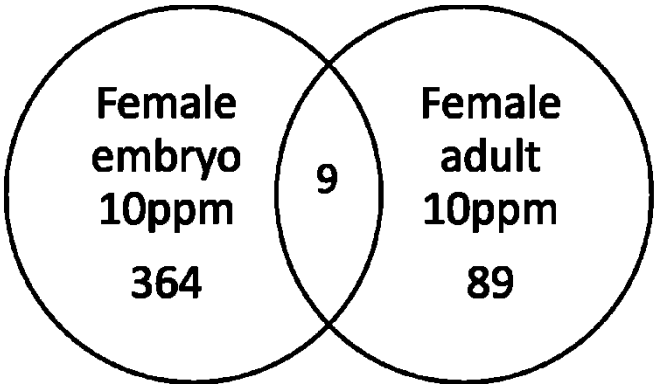
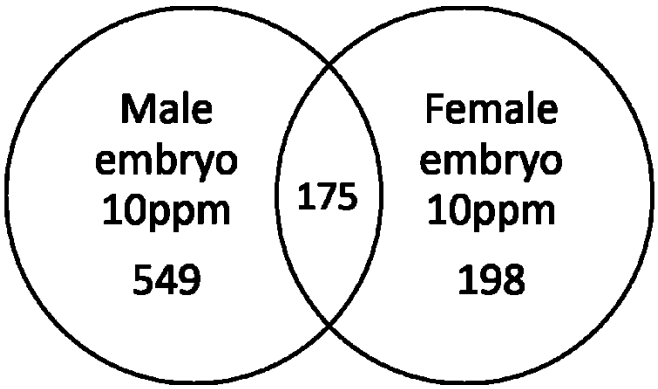


Figure 2.4: Number of significantly differentially expressed genes across male, and female Japanese quail exposed to 10 ppm 17β-trenbolone. (A) Embryos; (B) Adults

A)



B)

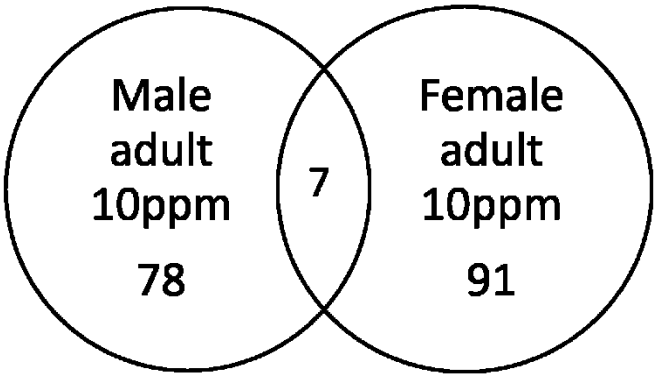


Figure 2.5: Biological process (BP) and molecular function (MF) grouping of differentially expressed hepatic genes in Japanese quail exposed to 10 ppm 17β-trenbolone. Fig A) Male (black) and female (grey) embryos; Fig B) Male (grey with black outline) and female (white with black outline) adults.

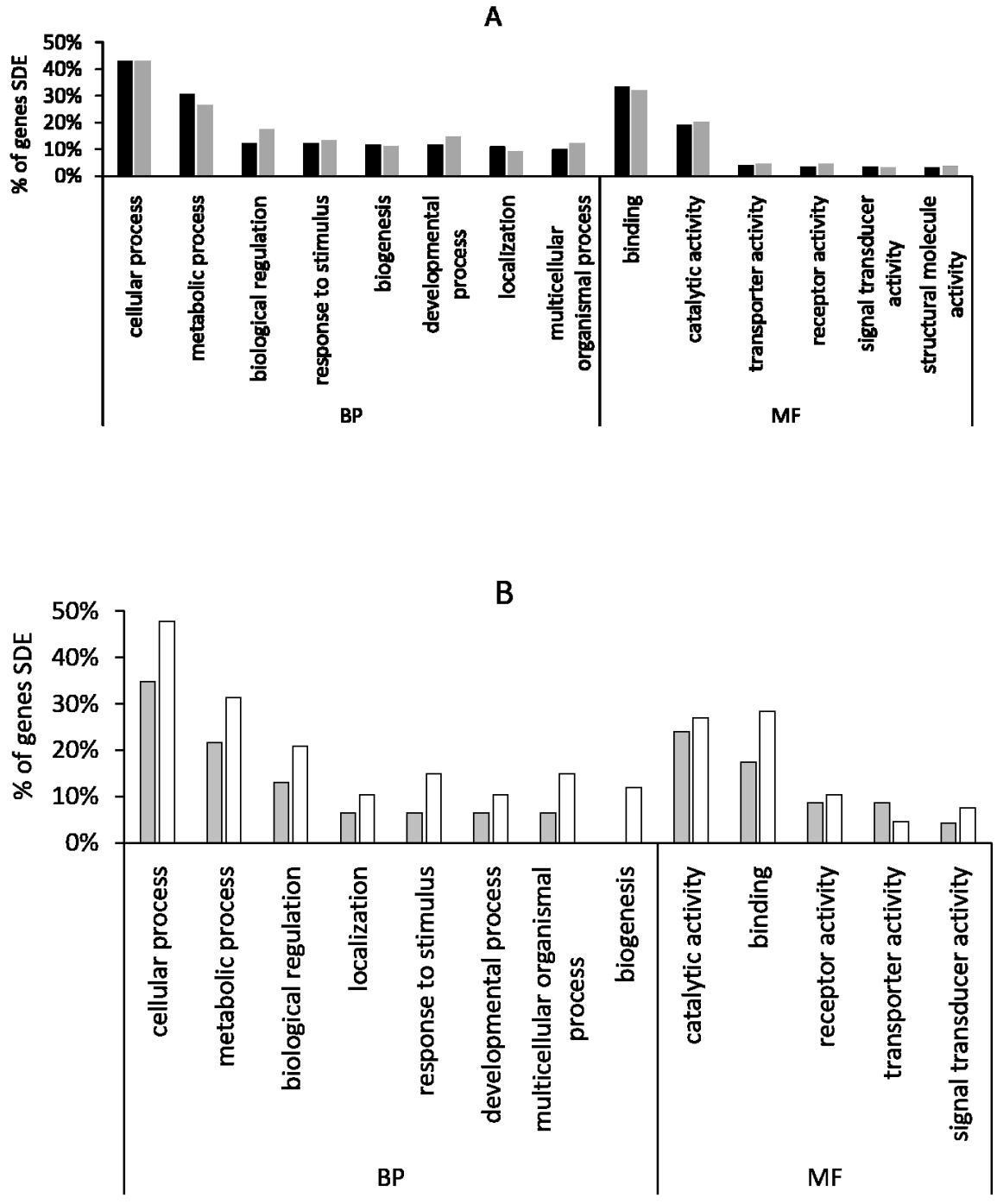
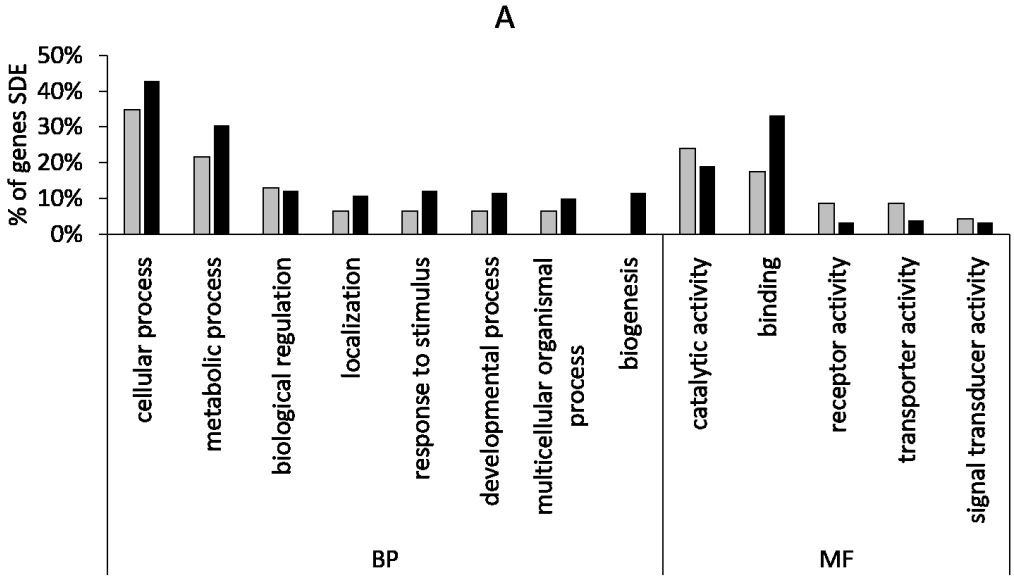
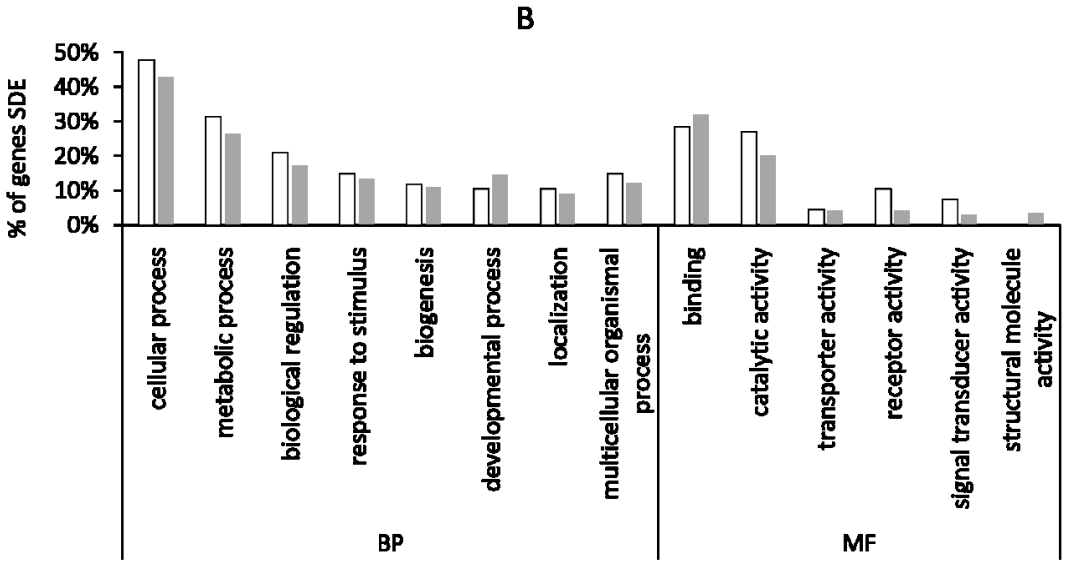


Figure 2.6: Biological process (BP) and molecular function (MF) grouping of differentially expressed hepatic genes in Japanese quail exposed to 10 ppm 17β-trenbolone. Fig A) Male adults (grey with black outline) and embryos (black); Fig B) Female adults (white with black outline) and embryos (grey).





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Preface to Chapter 3

Chapter 2 studied effects on global hepatic gene expression in Japanese quail (*Coturnix japonica)* exposed to *in ovo* and long-term dietary 17β-trenbolone (17βT). Differential effects on sex (male vs female) and developmental stage (embryo vs adult) were examined. However, effects during early stages of chemical exposure and sexual maturation are not yet clearly understood.

Chapter 3 investigates the short-term effects of dietary 17βT on molecular and biochemical endpoints relevant to the endocrine system in 6-9 week old Japanese quail (JQ). This chapter describes a laboratory study which examines changes in expression of genes in tissues (liver and gonads) relevant to the endocrine pathway and explores the effect on plasma concentrations of estradiol, testosterone, and vitellogenin. Emphasis was placed on understanding endocrine response in JQ within the first three weeks of *in vivo* exposure to 17βT.

This chapter is authored by the candidate and coauthored by Drs. Niladri Basu, Natalie K. Karouna-Renier, Paula F.P. Henry, Brandon Armstrong, and Cheryl Murphy. The candidate, in collaboration with the coauthors, was responsible for the study design and development, and sample collection; the candidate was responsible for sample analysis, data analysis and interpretation, discussion of the results, and preparation of the manuscript, and was provided advice on all aspects of the study by the candidate's supervisor Dr. Niladri Basu. It is planned for submission to Environmental Toxicology and Chemistry.

Chapter 3

3 Assessing Short-Term Effects of 17β-trenbolone on the Japanese Quail Hypothalamic-Pituitary-Gonadal-Liver Axis

3.1 Abstract

Endocrine disrupting chemicals (EDCs) have been long known to affect processes related to reproduction, however studies have focused mainly on fish and mammalian species. The few studies looking at effects of 17β-trenbolone (17βT, an anabolic androgenic steroid used as a growth promoter in the livestock industry) on Japanese quail (JQ) (*Coturnix japonica*) examined effects from long-term exposures (>8 weeks). We investigated effects of dietary 17βT (0, 5, 20 ppm), on the JQ (starting at 43 day old) hypothalamic-pituitary-gonadal-liver (HPGL) axis at days (D) 2, 7, 14 and 21 from the start of exposure. Morphometric measurements, including body weight, tarsal length, tissue weights, and the number of mature follicles were obtained from all birds during sampling. We examined plasma concentrations of luteinizing hormone, estradiol, testosterone and zinc (as a proxy for vitellogenin - VTG), and mRNA expression of genes relevant to the endocrine pathway in gonads and liver. Egg production was tracked throughout the exposure period. No change was observed in body weight across any treatment groups. A significant decrease was observed in the hepatosomatic index and total follicle weight in 20 ppm female birds at exposure D21 ($P < 0.01$). While the 20 ppm treated females produced fewer eggs, results were not statistically significant. 17βT significantly decreased plasma testosterone

concentration in treated females (D7 and 14; $P < 0.01$); significantly increased plasma estradiol concentration in treated males (days 8 and 21; $P < 0.01$) and females (D2, 14 and 21; $P < 0.05$); significantly decreased plasma Zn concentration in treated females (D21; $P < 0.01$). No statistically significant changes were observed in the expression of any of the genes measured in the liver and ovaries in any treatment groups or exposure days. This study helps to further characterize the disruptive effects of 17βT on the HPGL axis in birds.
Keywords: Endocrine disruption, 17β-trenbolone, HPGL axis, ecotoxicology, EDC

3.2 Introduction

Endocrine disrupting chemicals (EDCs) are chemicals that can interfere with hormonally regulated physiological processes by affecting normal signaling in the hypothalamic-pituitary-gonadal-liver (HPGL) axis, thus impairing reproductive functions (1). Depending on the stressor, effect on the endocrine system can be minor enough that normal physiological plasticity is able to re-establish homeostasis through regulatory feedback mechanisms (2,3). However, when the exogenous stressor is high enough to sufficiently impair feedback mechanisms, the organism may be unable to maintain homeostasis. The multitude of hormones controlling the HPGL axis and the intricacies of their regulation make the HPGL axis a prime target of EDCs (4). Such chemicals have long since been known to adversely affect the reproductive system in wildlife, ultimately impacting whole populations (5–7). Effects on hormonal and genomic biomarkers within the HPGL axis have been investigated in various species exposed to putative EDCs (8–11), however, overall mechanisms of action in birds have not been completely elucidated. Additionally, while evidence of endocrine disruption was initially observed in avian species, recently, research has been focused on mammalian and fish species (Figure 3.1).

Trenbolone, commercially available as trenbolone acetate (TbA), is a synthetic androgenic steroid used as a livestock growth promoter. Upon ingestion, TbA is hydrolyzed to 17β-trenbolone (17βT) (12,13), which is known to have a greater affinity for androgen receptors (AR) than endogenous testosterone (14). 17β-trenbolone has a long half-life, and thus higher potential to accumulate in higher trophic level organisms and soils treated with manure from animals that have been prescribed TbA implants (12). Wildlife can be exposed to 17βT in waterways, livestock fields, or feedlots that have been fertilized with manure from livestock.

Previous research has shown that 17βT disrupts key endocrine processes in fish species (15–17); however, few studies have looked at reproductive effects of 17βT in avian species, and these have been in Japanese quail (*Coturnix japonica*, JQ). Quinn et al. (18) studied the effects of a one-time *in ovo* injection of 17βT on reproductive behavior in adult JQ (18 weeks) and observed several traits including onset of puberty and successful copulations to be significantly impaired in treated birds. Henry et al. (19) reported lower female plasma testosterone (T) concentration and a decrease in the number of maturing yellow follicles and overall egg production in 12.5-week old JQ exposed to dietary 17βT. Karouna et al. (20) investigated effects of *in ovo* and long-term dietary exposure to 17βT across multiple generations of JQ and reported decreased plasma T in females, and increased plasma 17β-Estradiol (E2) in males and females. Quantitative polymerase chain reaction (qPCR) analyses showed that genes in the HPGL axis such as aromatase were differentially expressed in treated birds.

While these studies demonstrate the endocrine disrupting effects of 17βT in JQ, they were either based on one-time embryonic, or long-term (>8 weeks) dietary exposures in adult birds, and tissue samples were collected at a single time point. Ankley and Villeneuve (3) underscored the importance of duration of exposure while examining significant effects of EDCs

suggesting that investigating endpoints at multiple times is warranted. They discussed that due to the dynamic and transitory nature of molecular and biochemical endpoints, short-term time-course studies may be better suited for detecting EDCs when examining these types of biomarkers. Thus far, studies of responses to 17βT at multiple points during exposure have focused on fish and mammals and have not been examined in birds. This study builds upon previous research and exposed JQ to 17βT via feed, *ad libitum*, to characterize molecular (gene expression) and biochemical (plasma hormone levels) effects and egg production, by sampling birds at four time-points within the first 3 weeks of exposure. We hypothesize that 17βT exposed birds will exhibit changes in plasma hormone (including luteinizing hormone, E2, and T) levels and expression of associated genes (including those involved in T and E2 biosynthesis), and that these changes will vary over the exposure duration and will be different between males and females. This study aims to deepen our understanding of effects on hormone production and associated molecular changes that could potentially disrupt reproductive function such as egg production.

3.3 Methods

3.3.1 Animal Husbandry and 17β-trenbolone Exposure

A diagrammatic overview of the 17βT exposure is provided in Figure 3.2. All procedures involving the handling of animals were reviewed and approved by the USGS Patuxent Wildlife Research Center's (PWRC) Animal Care and Use Committee, Laurel, MD. The methods for animal husbandry and 17βT exposure were based on those described by Karouna-Renier et al. (20), with some modifications. Fertilized JQ eggs were incubated and hatched out in cabinet incubators (Kuhl Corp, NJ), set at 99.5 °F/37.5-37.8 °C and 55% relative humidity, with hourly

turning. Hatchlings were obtained 18-19 days after egg incubation and housed in 81.3 cm x 96.5 cm brooder pens (GQF Manufacturing Company Inc., Savannah, GA, USA) at 37.2˚C. Over the subsequent 6 weeks, daily photoperiod was progressively decreased from 24 h light at hatch to 16 h light/8 h dark and maintained as such for the remainder of the experiment. Feed and water were provided *ad libitum* during the entire period of the experiment. Birds were provided Wildlife International Gamebird Ration (Cargill # 108564-WI; Wildlife International Ltd. Easton, MD, USA). The Gamebird Ration contained ≥ 27% protein, ≥ 2.5% crude fat, ≤ 3.8% crude fiber, and approximately 1.0% calcium. Calcium was supplemented to 3% in the diet to meet the minimum required for breeding quail.

At day 33 post hatch, five females and one male were placed in each side of brooder pens divided in half by a plywood board and allowed to acclimate for 10 days. On d43 post hatch, that is, around the time of sexual maturation as determined by the onset of foam production, birds were randomly assigned to one of three treatment groups – 0, 5 or 20 ppm 17βT (CAS 10161-33-8; Sigma, St. Louis, MO, USA). These concentrations were chosen to represent a low and a high dose from a range of concentrations used in the previous multi-generation study (20). 17β-trenbolone was mixed into the feed and provided *ad libitum*. Daily feed consumption was estimated for assessment of 17βT uptake. Each bird was closely observed daily for signs of injuries and disease as well as for signs of aggressive behaviors towards their pen mates. No mortality was observed in the treatment groups used for this study

3.3.2 Sample Collection

Females from each treatment were euthanized by decapitation, at four time-points: exposure days 2, 7, 14, and 21 after 17βT treatment was commenced, corresponding to 45, 50, 57, and 64-day

old JQ, respectively. Within each sampling day, dissections were performed in three batches staggered by 2 h to try and capture the daily estradiol peak that corresponds to vitellogenesis (for details refer to Appendix 1). Thus, on sampling days, in each 2 h window, 5 females per treatment were dissected. Male JQ were euthanized, by decapitation, in two batches at exposure days 8 and 21, corresponding to 51 and 64 days of age, respectively.

Morphometric measurements were obtained including body weight, tarsal length, and the total weight of several tissues (brain, liver, spleen, testes or ovary, and thyroid gland), and the number of yellow follicles were counted. Whole trunk blood was collected from each bird into vacutainer tubes containing heparin beads (S-Monovette®, Sarstedt) and mixed thoroughly to prevent clotting. An aliquot of whole blood was stored for DNA damage analysis. Each blood sample was centrifuged at 300 g for 10 min to separate erythrocytes and isolate the plasma. Separate aliquots of the plasma were stored for estradiol and testosterone, thyroid hormone, steroid binding protein, vitellogenin, and luteinizing hormone analyses. Whole brain, ovaries, and thyroid gland were stored in RNALater (Qiagen) for mRNA analyses. A piece of the liver was stored in RNALater for mRNA analyses. Remaining liver and ~3 g muscle from each bird were stored in pre-cleaned amber jars (Amber wide mouth jars w/ PTFE cap, Scientific Specialties Service) for residue analyses. Tissues in RNALater were stored at 4 °C for at least 24 h before storing at -20 °C for RNA isolation. All plasma and whole blood aliquots were stored at -80 °C. Tissues for residue analyses were stored at -20 °C. Plasma aliquots for steroid binding protein and thyroid hormone, and the thyroid gland, brain, and spleen were stored for future analyses.

Approximately 15 g of liver (male and female) and muscle (female) samples were sent to Silliker Inc. (a subsidiary of Mereiux Nutrisciences) for 17βT residue analysis (Supplementary

Table 3.1). For females, ~1 g from 15 individuals from each treatment and time point were pooled for analysis. For males, 5 individuals from each treatment and time point were pooled for analyses. Samples were analyzed following Health Canada and Canadian Food Inspection Agency (CFIA) method CFIA CVDR-M-3017.16; internal reference for Silliker.Inc is M-P047. The method measured 17βT and 17αT, and also reported total trenbolone value.

3.3.3 Plasma Measurements

3.3.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

Plasma samples were assayed for luteinizing hormone (females only), 17β-estradiol (E2) and testosterone (T) at the Douglas Mental Health Institute (McGill University, Montreal, Quebec). Luteinizing hormone (LH) was measured by ELISA (Elabscience Chicken LH ELISA kit) following manufacturer's instructions. Due to low sample volume in the LH aliquots, some samples were assayed only in single replicates. Mean intra-assay CV was 14.9%; detection limit was 0.02 ng/ml. LH levels in 46 samples (24.7%) were below the detection limit and were not included in the analyses. Thus, this reduced the number of samples per group. Plasma T concentrations were measured by ELISA (Salivary Testosterone Enzyme Immunoassay Kit, Salimetrics, State College, PA, USA) with some modifications of the manufacturer's protocol. Each sample was run in duplicate. Female and male samples were diluted 10X and 50X, respectively. Mean intra- and inter-assay CVs were 4.12% and 9.67%, respectively; detection limit was 6.2 pg/ml. Plasma E2 concentrations were measured by ELISA (High Sensitivity Salivary 17β-Estradiol Enzyme Immunoassay kit, Salimetrics) with some modifications of the manufacturer's protocol. Each sample was run in duplicate. Since males have lower circulating E2 levels, these samples were subjected to diethyl ether extraction prior to ELISA analyses.

Female samples were diluted 10X. Mean intra- and inter-assay CVs were 2.75% and 2.79%, respectively; detection limit was 1.0 pg/ml (See Appendix 2 for further details on ELISA). The ratio of E2 to T was calculated based on the determined concentrations of E2 and T.

3.3.3.2 Total Reflection X-ray Fluorescence (TXRF) to measure zinc

There is a lack of commercial kits available to measure avian vitellogenin (VTG) concentration in plasma. Since VTG is known to be a zinc (Zn) protein, few studies in the past have developed and validated methods to measure plasma Zn levels as a proxy to measure VTG levels (21,22). Total reflection X-ray fluorescence (TXRF) analysis was performed to measure Zn levels in male and female plasma using the benchtop spectrometer S2 Picofox™ (Bruker AXS Microanalysis GmBH, Berlin, Germany) following the standard recommended protocol for serum/plasma analyses by the manufacturer (See Appendix 2 for further details on TXRF protocol). Gallium (Ga) was included in all samples as the internal standard; standard reference materials (SRMs) Q1316, Q1310 and Q1603 obtained from Institut National de Sante Publique (INSPQ) were used for this analysis. A standard curve was generated with linear fit using serially diluted concentrations of SRMs, and Zn concentrations were calculated by interpolation. Mean SRM recovery for all batches was 107.3% ± 7.47 standard deviation and RSD was 6.96%. Mean inter-assay RSD was 1.24% and 4.03% for males and females, respectively. Mean intra-assay RSD was 3.66%.

3.3.4 RNA Isolation and cDNA Preparation

RNA was isolated from ~20 mg livers and ovaries (and testes for males) using the RNeasy® mini plus kit following manufacturer's instructions (Qiagen, Valencia, CA, USA). On-column

DNase digestion was included to eliminate genomic DNA contamination using the RNase free DNase digestion kit (Qiagen). RNA from all livers was isolated manually using the Qiagen RNeasy spin-columns, while from ovaries was isolated using the automated process in a QIAcube (Qiagen). Total RNA concentration at 260 nm and A260/A280 was determined using QIAxpert (Qiagen). For livers mean 260/280 was 2.07 ± 0.03 (Mean ± standard deviation) and for ovaries mean A260/A280 was 2.08 ± 0.03. A subset of RNA samples were run on a gel following manufacturer's instructions using the Northern GlyMax loading gel to assess RNA quality by examining the 28s and 18s bands. The gel was run at 100V for 5 min followed by 55V for 50 min and viewed under a UV transilluminator to examine the resulting bands. Total RNA (400 ng) was used to prepare cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies) following the manufacturer's instructions in a 20 µl reverse transcription reaction. No-reverse transcription (NRT) control reactions were included for a subset of the samples.

3.3.5 Quantitative Polymerase Chain Reaction (qPCR)

For liver and ovary samples, primer pairs for genes of interest (GOI), including reference genes, were obtained from previous research conducted by our group and from literature (20,23). Each primer pair was validated using pooled cDNA from 20 individual samples. Primer validation steps included primer specificity analysis using BLAST, melt curve analysis, thermal gradient, primer titration, and standard curve to determine reaction efficiency and optimal cDNA concentrations. Primer pairs were deemed acceptable if efficiency was within 90-110%. All primer sequences, melting temperatures and reaction efficiencies are provided in Table 3.1.

For all qPCR reactions, 4 µl cDNA at appropriate dilution + 6 µl Master Mix containing ssoAdvanced SYBR green Supermix (Bio-Rad) was pipetted into each well of a 384-well qPCR plate for a total reaction volume was 10 µl. Each sample was assayed in duplicate. Male liver and testes qPCR plates were prepared by manual pipetting; Female liver and ovaries qPCR were prepared using QIAgility® (Qiagen), an automated pipetting instrument. Since the number of female liver and ovary samples were more than could fit on one 384-well plate, inter-plate calibrators (IPCs) were included in each plate. Three IPCs were created for each dilution used, for both the liver and ovary samples, by pooling 15 different individuals together. After pipetting, plates were sealed with optical tape and centrifuged briefly to spin down contents in each well. Following this qPCR runs were performed on the CFX384 (Bio-Rad) with the following protocol: 95 °C heat activation for 3 min, followed by 95 °C for 10 sec and incubated at the melting temperature corresponding to the gene for 30 sec for 40 cycles. The melt curve was run from 65 °C to 95 °C with a 0.5 °C increment. Each qPCR run included no-reverse transcription controls (NRT) and no template controls (NTC) for each gene, representing negative controls. Data were analyzed using the CFX Maestro 1.1 (Bio-Rad). In each case, data were normalized to one or both of the corresponding reference genes. Relative normalized expression was calculated for each gene for a treatment relative to the control group of the corresponding exposure day based on the ΔΔCq method.

3.3.6 Data Analyses

Statistical analyses were performed using JMP Pro V13 and XLSTAT 2017 (24,25). Summary statistics were calculated as means ± standard error. All morphometric measurements, plasma hormone concentrations and gene expression (gene expression data were log2 transformed and

results were tested for statistical significance in cases where the log2 fold change relative to control was greater than 1 or -1) data were checked for normality using Shapiro-Wilk's test, and Grubb's test was used to check for outliers. Data were log transformed in the cases where the assumptions were violated and re-tested to confirm the effectiveness of the transformation. If data passed the normality test (female – HSI, T and E2), we performed one-way Analysis of Variance to check for statistical significance among treatment groups. If significant differences were found, means were subjected to a Tukey's HSD post hoc test to determine statistical significance among treatment pairs. In cases where data failed the normality test (female – egg production, remaining morphometric measurements, gene expression, E2/T and Zn; male – all data), we performed Kruskal-Wallis Wilcoxon test to check for statistical significance among treatment groups. If significant differences were found, we performed Steel-Dwass post hoc test for comparisons between all pairs to check for statistical significance. Results were considered significant at $\alpha < 0.05$.

3.4 Results

3.4.1 Morphometrics and Egg Production

No mortality was observed in any of the treatment groups in males or females. There was no change in body weight, tarsal length, weights of gonads, thyroid and spleen in males or females, and number of maturing follicles (in females). We observed a significant decrease in liver weight, shown as hepatosomatic index (HSI, liver weight as a function of body weight; $P = 0.03$ Figure 3.3A) and total follicle weight ($P = 0.007$; Figure 3.3B) in the 20 ppm treated female birds compared to the control birds, at exposure day 21. Egg production was decreased in the 20

ppm group compared to the 5 ppm and control groups, however this decrease was not statistically significant (Figure 3.4).

3.4.2 Male Plasma Hormone and Gene Expression Analyses

Summary statistics for all plasma analytes including T, E2, and Zn measured in male JQ are provided in Supplementary Table 3.2. No significant effect of dietary 17βT exposure was observed on male plasma T concentration at either exposure day. There was a significant increase in plasma E2 concentration in male birds treated with 5 and 20 ppm 17βT at both exposure days (D8 P=0.001; D21 P=0.002) as shown in Figure 3.5. No significant changes in gene expression were found in male JQ testes and liver (Supplementary Table 3.3 A and B).

3.4.3 Female Plasma Hormone and Gene Expression Analyses

Summary statistics for all plasma analytes measured in females including LH, T, E2, and Zn are provided in Supplementary Table 3.4. Since we were unable to capture the true daily E2 peak we considered all samples for each treatment group within a sampling day as a single measure. No consistent pattern was observed in plasma LH concentrations in treated female JQ compared to controls in either of the four sampling days. Female JQ exposed to 17βT had significantly lower levels of plasma T compared to the control birds (D2 P = 0.0163; D7 P = 0.0056; D14 P = 0.0283; D21 P = 0.0186). At D2 the plasma T of the 5 ppm group was significantly lower than the control; at D7 plasma T of both the 5 and 20 ppm groups were significantly lower than the control; at D14 plasma T of the 20 ppm group was significantly lower than the control; and at D21 plasma T of neither treatment group was significantly lower than the control (Figure 3.6A). Dietary 17βT significantly increased plasma E2 concentration in female (D2 P < 0.0001; D7 P =

0.0448; D14 P = 0.0008; D21 P = 0.0004) birds across all sampling days (Figure 3.6B). At D2 plasma E2 of the 20 ppm group was significantly higher than both the control and 5 ppm groups; at D7 there were no significant differences in plasma E2 among treatment groups; at D14 plasma E2 of the 20 ppm group was significantly higher than both the control and 5 ppm groups; and at D21 plasma T of both 5 and 20 ppm groups were significantly higher than the control (Figure 3.6B). The ratio of E2 to T was higher in the 17βT treated groups compared to the controls (D2 P < 0.0001; D7 P < 0.0001; D14 P < 0.0001; D21 P = 0.0001). At D2, E2/T in the 20 ppm group was higher than the control; at D7 and D14 E2/T in both the 5 and 20 ppm groups were higher than control, and at D21 E2/T in the 20 ppm group was higher than the control (Figure 3.6C). A general decrease in plasma Zn concentrations was observed in treated birds compared to the control at all exposure days, but the decrease was statistically significant at D21 (P = 0.0007; Figure 3.6D). No significant differences in gene expression were observed in the liver and gonads of female birds exposed to either dose of 17βT across any of the exposure days (Supplementary Table 3.3 C and D).

3.5 Discussion

3.5.1 Circulating Hormones and Gene Expression in Males

Similar to the present study, previous studies on 17βT effects in male JQ observed no changes in plasma T concentration (19,20). While these studies looked at JQ exposed to 17βT for longer than 8 weeks, our study examined effects at days 8 and 21 of exposure. Collectively, these studies indicate that 17βT exposure does not directly affect T production in male JQ at early or later stages of exposure. Indeed, this is similar to other studies that examined effects of 17βT in male fathead minnow (FHM; *Pimephales promelas*) (15,16). However, we did observe an

increase in E2 concentrations in treated JQ compared to the controls, which is similar to results in the long-term 17βT exposure in male JQ and FHM (15,20). The authors postulated that this increase in E2 concentrations might be due to the organism attempting to maintain optimal E2/T which is critical for normal reproductive functions (26,27). Thus, it is thought that higher E2 levels are needed to compensate for the perceived higher androgen levels (endogenous testosterone plus exogenous 17βT) in the body. Examining E2/T ratio revealed no changes between control and treated males indicating that perhaps treated males are able to maintain the ratio similar to controls. Despite the increase in E2, we did not observe any changes in plasma Zn concentration which was used as a proxy to measure plasma VTG. Similar to this study, neither the time-course 8-day nor the 21-day exposure in FHM observed significant differences in plasma VTG levels (15,16).

Concomitant with the lack of changes in plasma T and Zn, we did not observe any change in mRNA expression of genes related to the T biosynthesis pathway (CYP11A, 17βHSD, CYP17A1), or VTG. Despite the 17βT exposure, we did not observe any change in AR gene expression. It is possible that AR mRNA level alone might not be an adequate endpoint, rather receptor binding levels could provide more information on AR response. We also did not observe any changes in CYP19A1 aromatase expression. This lack of change in CYP19A1 mRNA in males is similar to what was observed in the JQ and FHM studies discussed above. Thus, the cause for the increased plasma E2 in male JQ is still unclear. It has been suggested that the brain is a primary site for estrogen synthesis, for example, Schlinger et al. (28,29) reported that there was no CYP19A1 activity detected in male testes in zebra finch (20). Thus, it is possible that studying mRNA expression and enzyme activity of CYP19A1 in the brain might provide a better explanation for the observed increase in plasma E2 concentrations.

3.5.2 Circulating Hormones and Gene Expression in Females

Similar to previous studies, the effects of 17βT exposure in females were different from males. We saw a decrease in circulating T levels in 17βT treated females compared to controls as has been documented in previous research with JQ and FHM (15,20). The authors suggested that the perceived higher total androgen levels might be responsible for triggering the negative feedback loop in the HPGL axis, which could lead to a decrease in T levels. This negative feedback could occur at the level of the hypothalamus and pituitary by affecting the production of gonadotropins (2,30). Accordingly, we measured circulating levels of luteinizing hormone (LH), however, we did not observe any consistent or statistically significant differences in plasma LH concentration. Regardless, we saw a temporal change in plasma T levels: a decrease in the low dose at D2, a decrease in both doses at D7, followed by a decrease in only the high dose at D14. By D21, the level of T in the low dose was back to control levels, and while there was a decrease in the high dose, it was not statistically different. This temporal trend is similar to what was observed by Ekman et al. (16) in their time-course study. They discussed that the initial decrease in T could serve to compensate for the higher androgen levels perceived by the organism which was followed by recovery to control levels further into the exposure period. However, while they observed a decrease in T in both treatment groups after just 24 h exposure, we observed a decrease at both doses at D7. This could be indicative of a lag in the response to high androgen levels. Despite changes in plasma T levels, we did not observe any changes in gene expression in the ovaries. There is a lack of information on the effect of androgenic chemicals on the enzymes involved in the T biosynthetic pathway in avian species. However, previous studies in FHM and

rare minnow (*Gobiocypris rarus*) found a suppression of CYP11A and CYP17A1 in response to androgenic chemicals such as 17α-methyltestosterone and 17βT (16,31).

In addition to the changes observed in T levels, we observed an increase in plasma E2 concentration following 17βT exposure. This was somewhat contradictory to the time-course study in FHM where they saw a decrease in E2 after just 24 h exposure that lasted for 48 h followed by a return to control levels and a subsequent decrease in the high dose (16). The authors suggested that the decrease immediately following exposure might be due to the decrease in plasma T levels resulting in less T available to be aromatized to E2 (20). However, we noticed a lag in the decrease in T concentration following exposure. At D2, consistent with a lack of changes in T level at 20 ppm, we noticed an increase in E2, indicating that E2 production may have been increased to maintain optimal E2/T levels. This increase in E2 levels could also be a contributing factor in the subsequent decrease in T production at D7, since E2, as one of its functions, is involved in a negative feedback loop with the hypothalamus and pituitary which could result in decreased gonadotropin release and subsequent decrease in T production (2,32). Paralleling the decrease in T levels on D7, we saw a decrease in E2 in the 20 ppm group compared to D2 with a return to control levels. At D14, following the decrease in T in the 20 ppm one might have expected a concomitant decrease in E2 levels; however, we noticed a sustained increase in E2 levels at D21. This may be indicative of an overshoot response or an overcompensation resulting in a sustained increase in E2 production. This type of an overcompensation has been observed in female FHM as discussed by Ankley and Villeneuve (3). However, Karouna-Renier et al. (20) showed that female quails from long-term exposure at comparable 17βT treatments did not show any significant increase in E2. This could imply that over prolonged exposure, regulatory mechanisms in the female JQ, are able to return E2

production back to control levels. Despite the observed increases in E2, there was no change in CYP19A1 mRNA levels in the ovaries; however, previous research in FHM have found modest increases in CYP19A1 expression corresponding to increases in plasma E2 (16). Similar to the males, measuring CYP19A1 mRNA and activity in the brain in addition to the gonads might provide a better explanation (28).

The next plasma measurement along the HPGL axis was VTG (Zn) concentration, which is produced by E2 binding to ER (33,34). Since we observed an increase in E2 following exposure, one might have expected to observe an increase in VTG as well, rather, we saw a significant decrease on exposure day 21. Analyses of VTG, Apo, and ER (α and β) mRNA expression in the liver resulted in no significant changes, providing no explanation for this discord between plasma E2 levels and Zn-VTG levels. Perhaps ER expression alone is not an adequate endpoint to study VTG synthesis, rather estimating estrogen receptor levels could provide additional explanation. Following this, we observed a decrease, albeit not significant, in egg production in the 20 ppm group. Because of its role as a precursor to egg yolk proteins, the decrease in VTG may be implied in the decrease in egg production. While the increase in E2 levels did not parallel VTG levels or egg production, a study in European starlings (*Sturnus vulgaris*) found that exogenous E2 caused a decrease in egg yolk protein and lipid mass (35). The authors suggested that this could be due to higher level negative feedback mechanisms whereby estradiol may have lowered follicle stimulating hormone secretion at the level of the pituitary thus reducing endocrine stimulation of oocyte growth (35). Karouna-Renier et al. (20) observed a significant decrease in egg production from long-term exposure to 17βT indicating that while the short-term exposure to 17βT elicited effects at the biochemical level, this exposure may not have been sufficient to cause a significant impairment in egg production.

Overall, despite 17βT eliciting a change in plasma hormone levels, there were no changes in the mRNA levels of the key nuclear receptors - AR and ER. Studies on endocrine disruption have suggested that while these receptors are one of the mechanisms, there are other receptors and steroid hormone metabolic pathways to be considered as well (36,37). Androgens can bind to steroid hormone binding globulin (SHBG) and activate the SHBG receptor thus leading to a change in various enzymatic pathways (38). Cell membrane binding sites for testosterone different from the classic nuclear receptors have been identified in various cell types further identifying non-genomic mechanisms by which androgens can affect signal transduction (39,40). There is additional evidence indicating that steroid mimicking EDCs can modulate cytoplasmic and cell membrane-bound regulatory proteins, ion channels, and G protein-coupled receptors, and trigger intracellular signaling cascades (36,39,40). Steroid and xenobiotic receptor (SXR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have been implicated in steroid hormone metabolism. Activation of these molecules by EDCs could alter the local bioavailability of endogenous androgens and estrogens and represent other avenues by which EDCs could affect the activity without directly binding to the steroid receptors (41). Studies have shown that EDCs can impact cytochrome P450 enzymes and metabolic processes such as sulfation and glucuronidation which are involved in estrogen metabolism and are important routes for the clearance of active metabolites (42–45). Skilton et al. (46) examined hepatic sulfotransferase activity in two benthic fish species in response to EDCs and observed that different compounds sulfated estrogens to varying degrees. An impact on these enzymes and processes could thus alter the levels of active hormones available to bind to receptors. Collectively, these show that while steroid mimicking EDCs have been known to affect

biological processes through AR and ERs there are various other pathways involving metabolic enzymes and receptors that could be responsible for disruptive effects.

3.6 Limitations

While in this study we were able to characterize the effects of acute 17βT exposure on numerous endpoints in the JQ endocrine system, the mechanistic reasons for the observed responses still remain unclear. The dynamic nature of gene expression may render it insufficient to explain all the responses observed and thus including endpoints such as receptor binding levels and enzyme activity, and including additional target tissues such as the brain might provide a better explanation. However, there has been great interest in the use of transcriptomic changes to screen chemicals for endocrine disrupting potential. Thus, rather than measuring effects on expression of a few key genes, studying global transcriptome-wide changes using RNA Sequencing and covering a wider biological space could further explain changes at the molecular level.

3.7 Conclusion

Through this study, we investigated changes along the JQ endocrine system at four time-points during the first three weeks of exposure to dietary 17βT, including biochemical (plasma hormone levels), molecular (gene expression) endpoints and egg production. We observed distinct differences in both males and females, but also in the current study and the previous long-term study in JQ. For example, the significant increase in female plasma E2 seen here over the first 21 days, was not observed in the long-term study at comparable concentrations (20), indicating that the increase in E2 in the early stages could be a direct response to the androgenic 17βT.

Additionally, we saw a non-significant decrease in egg production in the 20 ppm dose, however, they observed significant decreases in egg production in the 10 ppm and higher treatments. This might imply that while the 17βT treatment elicited changes in plasma hormone levels, this short-term exposure may not yet extend to an impairment of egg production. Gene expression analysis revealed no changes in the liver and gonads demonstrating a lack of agreement with plasma hormone levels. The interactions of steroids and steroid mimics have been shown to extend beyond the androgen and estrogen receptors to numerous signaling pathways, non-nuclear receptors and steroid metabolizing enzymes. Taken together these results further demonstrate the continuous and complex nature of the response to EDCs and imply that more research is warranted on the mechanisms by which 17βT impairs the avian endocrine system. Data generated from this study are also being used to develop the first avian computational vitellogenesis model which will link molecular and biochemical level changes within the HPGL axis to adverse effects on egg production.

3.8 Acknowledgements

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3.9 Tables and Figures

Table 3.2: List of genes measured in A) gonads, B) liver.

A)

Genes	Forward/ Reverse	Sequence (5'-3')	Efficiency	T_m (°C)
PPIA (ref)	F	TCGCCGATGAGAACTTCATCCTGA	109.2%	60
	R	AAGAACTGGGAGCCGTTTGTGTTG		
HPRT1 (ref)	F	ACGGGCTCATCATGGACAGGACA	107.4%	60
	R	GCCGCCCTTGAGCACACAGA		
17β-HSD	F	TCTTGGTGTGGGAATGTGAA	107.6%	60
	R	CCGGAATAGAAGGAACACCA		
CYP11A	F	AGGTGAGCGAGGACTTTGTG	101.4%	60
	R	TTGCAGAGTCATGGAAGTCG		
CYP17A1	F	CTGTGAGGGACCTGATGGAT	99%	60
	R	CCACTCCTTCTCATCGTGGT		
ERα	F	GTTCCTTGCTCACTGCCATTAG	97.7%	60
	R	GAATCTTCAGCTGAGGTCTGC		
AR	F	GGATCTGCATCAGTTTACATTTG	100.6%	60
	R	GGACCTGCACAGAGATGATC		
CYP19A1	F	ACCAGCTGCCACAGTGCCT	109.3%	64
	R	CCCATACAGTATCCTGGCCCTGGT		

B)

Genes	Forward/ Reverse	Sequence (5'-3')	Efficiency	T_m (°C)
B2M (ref)	F	CAAGATCTCCATCACGCTGCAGAAG	98.1%	60
	R	AGTCGTCGCTGAAGGACATGTCTGA		
EEF1A1 (ref)	F	GAAGCCAGGCATGGTTGTCACATT	105.6%	60
	R	TAAGGGCCTCATGGTGCATCTCAA		
VTG	F	GAAAACCCTGAGCAACGGATAG	105.6%	60
	R	TGGAACATCATCATGGAAATCTTG		
Apo	F	AACCCAGCATCAGAGACCAAAGGA	100.6%	60
	R	AGGGTCAATGGCCAAGTCATTCAG		
ERα	F	GTTCCTTGCTCACTGCCATTAG	108%	60
	R	GAATCTTCAGCTGAGGTCTGC		
ERβ	F	CTCCATGATGATGTCCCTGAC	108.3%	60
	R	CTGAGATCAATGAAGCCAGGA		
AR	F	GGATCTGCATCAGTTTACATTTG	99%	60
	R	GGACCTGCACAGAGATGATC		

Supplementary Table 3.1: Results of residue analyses in livers from A) male and B) female Japanese quail (JQ), exposed to 17β-trenbolone (17βT) (0, 5, 20 mg/kg or ppm) are presented in mg/kg. Residue in all female muscle samples were below 0.002 mg/kg.

A)

Exposure Day	8			21		
Analyte	α	β	total	α	β	total
17βT dose						
0 mg/kg	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002
5 mg/kg	<0.002	<0.002	<0.002	<0.002	0.002	0.002
20 mg/kg	<0.002	<0.002	0.002	<0.002	0.002	0.002

B)

Exposure Day	2			7			14			21		
Analyte	α	β	total	α	β	total	α	β	total	α	β	total
17βT dose												
0 mg/kg	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002
5 mg/kg	<0.002	<0.002	<0.002	<0.002	<0.002	0.002	<0.002	0.003	0.003	<0.002	0.003	0.004
20 mg/kg	<0.002	0.003	0.004	<0.002	0.003	0.004	<0.002	0.003	0.004	<0.002	0.004	0.006

Supplementary Table 3.2: Summary statistics for plasma analytes in male Japanese quail (JQ) exposed to 17β-trenbolone (0, 5 and 20 mg/kg). T = Testosterone; E2 = 17β-estradiol; Zn = zinc; VTG = Vitellogenin; * = significantly different from controls ($p < 0.05$).

	ExposureDay	Dose (mg/kg)	Mean ± SE	Range	N
T(ng/ml)	8	0	4.81 ± 1.74	1.07 - 12.28	6
		5	1.74 ± 0.74	0.04 - 4.07	6
		20	3.94 ± 1.46	0.16 - 7.79	5
	21	0	4.85 ± 0.89	2.36 - 7.57	5
		5	6.20 ± 1.92	0.28 - 14.3	6
		20	3.35 ± 1.58	0.26 - 8.81	6
E2 (ng/ml)	8	0	0.006 ± 0.0002	0.006 - 0.007	6
		5	0.008 ± 0.0003 *	0.007 - 0.009	6
		20	0.014 ± 0.0019 *	0.009 - 0.020	6
	21	0	0.006 ± 0.0004	0.005 - 0.008	6
		5	0.011 ± 0.0015 *	0.007 - 0.016	6
		20	0.017 ± 0.0021 *	0.012 - 0.022	6
E2/T	8	0	0.002 ± 0.0008	0.001 - 0.005	6
		5	0.037 ± 0.0284	0.020 - 0.178	6
		20	0.024 ± 0.0179	0.012 - 0.094	5
	21	0	0.001 ± 0.0003	0.010 - 0.024	5
		5	0.008 ± 0.0061	0.009 - 0.038	6
		20	0.017 ± 0.0068	0.013 - 0.048	6
Zn (VTG) (µg/ml)	8	0	2.00 ± 0.16	0.99 - 3.55	6
		5	1.92 ± 0.25	1.54 - 2.62	6
		20	1.70 ± 0.1	1.41 - 2.61	6
	21	0	2.18 ± 0.38	1.44 - 2.57	6
		5	2.12 ± 0.19	1.32 - 3.07	6
		20	1.86 ± 0.13	1.20 - 1.90	6

Supplementary Table 3.4: Summary of gene expression results in Japanese quail exposed to 17β-trenbolone treated Japanese quail (0, 5 and 20 mg/kg). A) Testes, B) Male liver, C) Ovary, D) Female liver. ERα = estrogen receptor α; ERβ = estrogen receptor β; AR = androgen receptor; CYP11A = cholesterol side chain cleavage enzyme; CYP17A1 = steroid 17α-monooxygenase; 17βHSD = 17β hydroxysteroid dehydrogenase; CYP19A1 = aromatase; VTG = vitellogenin; Apo = apolipoprotein.

A)

Gene	Exposure Day	Dose (mg/kg)	Mean log2(FC)	SEM	N
ERα	8	0	0.000	0.317	6
		5	-0.291	0.283	6
		20	-0.592	0.825	5
	21	0	0.000	0.170	6
		5	0.063	0.140	6
		20	0.454	0.186	6
AR	8	0	0.000	0.113	6
		5	0.027	0.094	6
		20	0.028	0.181	5
	21	0	0.000	0.158	6
		5	-0.123	0.088	6
		20	-0.254	0.126	6
CYP11A	8	0	0.000	0.114	6
		5	-1.712	1.045	6
		20	0.859	0.371	5
	21	0	0.000	0.216	6
		5	-0.787	0.896	6
		20	0.097	0.271	6
CYP17A1	8	0	0.000	0.119	6
		5	-0.303	0.320	6
		20	0.922	0.143	5
	21	0	0.000	0.294	6
		5	-1.294	1.623	6
		20	0.408	0.283	6
17βHSD	8	0	0.000	0.098	6
		5	0.084	0.120	6
		20	-0.049	0.182	5
	21	0	0.000	0.120	6
		5	-0.048	0.101	6
		20	-0.093	0.052	6
CYP19A1	8	0	0.000	0.359	6
		5	-0.684	0.449	6
		20	0.652	0.335	5
	21	0	0.000	0.321	6
		5	0.386	0.121	6
		20	-0.181	0.172	6

B)

Gene	Exposure Day	Dose (mg/kg)	Mean log2(FC)	SEM	N
ERα	8	0	0.000	0.290	6
		5	-0.329	0.078	6
		20	-0.102	0.240	5
	21	0	0.000	0.243	6
		5	-0.057	0.265	6
		20	0.207	0.181	6
AR	8	0	0.000	0.168	6
		5	-0.079	0.213	6
		20	-0.066	0.185	5
	21	0	0.000	0.236	6
		5	-0.402	0.195	6
		20	-0.566	0.112	6
VTG	8	0	0.000	0.350	6
		5	-0.435	0.265	6
		20	0.965	0.641	5
	21	0	0.000	0.498	6
		5	0.081	0.283	6
		20	-0.089	0.454	6
Apo	8	0	0.000	0.574	6
		5	0.575	0.239	6
		20	0.100	0.450	5
	21	0	0.000	0.504	6
		5	0.467	0.325	6
		20	-0.247	0.661	6

C)

Gene	Exposure Day	Dose (mg/kg)	Mean log2(FC)	SEM	N	Gene	Exposure Day	Dose (mg/kg)	Mean log2(FC)	SEM	N
ERα	2	0	0.000	0.094	15	CYP17A1	2	0	0.000	1.099	15
		5	0.066	0.103	15			5	0.268	0.937	15
		20	0.131	0.115	15			20	0.189	0.953	15
	7	0	0.000	0.148	15		7	0	0.000	1.039	15
		5	0.405	0.130	15			5	0.928	0.703	15
		20	0.345	0.150	14			20	-1.091	1.054	14
	14	0	0.000	0.129	17		14	0	0.000	1.092	17
		5	0.114	0.170	15			5	1.412	1.073	15
		20	0.245	0.117	18			20	0.801	0.872	18
	21	0	0.000	0.224	15		21	0	0.000	0.661	15
		5	0.099	0.108	15			5	-0.292	0.573	15
		20	0.330	0.159	16			20	1.216	0.875	15
AR	2	0	0.000	0.105	15	17βHSD	2	0	0.000	0.169	15
		5	-0.109	0.135	15			5	-0.467	0.245	15
		20	-0.105	0.076	15			20	-0.708	0.205	15
	7	0	0.000	0.131	15		7	0	0.000	0.133	15
		5	0.289	0.125	15			5	0.151	0.109	15
		20	0.056	0.086	14			20	-0.062	0.103	14
	14	0	0.000	0.156	17		14	0	0.000	0.141	17
		5	-0.038	0.147	15			5	0.327	0.155	15
		20	-0.003	0.130	18			20	0.450	0.240	18
	21	0	0.000	0.170	15		21	0	0.000	0.185	15
		5	-0.152	0.088	15			5	-0.224	0.108	15
		20	0.320	0.168	16			20	-0.025	0.171	16
CYP11A	2	0	0.000	0.757	15	CYP19A1	2	0	0.000	1.015	15
		5	-0.273	0.854	15			5	0.601	0.987	15
		20	0.295	0.421	15			20	1.664	0.819	15
	7	0	0.000	0.593	15		7	0	0.000	0.236	11
		5	0.529	0.436	15			5	0.105	0.213	14
		20	-1.615	0.875	14			20	0.577	0.152	11
	14	0	0.000	0.689	17		14	0	0.000	0.340	13
		5	0.063	0.724	15			5	-0.207	0.365	12
		20	-0.490	0.933	18			20	-0.466	0.339	15
	21	0	0.000	0.676	15		21	0	0.000	1.198	11
		5	-1.506	0.681	15			5	0.920	0.940	11
		20	0.734	0.910	16			20	2.617	1.193	11

D)

Gene	Exposure Day	Dose (mg/kg)	Mean log_2(FC)	SEM	N	Gene	Exposure Day	Dose (mg/kg)	Mean log_2(FC)	SEM	N
ERα	2	0	0.000	0.224	14	VTG	2	0	0.000	1.597	14
		5	0.148	0.197	15			5	-0.867	1.553	14
		20	0.080	0.189	14			20	2.292	0.955	15
	7	0	0.000	0.149	15		7	0	0.000	1.207	15
		5	-0.388	0.162	14			5	-2.638	1.356	14
		20	-0.687	0.168	15			20	-4.411	1.729	14
	14	0	0.000	0.195	17		14	0	0.000	0.490	17
		5	-0.451	0.187	14			5	-1.747	1.342	14
		20	-0.261	0.209	18			20	-0.492	0.762	18
	21	0	0.000	0.189	15		21	0	0.000	0.595	15
		5	-0.008	0.103	14			5	0.786	0.093	12
		20	-0.018	0.109	14			20	-0.222	0.496	14
ERβ	2	0	0.000	0.218	14	Apo	2	0	0.000	1.642	14
		5	0.514	0.215	15			5	-0.869	1.530	14
		20	0.401	0.160	14			20	2.129	0.913	15
	7	0	0.000	0.195	15		7	0	0.000	1.244	15
		5	0.387	0.184	14			5	-3.603	1.348	15
		20	0.675	0.266	15			20	-4.285	1.575	14
	14	0	0.000	0.241	17		14	0	0.000	0.710	17
		5	-0.191	0.198	14			5	-0.805	1.446	14
		20	-0.223	0.226	18			20	-0.956	0.761	18
	21	0	0.000	0.130	15		21	0	0.000	0.655	15
		5	0.108	0.105	14			5	1.313	0.087	12
		20	-0.698	0.808	14			20	-0.217	0.760	13
AR	2	0	0.000	0.123	14						
		5	-0.166	0.129	15						
		20	-0.225	0.184	14						
	7	0	0.000	0.198	15						
		5	-0.156	0.120	14						
		20	-0.166	0.176	15						
	14	0	0.000	0.207	17						
		5	-0.531	0.174	14						
		20	-0.907	0.259	18						
	21	0	0.000	0.206	15						
		5	0.142	0.123	14						
		20	-1.031	0.725	14						

Supplementary Table 3.4: Summary statistics for plasma analysis of 17β-trenbolone treated female Japanese quail (0, 5 and 20 mg/kg). LH = Luteinizing Hormone; E2 = 17β-estradiol; Zn = Zinc; T = Testosterone; VTG = Vitellogenin; * = significantly different from controls ($p < 0.05$).

	ExposureDay	Dose (mg/kg)	Mean ± SE	Range	N
LH (ng/ml)	2	0	0.16 ± 0.03	0.03 - 0.29	8
		5	0.28 ± 0.05	0.07 - 0.59	11
		20	0.24 ± 0.04	0.11 - 0.46	12
	7	0	0.38 ± 0.07	0.13 - 1.10	14
		5	0.57 ± 0.16	0.12 - 2.64	15
		20	0.46 ± 0.11	0.09 - 1.15	10
	14	0	0.36 ± 0.06	0.06 - 0.82	13
		5	0.44 ± 0.08	0.08 - 0.85	9
		20	0.29 ± 0.05	0.02 - 0.50	13
	21	0	0.25 ± 0.08	0.06 - 0.90	9
		5	0.77 ± 0.15	0.11 - 1.92	11
		20	0.46 ± 0.05	0.15 - 0.66	14
T (ng/ml)	2	0	0.84 ± 0.16	0.11 - 2.09	15
		5	0.39 ± 0.08	0.08 - 0.98	14
		20	0.58 ± 0.08	0.14 - 1.46	15
	7	0	0.61 ± 0.09	0.14 - 1.25	14
		5	0.32 ± 0.07 *	0.06 - 0.89	15
		20	0.29 ± 0.10 *	0.09 - 1.44	13
	14	0	0.91 ± 0.16	0.04 - 2.96	17
		5	0.48 ± 0.08	0.03 - 1.04	15
		20	0.37 ± 0.04 *	0.06 - 0.76	17
	21	0	0.78 ± 0.18	0.05 - 2.35	14
		5	0.72 ± 0.09	0.33 - 1.63	15
		20	0.34 ± 0.03	0.12 - 0.50	16
E2 (ng/ml)	2	0	0.024 ± 0.004	0.010 - 0.060	15
		5	0.026 ± 0.006	0.010 - 0.100	15
		20	0.054 ± 0.006 *	0.020 - 0.110	15
	7	0	0.025 ± 0.004	0.010 - 0.053	15
		5	0.027 ± 0.005	0.010 - 0.071	15
		20	0.038 ± 0.005	0.018 - 0.075	15
	14	0	0.023 ± 0.002	0.010 - 0.034	17
		5	0.030 ± 0.004	0.011 - 0.062	15
		20	0.041 ± 0.004 *	0.022 - 0.092	18
	21	0	0.024 ± 0.003	0.010 - 0.045	15
		5	0.038 ± 0.004 *	0.019 - 0.071	15
		20	0.044 ± 0.004 *	0.017 - 0.081	16
E2/T	2	0	0.035 ± 0.005	0.013 - 0.090	15
		5	0.097 ± 0.018 *	0.028 - 0.229	14
		20	0.115 ± 0.019 *	0.045 - 0.325	15
	7	0	0.053 ± 0.010	0.013 - 0.129	14
		5	0.134 ± 0.026 *	0.033 - 0.387	15
		20	0.161 ± 0.014 *	0.045 - 0.260	13
	14	0	0.046 ± 0.016	0.008 - 0.289	17
		5	0.101 ± 0.026 *	0.025 - 0.412	15
		20	0.147 ± 0.025 *	0.047 - 0.461	17
	21	0	0.086 ± 0.036	0.015 - 0.432	14
		5	0.061 ± 0.007	0.024 - 0.122	15
		20	0.138 ± 0.011 *	0.069 - 0.225	16
Zn (VTG) (µg/ml)	2	0	3.69 ± 0.39	1.98-6.16	15
		5	3.21 ± 0.33	1.61-6.06	15
		20	3.00 ± 0.26	1.57-5.10	15
	7	0	4.07 ± 0.52	1.35-7.19	15
		5	3.27 ± 0.43	1.97-7.74	15
		20	3.17 ± 0.28	1.82-5.60	15
	14	0	4.75 ± 0.39	2.28-7.77	17
		5	4.19 ± 0.40	1.72-6.34	15
		20	3.81 ± 0.41	1.60-8.05	18
	21	0	5.35 ± 0.41	2.33-7.85	15
		5	5.64 ± 0.39	3.81-8.84	15
		20	3.92 ± 0.32 *	1.87-6.56	16

Figure 3.1: Number of publications related to endocrine disruption separated by species from Web of Science (1987-2017). Search terms were: (toxic* AND ("endocrine disruption" OR "endocrine disruptor" OR "endocrine disruptors" OR EDC* OR reproduct* OR androgen* OR estrogen*)). Results within this search were refined for human/mammal studies: (human* OR rat* OR mice OR mammal*); fish studies: (fish* OR minnow* OR medaka* OR trout* OR perch* OR walleye* OR sturgeon*); avian studies: (bird* OR avian* OR chick* OR quail* OR finch* OR duck* OR mallard*). Black circles = all studies related to endocrine disruption; Open diamonds = human/mammalian studies; Grey triangles= fish studies; Black squares = bird studies. Accessed March 13th, 2018.

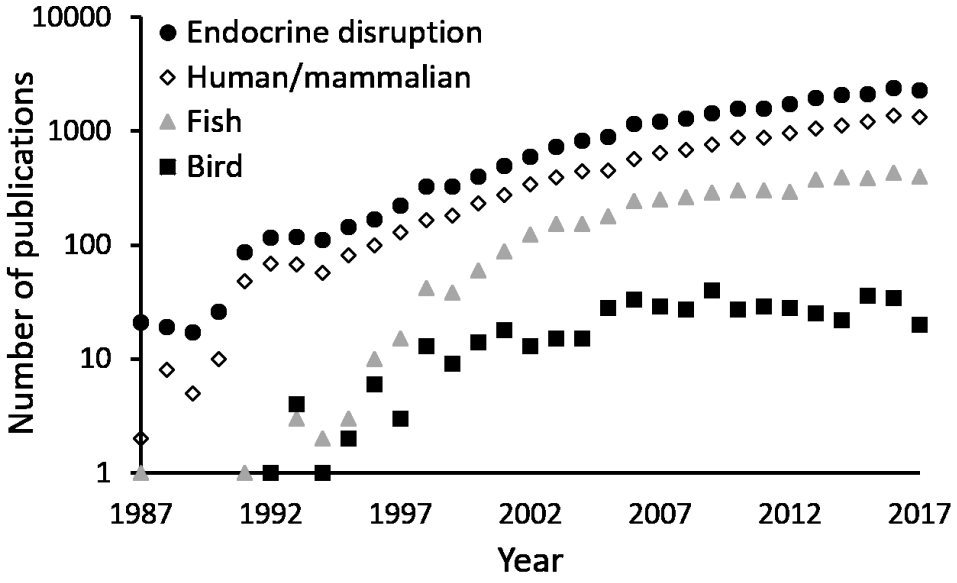


Figure 3.2: Diagrammatic representation of Japanese quail (JQ) 17β-trenbolone (17βT) exposure (0, 5, 20 ppm); n = 15 per treatment per time-point.

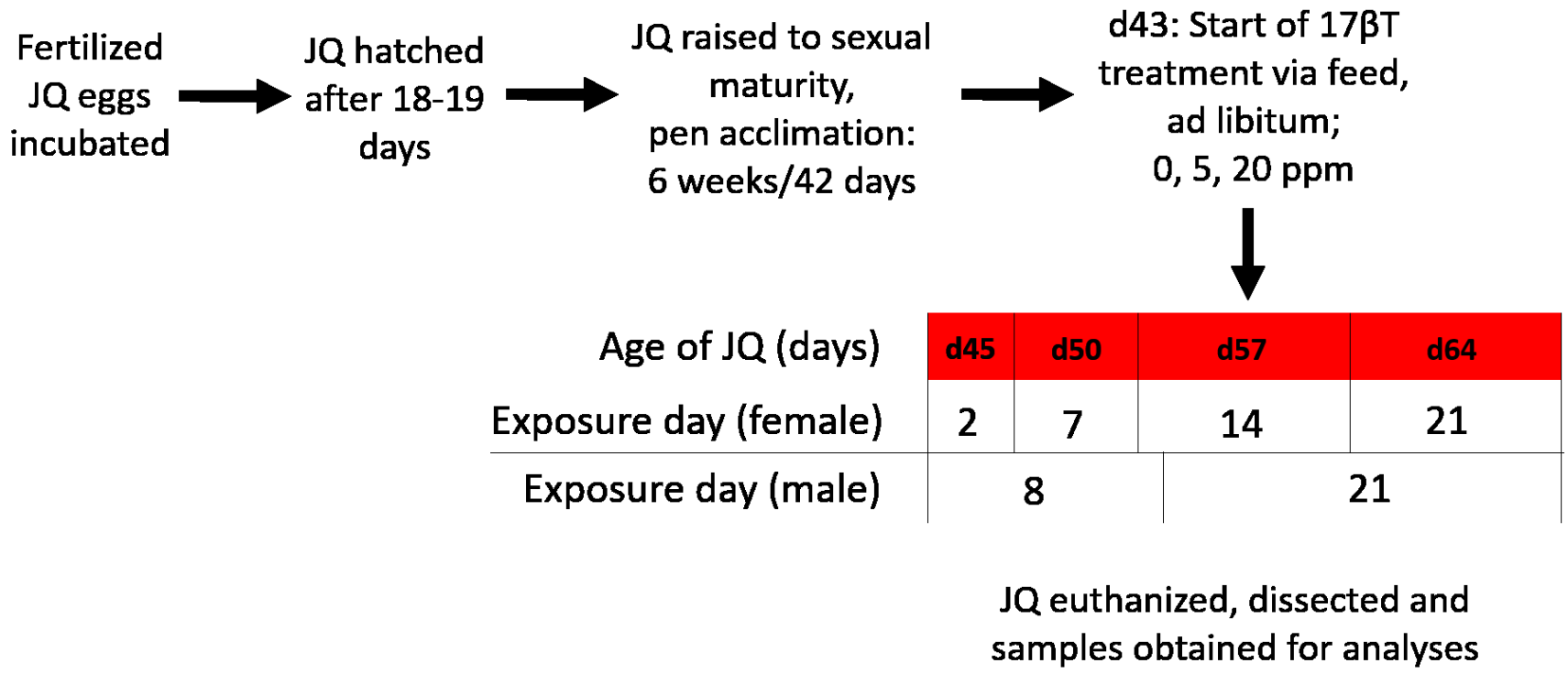
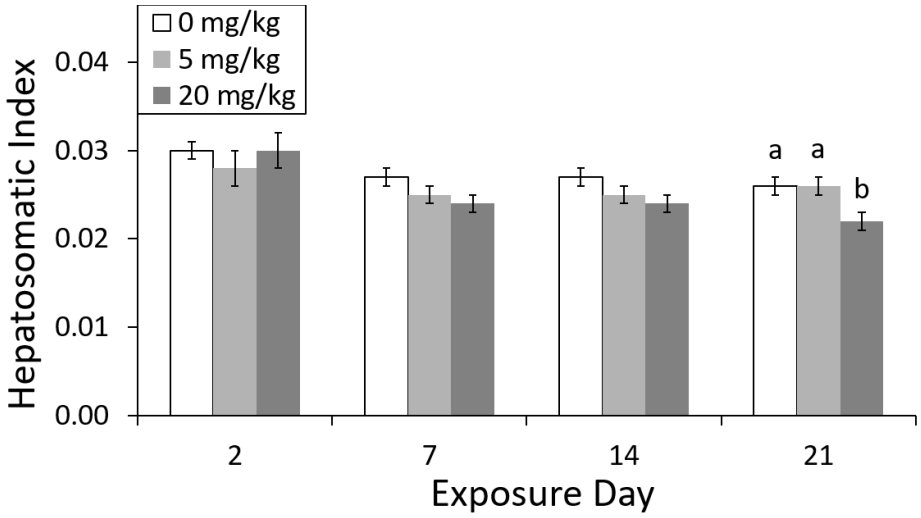


Figure 3.3: A) Liver weight for female Japanese quail (JQ) exposed to 17β-trenbolone (0, 5, 20 mg/kg); B) Total follicle weight for female JQ exposed to 17βT. Data are presented as means ± standard error. Letters represent significant differences in treatment groups from controls within each exposure day at $P < 0.05$

A)



B)

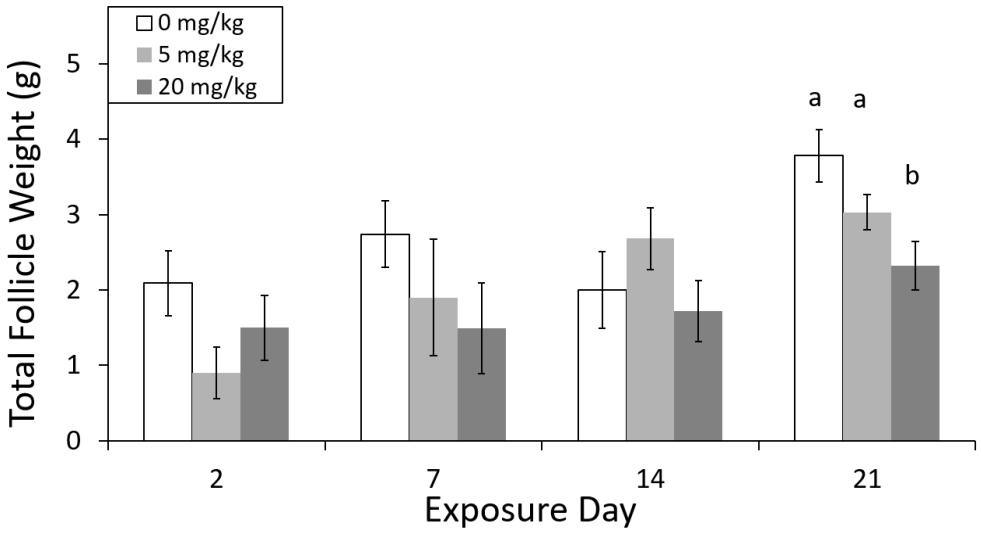


Figure 3.4: Cumulative eggs produced per female Japanese quail per day, exposed to 17β-trenbolone (0, 5, 20 mg/kg) throughout the exposure period.

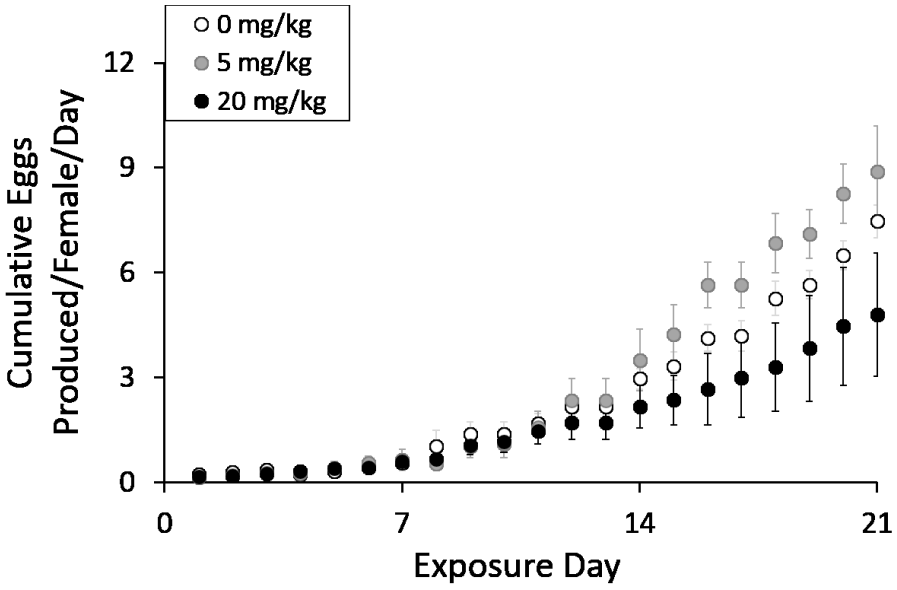


Figure 3.5: Plasma 17β-estradiol (ng/ml) concentrations in male Japanese quail (JQ) exposed to 17β-trenbolone (0, 5, 20 mg/kg). N = 5-6; Data are presented as means ± standard error. Letters represent significant difference from control within each exposure day at $P < 0.05$.

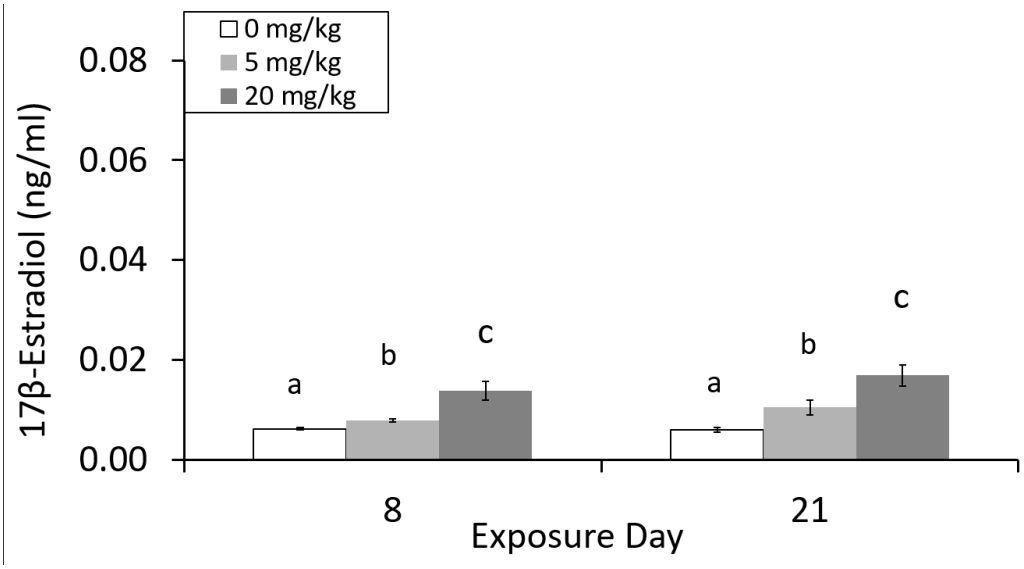
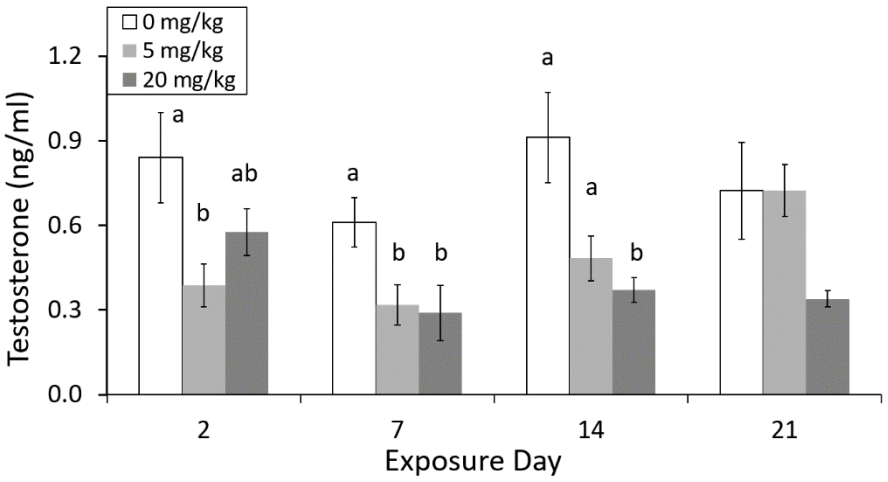
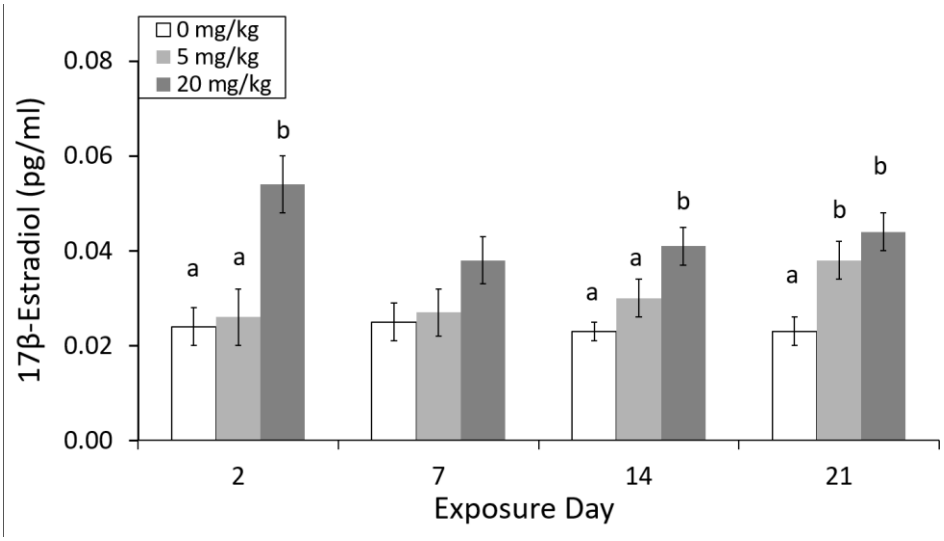


Figure 3.6: Plasma hormone concentrations in female Japanese quail (JQ) exposed to 17β-trenbolone (0, 5, 20 mg/kg). N = 13-18; Data are presented as means ± standard error. Letters represent significant difference from control within each exposure day at $P < 0.05$; A) Testosterone (ng/ml) (T); B) 17β-estradiol (ng/ml) (E2); C) E2/T; D) zinc (µg/ml) (Zn).

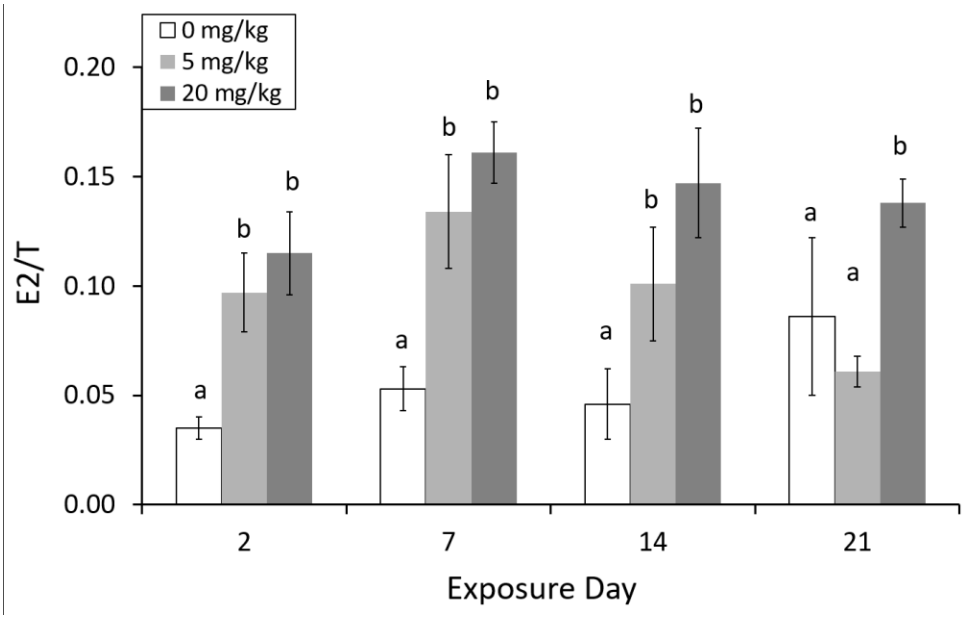
A)



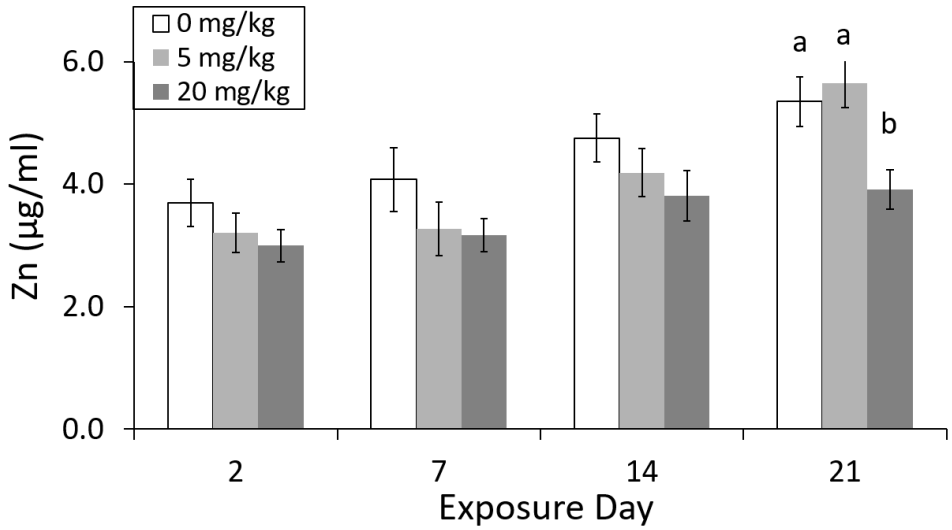
B)



C)


0 mg/kg
5 mg/kg
20 mg/kg
E2/T
0.20
0.15
0.10
0.05
0.00
a
b
b
a
b
b
a
b
b
a
a
b
2
7
14
21
Exposure Day

D)


0 mg/kg
5 mg/kg
20 mg/kg
Zn (µg/ml)
6.0
4.0
2.0
0.0
a
a
b
2
7
14
21
Exposure Day

3.10 Appendix

3.10.1 Appendix 1: Staggered dissections

Doi et al. (47) identified two LH and E2 peaks in circulating plasma levels of JQ. The first peak is thought to initiate vitellogenesis, while the second peak occurs post vitellogenesis and initiates ovulation of the largest mature follicle (48). Due to the importance of VTG in egg production and quality, we sought to characterize hormone concentrations and gene expression prior to the first E2 peak, at the peak, and after the peak. Thus, within each sampling day, dissections were performed in three batches staggered by two hours. Preliminary experiments were carried out to identify the E2 peak to determine the dissection window based on E2 concentrations measured in fecal samples from JQ samples every hour within a 24 hour period. However, the results were rather variable between individuals, and based on these observations we attempted to narrow down the time for the first E2 peak, and sampled birds 2 hours prior to this window, at the peak window, and 2 hours after the peak. However, plasma measurements from the samples did not represent the true peak and hence all subsequent analyses groups the birds from the three windows into one time point.

3.10.2 Appendix 2: Plasma LH, Testosterone, Estradiol and Zn measurements

Luteinizing hormone (LH)

LH concentration in female Japanese quail (JQ) plasma was determined using the chicken LH ELISA kit (Elabscience®) with some modifications of the manufacturer's protocol. Samples with sufficient volume were assayed in duplicate, remaining samples were assayed as single replicates. Since LH concentrations were low, the standard solutions provided were diluted down to 0.02 ng/ml. Equal volumes of standards and unknown samples (100 µl) was added to each well and incubated at 37 °C for 90 mins. Liquid was discarded from the wells and this was followed by incubation steps with detection Ab working solution (100 µl), horseradish peroxidase (HRP) conjugate working solution, and substrate reagents with alternating washing steps in between each incubation. Following this stop solution was added to each well (50 µl) and optical density was read at 450 nm. A standard curve was prepared for each plate and fit using a 4- parameter sigmoid minus curve. Concentration of LH was determined by interpolating from the standard curve.

Testosterone

Plasma testosterone (T) concentrations were measured by ELISA (Salimetrics salivary testosterone Kit, 1-2402-5) with some modifications of the manufacturer's protocol. Each sample was assayed in duplicate. Male samples were diluted 50X and female samples were diluted 10X. Equal volume of unknown samples, controls or standards (25 µl) were added along with 150 µl of enzyme conjugate solution to corresponding wells of an antibody coated microplate, mixed for 5 min at room temperature (RT) and incubated at 21.5˚C for 55 min. The mixture was then discarded, and the plate was washed 4X using the wash solution included in the kit. Substrate

solution (200 ml) was added to the wells, mixed for 5 min at RT, and incubated for in the dark at RT for an additional 25 min. The reaction was stopped by the addition of 50µl stop solution and the optical density of each well was read at 450 nm within 10 min of addition of stop solution. A standard curve was prepared for each plate and fit using a 4- parameter sigmoid minus curve. T concentrations were calculated by interpolation.

Estradiol

Plasma estradiol (E2) concentrations were measured by ELISA (Salimetrics salivary estradiol kit, 1-3702) with some modifications of the manufacturer's protocol. Each sample was run in duplicate. Female samples were diluted 10X. All standards and controls were prepared to contain 10% charcoal stripped chicken plasma (Bioreclamation, LLC, Hicksville, NY, USA) in the final mixture to be consistent in components with the diluted quail plasma samples. Since E2 concentration in adult male plasma may be under detection limit, extraction was performed with diethyl ether (Sigma) to obtain more concentrated samples to assay. Equal volume of unknown samples, controls or standards (100 µl) were added along with 100 µl of enzyme conjugate solution to corresponding wells of an antibody coated microplate, mixed for 5 min at RT and incubated at 21.5˚C for 115 min. The mixture was then discarded, and the plate was washed 4X using the wash solution included in the kit. Substrate solution (200 ml) was added to the wells, mixed for 5 min at RT, and incubated in the dark at RT for an additional 25 min. The reaction was stopped by the addition of 50µl stop solution and the optical density of each well was read at 450 nm (and 630 nm for correction), within 10 min of addition of stop solution. A standard curve was prepared for each plate and fit using a 4- parameter sigmoid minus curve. Estradiol concentrations were calculated by interpolation.

Zinc

Total reflection Z-ray fluorescence (TXRF) is an analytical method used to measure element levels in various matrices including plasma, urine, and blood. Since VTG is known to be a Zn protein, a few studies in the past have developed and validated methods to measure plasma Zn levels as an indicator of VTG levels (21,22). Thus, Zn was measured in male and female plasma using the benchtop spectrometer S2 Picofox™ (Bruker AXS Microanalysis GmBH, Berlin, Germany). Male and female plasma were diluted 5X and 10X, respectively, with Ga standard solution (used as the internal standard). Serum standard reference material (SRM) Q1316, Q1310 AND Q1603 obtained from Institut National de Sante Publique (INSPQ) were used for this analysis. Quartz discs were cleaned prior to analysis through various steps including rinsing with ethanol, wiping with acetone, rinse with cleaning solution 5% RBS™ 50 (Sigma) followed by Milli-Q, heating with 10% HNO_3, and rinsing again with Milli-Q. Washed discs were allowed to dry after which 10 µl silicon solution – Serva (Helixx Technologie) was pipetted onto the center of the discs and allowed to dry on a hot plate at ~40 °C for 5 mins. Clean discs were run through the instrument to check for contamination by checking peaks for metals other than argon, silicon and molybdenum and ensuring that all other peaks were less than half of the argon peak. Once checked, 10 µl of sample with Ga standard was pipetted on to the clean discs treated with Serva and placed on a hot plate at 50 °C to dry and subsequently analyzed on the S2 Picofox. Each TXRF batch typically contained 15 samples including 3 random samples run in duplicate, 2 blanks and 3 standard reference materials (SRMs). A linear standard curve was created using dilutions of the SRMs and corrected Zn were obtained by interpolating from the standard curve equation and accounting for appropriate dilution factor for males or females.

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Preface to Chapter 4

Chapters 2 and 3 investigated adverse effects of a model EDC (17β-trenbolone) on the avian reproductive pathway using traditional *in vivo* studies. In these chapters we studied sex- and developmental stage-related differences, from short- and long-term exposures. However, these whole animal studies are not feasible for every chemical-species scenario. Thus, alternative testing methods are needed to screen and prioritize chemicals for further animal testing.

Despite great interest in alternative testing methods, we do not yet know how results from alternative methods compare to those in the whole organism. Several alternative methods have been developed, each with inherent limitations and advantages, making it difficult to determine which among these methods might be most effective for chemical screening. It is important to examine differences across these methods by comparing results for common endpoints. Using a model avian species, White Leghorn Chicken (*G. gallus domesticus*), chapter 4 compares effects of model test chemicals on hepatic gene expression across three alternative toxicity testing methods: primary hepatocyte and liver slice culture, and liver from *in ovo* exposed embryos.

This chapter is authored by the candidate and coauthored by Dr. Niladri Basu and Doug Crump. The candidate was responsible for study design and development, sample collection and analysis, analysis and interpretation of the data, discussion of the results, and preparation of the manuscript, and was provided advice on all aspects of the study by the candidate's supervisor Dr. Niladri Basu. Doug Crump provided guidance on the study design, qPCR arrays, and preparation of the manuscript. It is planned for submission to Environmental Toxicology and Chemistry.

Chapter 4

4 A Comparative Study of Alternative Toxicity Testing Methods: Effects on Hepatic Gene Expression in the Chicken Embryo

4.1 Abstract

Toxicity testing is an integral aspect of environmental hazard identification and risk assessment and is traditionally conducted by exposing whole animals to test chemicals and observing adverse outcomes. However, this approach may not be logistically feasible for every species-chemical scenario and raises ethical concerns pertaining to animal use. Hence there has been growing interest in developing alternative toxicity testing methods to screen and prioritize chemicals in an effort to reduce whole animal testing. The objective of this study was to compare three alternative methods among each other: primary hepatocyte culture, liver slice culture, and liver from *in ovo* injected embryos, using a model avian species, White Leghorn Chicken (*Gallus gallus domesticus*). We examined changes in hepatic gene expression upon exposure to three model test chemicals: 17β-trenbolone (17βT), 17β-Estradiol (E2), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). A custom designed qPCR array with seven genes relevant to avian reproductive and xenobiotic metabolism pathways: vitellogenin (VTG), apolipoprotein (Apo), cytochrome P450 1A4 (CYP1A4), liver basic fatty acid binding protein (LBFABP), 3β-hydroxysteroid dehydrogenase (3βHSD1), stearoyl-CoA desaturase (SCD), and estrogen sulfotransferase 1E1 (SULT1E1). Hierarchical clustering was used to examine differences in

expression across the methods. CYP1A4 was consistently upregulated in response to TCDD in all three methods, and the magnitude was higher in hepatocytes (>150-fold) compared to slices (>31-fold) and *in ovo* liver (>27-fold). SCD and VTG were upregulated in the hepatocytes (>4-fold and >16-fold, respectively) in response to 17βT and E2. But aside from an upregulation of SCD (32-fold) in the *in ovo* liver in response to the high dose of E2, no changes were observed in the slices or *in ovo* liver. Overall, significant changes in expression were observed in more cases in the hepatocytes than in slices and *in ovo* liver. Hierarchical clustering of gene expression data grouped liver slice culture and *in ovo* liver as more similar to each other, while hepatocytes were grouped separately from the *in ovo* liver. Overall, this study indicated that despite similarities between slice culture and *in ovo* method, no clear pattern was observed across all the genes that were examined. There is a need for such introspective studies to understand how and why alternative methods differ and aid in establishing these methods as effective substitutes for whole animal methods.

Keywords: Alternative, 21st century toxicity testing, slice culture, hepatocyte, *in ovo*, *in vitro*

4.2 Introduction

Toxicity testing is an integral aspect of hazard identification and risk assessment (1–3). Testing chemicals for adverse effects has traditionally been performed using whole animals exposed to test chemicals, and measuring outcomes including growth, reproduction, and survival. However, such *in vivo* studies tend to be time-consuming, costly, and require large numbers of animals thus raising ethical concerns (4,5). As a result, few chemicals undergo extensive toxicity testing and it has been estimated that thousands of chemicals currently lack basic toxicity information, with over 700 new chemicals being introduced annually (6–8). The landmark 2007 National Research

Council (NRC) report (Toxicity Testing in the 21st century: A Vision and a Strategy) examined the status quo in toxicity testing and concluded that it may not be equipped to meet current chemical testing needs (9). It proposed a tiered strategy focused on screening and prioritizing chemicals via mechanistically based suites of alternative assays (9). The need for an improved testing framework, and advances in toxicogenomics, molecular biology, and bioinformatics have led to the development of various alternative toxicity testing methods (4,9).

Alternative toxicity testing is described as methods that incorporate the 3Rs concepts of: A) reducing the number of animals used, B) replacing animals with non-animal components (*in vitro*) or C) refining a study to be less stressful or painful to animals (10,11). A bibliometric search shows that there has been a substantial increase in the number of publications related to alternative toxicity screening following the 2007 NRC report (Figure. 4.1). However, most of these advances have been related to human health and focused on mammalian species (12–15). There are few alternative toxicity testing options for wildlife and even fewer for avian species (Figure 4.1). Three common alternative testing methods are primary hepatocyte culture (isolated from liver tissue), liver slice culture (slices prepared from liver tissue) and the use of early life stages (i.e. embryos). These three methods provide contrasting advantages for toxicity testing, for instance, while isolated hepatocytes are convenient for screening several chemicals at a time, they may not retain all the characteristics of an intact liver (16), tissue slices tend to better represent the complexity of the intact organ and have better structural retention than regular 2-D cell culture (16–18); additionally, embryos represent whole organisms, however, the use of embryos is considered as an alternative method since they are nonself-feeding organisms. While hepatocyte culture and embryos have been previously used in avian toxicity studies (19–24), liver slice culture has not yet been explored for avian toxicology.

At present, it is unclear how results from alternative methods compare with results in the whole organism. Additionally, varying methods have inherent advantages and disadvantages making it unlikely that any single alternative method alone could be used for screening chemicals (25). Thus, a comparison of alternative methods is important to understand how they differ in their response to chemical exposure (25). de Jong et al. (26) compared developmental toxicity using mouse embryonic stem cell test (EST), zebrafish embryotoxicity test (ZET) and rat whole embryo culture (WEC) and found that the ZET gave the best correlation with *in vivo* studies. Boess et al. (27) compared gene expression in rat liver slices, primary hepatocyte culture, and hepatic cell lines to *in vivo* rat liver and determined that slices showed the strongest similarity to *in vivo* liver tissue. Previous studies have not examined differences across *in vitro* and *in ovo* alternative testing methods in avian species.

In the present study, we aimed to compare gene expression responses using White Leghorn Chicken (*Gallus gallus domesticus*) embryo or embryonic liver as the tissue source for the three methods: primary hepatocytes, slice culture, and liver *in ovo* following exposure to model chemicals. Three model chemicals were used in this study: 17β-trenbolone (17βT) and 17β-Estradiol (E2) which are both endocrine active chemicals and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) which is a dioxin and a positive control of CYP1A4 induction.

4.3 Methods

4.3.1 Overall Study Design

In this study, three toxicity testing methods were used (two *in vitro* methods: hepatocyte culture and liver slice culture; and one *in ovo* egg injection method) to examine expression of seven genes exposed to three chemicals at three concentrations. Thus, there were 3*3 = 9 chemical

treatments and a solvent control, i.e. in total 10 treatment conditions. The overall study design is provided in Table 4.1.

4.3.2 Chemicals

All chemicals were purchased from Sigma unless otherwise indicated. The three test chemicals used were 17β-trenbolone (17βT; Toronto Research Chemicals; > 96% purity), 17β-Estradiol (E2; > 98% purity), and 2,3,7,8-Tetrachlorodibenzodioxin (TCDD, Wellington Laboratories; > 98% purity) were dissolved in the solvent dimethyl sulfoxide (DMSO). The dilutions of 17βT (final concentration 3 µM, 15 µM and 60 µM), E2 (final concentration 10 nM, 100 nM and 1000 nM) and TCDD (final concentration 0.01 nM, 0.1 nM and 0.5 nM) were stored at room temperature. The concentrations used here were based on the LD50 of the chemical or those concentrations used in previous studies.

4.3.3 Chicken Liver Tissue Source

Fertilized chicken eggs obtained from Couvoir Simetin, Inc., hatchery (Mirabel, QC, Canada) and Ferme, GMS (St.Liboire, QC, Canada), were incubated at 37 °C and 60% humidity for 19 days. On day 19 of incubation, eggs were opened, and embryos were euthanized by cervical decapitated. Liver tissues were dissected out of the embryos and processed as outlined below for the hepatocyte cultures and the liver slices cultures.

4.3.3.1 Hepatocyte culture

A chicken embryo hepatocyte pool from 18 individuals was prepared under sterile conditions according to the methods described by Lorenzen et al. (28) with slight modifications. Briefly,

after dissections whole livers were rinsed in Krebs-Ringer Buffer (KRB) and transferred to a petri dish containing collagenase dissolved in KRB (0.5mg/ml). A few cuts were made in each lobe using a scalpel. Cut livers and collagenase were transferred to a capped 250-ml Erlenmeyyer flask which was shaken at 100 rpm for 90 min at 37 °C. Following collagenase digestion, the entire digestate was filtered through a filtration apparatus using light vacuum as needed. The filtrate was divided equally between two 50-ml centrifuge tubes and centrifuged at 300g for 5 min at room temperature. The cell pellets were pooled together in 50 ml of filter-sterilized M199 culture medium (supplemented with 2.24 g/L $NaHCO_3$, 0.2% L-Thyroxine and 0.2% Insulin (5mg/ml in 0.1N NaOH)), and 1% penicllin/streptomycin (pen/strep; Fisher) and resuspended by gently pipetting up and down. The resulting cell suspension was transferred to 50-ml centrifuge tubes containing a 24-ml mixture of 90% Percoll and 2.5M sucrose, mixed by inverting and centrifuged at 50g for 10 min. The resulting upper layer of cells was carefully removed and transferred to a pre-weighed 15-ml centrifuge tube with 10 ml DNaseI (Roche) to remove DNA contamination, gently mixed, and centrifuged at 50g for 5 min. Resulting cell pellet was rinsed with medium and centrifuged again at 50g for 5 min and the supernatant discarded. A volume of medium 32 times the mass of the pellet in the tube was added to resuspend the cells. The cell suspension (25 µl) was added to each well of 4, 48-well culture plates (Fisher) containing 500µl culture medium and incubated at 37 °C, 60% humidity and 5% CO_2. Twenty-four hours after incubation, old medium was aspirated and 500 µl fresh medium was added to cells along with 2.5 µl DMSO or test chemical at appropriate concentrations and incubated at same conditions. Twenty-four hours after treatment, the medium was aspirated from each well and plates were stored at -80 °C until RNA isolation and gene expression analyses.

4.3.3.2 Liver slice culture

Liver slices were prepared from four, day 19 chicken embryos based on methods described previously (28,29), with modifications for chicken embryonic liver as follows: embryos were dissected, one at a time. Both lobes of the liver (approximate weight 0.7 g) were extracted intact and immediately placed in ice cold phosphate buffered saline (PBS; Fisher) with 1% pen/strep. Each lobe was sliced using the manual mode of the McIlwain Tissue Chopper (Ted Pella, Inc.) into slices of ~300 µm thickness and each slice was immediately placed in ice cold PBS with 1% pen/strep. While one lobe was being sliced further, the prepared slices were weighed and placed in one of the wells of a 24-well tissue culture plate (Fisher) containing 700µl culture medium (prepared as described above). About one to three slices were added to each well such that the total weight of tissue in a well was ~15 mg. Liver from each embryo was sliced to provide sufficient slices for all treatments. Once the slices were added to the wells they were dosed with 3.5µl DMSO (0.5% DMSO) or test chemicals at appropriate concentrations and plates were placed in a 5% CO_2 incubator at 37 °C and 60% humidity for 24 hours. After the 24 hour period, slices were removed from the wells and homogenized in lysis buffer and stored at -80 °C until RNA isolation and gene expression analyses.

4.3.4 In ovo Injected Embryo

Fertilized chicken eggs from Ferme, GMS (98 eggs; St.Liboire, QC, Canada) were dosed with solvent or test chemicals following egg injection methods described in Rutkiewicz et al. (30). Briefly, prior to incubation, chicken eggs were candled to find the aircell and a hole was drilled into each egg. The solvent control eggs (8 eggs) were injected with 10µl DMSO. Treatment eggs (10 per treatment) were injected with 10µl of the test substance at appropriate concentrations.

Once injected the drilled holes were covered with Airpore microporous tape (Qiagen) and eggs were placed on their side in the incubator at 37 °C and 60% humidity and automatically turned every 45 min. Embryos were sampled just prior to hatch at day 19 of incubation. Four embryos per treatment were dissected (n=4 per treatment for qPCR) and stored at -80 °C until RNA isolation and gene expression analyses. There was no mortality in the 17βT or E2 treatment groups. In the TCDD group, there was 1 mortality (out of 10 eggs) in the 0.5 nM, and no mortalities in the other two concentrations. One egg was infertile in the DMSO group.

4.3.5 RNA Isolation and cDNA Preparation

RNA was isolated from hepatocytes, liver slices and *in ovo* liver tissue using the Nucleospin RNA plus isolation kit as per manufacturer's instructions (Macherey Nagel). Hepatocytes were lysed by repeatedly mixing cells with lysis buffer. Tissue slices and *in ovo* liver tissue were lysed using stainless steel beads (Qiagen) and TissueLyser (Qiagen) at 25Hz for 2 min. Total RNA concentration at 260 nm and A260/A280 was determined by spectrophotometry using QIAxpert (Qiagen). The A260:A280 of all RNA samples ranged from 1.9 - 2.2 (mean ± standard error was 2.11 ± 0.035). Total RNA (250ng) was reverse transcribed using Qiagen RT^2 First Strand reverse transcription kit as per manufacturer's instructions (Qiagen). On-column DNase digestion, and genomic DNA (gDNA) elimination buffer was included to eliminate (gDNA) (Qiagen).

4.3.6 Quantitative Polymerase Chain Reaction

A custom 384-well, RT^2 qPCR array (Catalog # CLAG25364; Qiagen) was created for this study that consisted of seven genes chosen based on pathways of interest related to the endocrine system and metabolism (Table 4.2). Three internal controls (gDNA, positive PCR control (PPC)

and reverse transcription control (RTC)), and two reference genes (EEF1A1 and RPL4) were included with every set of seven genes. Thus, the qPCR array contained 12 total genes (seven genes of interest + 5 control genes) in 32 replicates (12*32 = 384 wells). Each sample was run in a plate in single replicates; a mastermix was prepared by combining 4µl cDNA + 81.25µl RT^2 SYBR Green Mastermix (Qiagen) + 77.25µl H_2O. 10µl of the mastermix for each sample was loaded into a well containing one of the twelve genes, using a pipetting robot, QIAgility (Qiagen). Once the mastermix was loaded into each well, the plates were sealed with Optical Adhesive Film (Qiagen) and spun in the centrifuge for a few seconds. The plate was then placed in a CFX384 (Bio-Rad) and qPCR run was started with the following thermal profile: 95 °C for 10 min to activate the enzyme, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, and ending with a melting curve segment of 65 °C to 95 °C at 0.5 °C increments for 5 sec.

No amplification was detected in the gDNA controls confirming the lack of gDNA contamination. Difference between PPC and RTC was less than 5 in samples thus satisfying the manufacturer's qPCR array quality control parameters. Melt curves for all genes resulted in a single peak indicating amplification of a single qPCR product. Cycle number or Cq values were obtained from CFX Maestro Software Version 1.1 (Bio-Rad). Relative normalized expression of target genes was quantified as the difference in fold change normalized to one or both of the reference genes and relative to the corresponding control samples following the ΔΔCq method.

4.3.7 Data Analyses

Statistical analyses were performed using CFX Maestro Version 1.1 (Bio-Rad) and JMP Pro V13 (31). For each method and chemical, log 2 transformed gene expression data normalized to the reference gene and relative to the corresponding control, where the fold change (log_2FC) was

greater than 1 or -1, were checked for normality using the Shapiro-Wilk's test. If data passed the normality test, we performed one-way Analysis of Variance to check for statistical significance in gene expression changes. If significant differences were found, means were subjected to a Tukey's HSD post hoc test to determine statistical significance among treatment pairs. In cases where data failed the normality test, we performed Kruskal-Wallis Wilcoxon test to check for statistical significance among treatment groups. If significant differences were found, we performed Steel-Dwass post hoc test for comparisons to check for statistical significance among treatment pairs. Results were considered significant at $\alpha < 0.05$.

To perform a comparison among the three alternative methods with respect to differences in gene expression (based on the $\log_2$ transformed fold change data), hierarchical cluster analysis (HCA) was performed with Euclidean distance function and the centroid linkage rule, in R (32), and data were visualized using a heatmap. Variables used were the method*chemical vs genes.

4.4 Results and Discussion

Changes in the expression of individual genes are presented and discussed in the first section. This is followed by a comparison across the three alternative methods.

4.4.1 Individual Gene Expression

Expression profiles for method*chemical*gene scenarios are provided in Figure 4.2 and a summary of gene expression results are provided in Supplementary table 4.1. Results where a significant up- or down-regulation by a minimum fold change (FC) of 2 (i.e., $\log_2 FC = 1$ or -1) were observed are discussed below. No significant results were observed for 3β-hydroxysteriod dehydrogenase (3βHSD1). Apolipoprotein was not detected in any of the three methods and hence expression for this was not calculated.

4.4.1.1 Cytochrome P450 1A4 (CYP1A4)

A significant upregulation of CYP1A4 was observed in the hepatocytes, liver slices and *in ovo* liver exposed to TCDD. Compared to hepatocytes (>7 log_2FC in all tested concentrations), the magnitude of gene expression was lower in the slice culture (> 4 log_2FC in all tested concentrations) and the *in ovo* liver (>4 log_2FC in 0.1 and 0.5 nM). No consistent changes in CYP1A4 expression were observed in the three methods exposed to E2 or 17βT.

CYP1A4 is a gene involved in Phase 1 and II metabolism of compounds and its expression is regulated by the aryl hydrocarbon receptor (AhR), which is the main site for binding of dioxin-like compounds (33). Comparable up-regulation of CYP1A4 expression (5 log_2FC) in chicken (*in ovo* or hepatocytes) exposed to TCDD have been demonstrated in previous studies (34,35). As this gene was purposefully included here to assess the overall functionality of the three methods, the observation of CYP1A4 up-regulation in all three approaches indicate that the hepatocytes and liver tissue in these methods are metabolically functional. No change in expression of CYP1A4 was seen upon exposure to 17βT and E2 (except for down-regulation in 3 μM 17βT treated embryos, and 10 nM E2 treated slices). Based on a search of CTDbase.org, no prior studies have shown effects of E2 and 17βT on CYP1A4 expression.

4.4.1.2 Vitellogenin (VTG)

A significant upregulation of VTG was observed in hepatocytes dosed with 17βT (>4 log_2FC) and E2 (>3 log_2FC) in all tested concentrations, but not in the slice culture. No significant change in VTG expression was observed in hepatocytes or slice cultures dosed with TCDD. Vitellogenin

amplification was not detected in a consistent manner in the embryos hence no change in expression could be calculated for the *in ovo* method.

In oviparous or egg laying species, VTG, a key phosphoprotein is known to be a precursor to egg yolk proteins, and expression of the VTG gene has been shown to be directly mediated by the estrogen receptor pathway (36,37). As such VTG is expressed in high levels in adult females but not in embryos, juveniles and roosters. However, previous studies have shown that embryonic avian hepatocytes exposed to estrogenic chemicals such as E2 and moxesterol at comparable concentrations, induced VTG expression (28). 17β-trenbolone is an androgen mimicking EDC which has been shown to cause changes in plasma E2 and VTG levels, and VTG mRNA expression in fathead minnow (FHM; P*imephales promelas*) (38). VTG was included in the array as it is considered to be a valuable endpoint to test for EDCs that might affect the estrogenic pathway. In our study we did observe an upregulation of VTG in response to 17βT and E2, but only in the hepatocytes, and we were unable to observe changes in the slices and embryos. A previous study from 1978 (39) demonstrated trace VTG induction in day 13 and 15 chicken embryos, however the concentration used in this study was 25 mg/ml or around 91 mM, several magnitudes higher than the doses in our study. Thus, it is possible that the concentrations of E2 here did not induce VTG expression in the embryo or the liver slice but were sufficient to trigger VTG expression in the isolated hepatocytes.

4.4.1.3 Estrogen Sulfotransferase (SULT1E1)

There was a significant decrease in SULT1E1 expression in embryos dosed with 17βT (-1.84 log_2FC in 60 μM) and E2 (-1.68 log_2FC in 10 nM). In contrast, no significant changes in

expression were observed in the hepatocytes or the slice cultures. Additionally, no significant differences of SULT1E1 were observed in the three methods in response to TCDD.

SULT1E1 is a gene that codes for the enzyme estrogen sulfotransferase and is involved in the transfer of a sulfur moiety to and from estrone and is involved in the regulation of the level of estrogen receptors and is thus one of the enzymes implicated in estrogen metabolism (40). Due to this and the impacts of 17βT seen on plasma E2 levels, SULT1E1 was included in this qPCR array. While not much is known regarding the effect of 17βT or E2 on SULT1E1 expression in avian species, it was shown that primary cultures of mouse fetus prostate mesenchymal and rat ovarian cells exposed to estrogenic chemicals resulted in decreased SULT1E1 mRNA expression (41,42). This is similar to the decrease in expression we observed in the embryos, however, we did not observe a similar decrease in either *in vitro* method used. While these previous studies examined effects in ovarian and prostate cells, we examined the liver. Thus, perhaps any effects seen in the liver are due to changes further upstream in the endocrine pathway such as the ovaries. This might explain why we observed changes in the *in ovo* method which contains the intact endocrine pathway, but not in the isolated hepatocytes or liver slices, while previous studies observed changes in ovarian cells, even though both were *in vitro* tests.

4.4.1.4 Stearoyl CoA Desaturase (SCD)

A significant increase in SCD expression was observed in hepatocytes dosed with 17βT (>4 $\log_2$FC) and E2 (>2 $\log_2$FC), in all tested concentrations, and in liver slices dosed with 17βT (2.22 $\log_2$FC in 3 μM) and E2 (2.05 $\log_2$FC in 1000 nM). In contrast, there were no changes in embryos dosed with 17βT, but a significant increase was found in embryos treated with E2 (5.5

log_2FC in 1000 nM). Additionally, no changes in gene expression of SCD was observed in the three methods exposed to TCDD.

SCD is a gene which codes for the enzyme stearoyl coA desaturase, which catalyzes the rate limiting step in the formation of monosaturated fatty acids. These are major components of various biomolecules such as cholesterol esters (43). Cholesterol is a precursor to androgen biosynthesis and hence SCD can play a role in the steroid biosynthesis pathway. Due to the relevance of this enzyme in the endocrine pathway, it was included in this array. Not much is known about the effect of endocrine disruptors on SCD expression in avian species. However, two studies looking at effect of estrogens and phytoestrogens in human cell lines and pregnant rats observed a down-regulation of SCD (44,45). Stearoyl CoA desaturase expression was induced even at lower treatments of E2 and 17βT in the hepatocytes, and at lower treatment of 17βT in the liver slice, however an upregulation was not observed until the high dose in the embryonic liver. This further demonstrates a differential response between the three methods, indicating that hepatocytes might be affected by the chemical at lower treatments, whereas a response is not observed in the embryo until higher treatments.

4.4.1.5 Liver basic fatty acid binding protein (LBFABP)

A significant decrease in LBFABP expression was observed in hepatocytes treated with 17βT (<-1.23 log_2FC in all tested concentrations) and E2 (<-1.43 log_2FC in 100 nM and 1000 nM), and *in ovo* embryos dosed with 17βT (<-1.22 log_2FC in all tested concentrations) and E2 (<-1.53 log_2FC in 100 nM and 1000 nM). No changes in LBFABP expression were observed in response to TCDD.

LBFABP is the gene that codes for Liver Basic Fatty Acid Binding Protein which is involved in the binding, transport and metabolism of long chain fatty acids and lipids such as cholesterol (46). Due to the relevance of these processes in the endocrine pathway, LBFABP was included in this array. Not much is known about the effect of 17βT and E2 on LBFABP expression in avian species, however a study using human hepatoma cell lines showed that E2 results in suppression of LBFABP expression (47). While we observed a similar decrease in LBFABP expression in all three methods, results were significant only in the hepatocytes and *in ovo* liver, thus indicating that depending on the pathway being examined, perhaps there is potential to using hepatocytes as an alternative to whole liver studies.

4.4.2 Comparison of Alternative Methods

Clustering analysis of the gene expression data were performed to examine similar groupings across the three testing methods. No clear dose-response pattern was discernable in all three methods for any of the gene-chemical combinations (except for a consistent significant up-regulation only in CYP1A4-TCDD). Hierarchical clustering analysis of the gene expression data are presented by alternative method*chemical against the genes (Figure 4.3). Individual blocks in a row represent gene expression data normalized to the mean of the whole row with cells shaded in red and yellow representing lower and higher gene expression values, respectively, relative to this mean. Hierarchical clustering analysis showed groupings related to the alternative method and the chemical as highlighted by numbered regions in the heatmap (Figure 4.3)

In terms of comparing responses across the three models, HCA indicated that the slice culture and *in ovo* results were clustered together (region 1 in Figure 4.3) and separated from the hepatocyte culture (region 2 in Figure 4.3). This might be because slice cultures responded more

similarly to the *in ovo* liver, and the hepatocyte culture was more different from the *in ovo* liver. Previous studies using slices from rat (*Rattus norvegicus*) liver and rainbow trout (*Oncorhynchus mykiss*) testis have shown that slices can have high fidelity to and better functional representation of liver from a whole organism (16,48–50). Tissue slices are said to better retain all cell types of the tissue in their natural environment with good retention of intracellular and cell-matrix interactions (17,18). Additionally, unlike isolated hepatocytes, there might be delayed accessibility of the chemicals to the cells within the slice (51); this is also likely more representative of an intact liver. A number of biomedical and pharmacological studies have examined effects of chemicals on gene expression in slices and compared to *in vivo* studies. Boess et al. (27) compared a number of *in vitro* systems including hepatic cell lines, primary hepatocyte culture, and liver slice culture to *in vivo* rat liver, and observed that liver slices were best correlated to the *in vivo* liver. Elferink et al. (52) studied model toxic compounds such as paracetamol in rat liver slices and found that they accurately predicted toxicity in the *in vivo* liver.

Additionally, TCDD, (a dioxin and agonist of the AhR pathway), is a potent inducer of CYP1A4 expression (34,35), distinct from the other two chemicals chosen in this study, thus likely explaining why the TCDD results for the three methods clustered together (region 3 in Figure 4.3). Accordingly, we saw that the E2 and 17βT were distinct from TCDD, but there were no distinct groupings within E2 and 17βT, which might be expected since they both are chemicals active in the endocrine system and are involved in impacts on the estrogenic pathway (region 3 in Figure 4.3). In general, the magnitude of up-regulation was higher in the hepatocytes than in the slice cultures and *in ovo* method. In the hepatocytes, with the exception of LBFABP, genes tended to be up-regulated. In the liver slices and *in ovo* liver genes were up- and down-

regulated. In certain cases, such as SCD-E2 in the *in ovo* method, a down-regulation was observed in the low and medium doses, followed by an up-regulation in the high dose. A comparison with external studies looking at these chemical-gene interactions showed some agreement with our results (such as CYP1A4, VTG, and LBFABP) however differed in other cases (such as SCD, 3βHSD1). Such discrepancies between results from this study and previous research make external validation of these alternative approaches complicated. A summary of studies looking at effects of the test chemicals on mRNA expression levels of genes included in this study is provided in Supplementary Table 4.1.

Through this study, we examined three different alternative approaches including two *in vitro* and an *in ovo* method enabling us to compare *in vitro* results to a whole organism. Overall, this research represents the challenges faced with the development and use of alternative toxicity testing methods. Tissue slices and cell cultures are both *in vitro* methods and involve extracted target biological components which are directly dosed with the chemicals of interest. Conversely, in the *in ovo* method, the developing embryo is treated with the chemical of interest and thus the exposure in the liver is not known. Thus, the toxicokinetics of the test chemical (adsorption, distribution, metabolism, and excretion in an organism) is typically not or is only partially accounted for in the *in vitro* methods (53,54). In this study, as is often the case in ecotoxicological research, we are not able to determine actual concentrations of chemicals in the hepatocytes, slices and *in ovo* liver. Some studies in mammalian and fish species have investigated the toxicokinetics of chemicals through computational modeling approaches to be able to extrapolate from *in vitro* to *in vivo* methods (55–57). However, further research is needed to understand the differences in uptake of chemicals in the biological systems in alternative approaches (25,58).

Clustering analyses indicated that slice culture and the *in ovo* method were more similar to each other, however, no clear patterns could be observed across all the genes examined. In their study of alternative and *in vivo* methods in rats, Boess et al. determined that liver slice cultures were most similar to the *in vivo* liver but concluded that neither of the *in vitro* methods examined were directly comparable to the *in vivo* method (27). The effects on gene expression in the various methods can be affected by factors such as uptake of test chemicals, duration of exposure, and test species. These aspects preclude us from drawing direct gene-gene comparisons across the exposure methods (27). Additionally, while the genes chosen in this study represented relevant reproductive and xenobiotic metabolism pathways, they covered a rather small biological space. A more comprehensive and targeted set of genes might allow for better comparisons across differing alternative toxicity testing methods.

Other factors that can differ across various alternative methods are the resources needed (quantified as the costs associated with money, number of animals, and time needed for testing) and variability present in the results. With respect to resources, all three alternative methods were comparable in terms of time and money needed; each method took about 4 weeks to complete and required approximately $3,000. However, they differed with respect to the number of eggs required. Twenty four eggs were incubated for the hepatocyte cultures, and 12 eggs were incubated for the liver slice culture. In the *in ovo* method, a total of 98 eggs were used. This difference in the number of eggs needed demonstrates the reduction in animal usage even within methods that are considered alternatives, i.e., *in vitro* methods compared to the *in ovo* method. With respect to variability, the SE of the mean fold change in gene expression ranged from 0.027 to 1.40 in hepatocytes, 0.073 to 1.86 in liver slices and 0.061 to 2.02 in the *in ovo* method. In the hepatocyte culture, cells were isolated from liver tissues from multiple individuals that were

pooled together and plated in multiple wells of culture plates. While test chemicals were added to replicate wells, these represented technical rather than biological replicates thus likely not representing individual variability. In contrast, slices were prepared from each individual for each treatment, and in the *in ovo* method, embryos were exposed to respective test chemicals, and these thus are more likely to retain individual variability. While SE values overlapped between the three methods, in general the range was lower in the hepatocytes compared to the liver slices and *in ovo* embryos. This could be interpreted in that hepatocytes represent a pooled sample set and hence display lower variability, whereas the liver slices and *in ovo* embryos represent individuals and thus might display higher variability.

4.5 Conclusion

In this comparative study, we examined differences in gene expression across three alternative methods of toxicity testing (hepatocyte culture, liver slice culture and *in ovo* exposures) upon exposure to three chemicals of interest. Hepatocyte and slice cultures are both *in vitro* alternative methods; embryos, while representing a whole organism, are also considered to be an alternative method since the embryo is not a self-feeding organism. This allowed us to examine differences across alternative testing methods, but also between *in vitro* methods and an *in ovo* method. Hierarchical clustering of the gene expression results indicated that the slice cultures and *in ovo* results were grouped to be more similar to each other and that hepatocytes were more different from the *in ovo* embryo. However, no clear pattern could be observed across the three methods for all the genes analyzed highlighting the challenges in the use of alternative testing methods. Factors such as chemical toxicokinetics, duration of exposure, species, and toxicity pathways can further complicate the comparison between results from various methods. Since diverse

alternative methods can have inherent advantages and disadvantages, the absence of a best alternative testing method further emphasizes the possibility that no single method or assay is likely applicable as an alternative to an *in vivo* method, rather a carefully validated suite of methods is likely a better approach. Additionally, while we were able to study results in expression of relevant genes from exposure to three model chemicals, these represented a limited biological space. Thus, further introspective studies with more comprehensive sets of genes are necessary to understand how alternative approaches differ.

4.6 Acknowledgements

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4.7 Tables and Figures

Table 4.1: Overall study design is presented as a matrix of methods vs chemical treatments. Chicken embryos were used for liver tissue for each alternative testing method (hepatocyte culture, liver slice culture, and *in ovo* liver) and were tested against the three chemicals: 17β-trenbolone (3 µM, 15 µM, 60 µM), 17β-Estradiol (10 nM, 100 nM, 1000 nM), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 0.01 nM, 0.1 nM and 0.5 nM); dimethyl sulfoxide (DMSO) was used as a solvent control. There were four replicates per treatment for each method.

Method / **Chemical**	**Hepatocyte culture (concentrations)**	**Liver slice culture (concentrations)**	***In ovo* exposure (concentrations)**
Control	DMSO	DMSO	DMSO
17β-Estradiol	10 nM	10 nM	10 nM
	100 nM	100 nM	100 nM
	1000 nM	1000 nM	1000 nM
17β-Trenbolone	3 µM	3 µM	3 µM
	15 µM	15 µM	15 µM
	60 µM	60 µM	60 µM
TCDD	0.01 nM	0.01 nM	0.01 nM
	0.1 nM	0.1 nM	0.1 nM
	0.5 nM	0.5 nM	0.5 nM

Table 4.2: List of seven genes included in the custom 384-well qPCR array and their corresponding function. Genes chosen were based on relevant pathways ranging from xenobiotic metabolism to estrogenic, steroidogenic and fatty acid metabolism pathways.

GENE	Function
Cytochrome P450 1A4 - CYP1A4	Xenobiotic metabolism
Vitellogenin - VTG	Estrogen pathway
Apolipoprotein - APO	Estrogen pathway
Estrogen Sulfotransferase - SULT1E1	Estrogen metabolizing enzyme
Hydroxysteroid dehydrogenase - HSD3β1	Steroidogenesis
Liver basic fatty acid binding protein - LBFABP	Fatty acid pathway
Stearoyl CoenzymeA Desaturase - SCD	Fatty acid pathway

Supplementary Table 4.1: Summary of gene expression data for the three methods and chemicals. Data are presented as mean $\log_2$ transformed relative normalized fold change in expression. Values in bold are significantly different at $\alpha < 0.05$. Hepatocytes – hepa; Liver slices – slices; and *In ovo* liver – in ovo; 17β-trenbolone – 17βT; 17β-estradiol – E2; 2,3,7,8-tetrachlorodibenzodioxin – TCDD.

Method	Chemical	Dose	Genes					
			CYP1A4	HSD3β1	LBFABP	SCD	SULT1E1	VTG
hepa	TCDD	1	**7.59**	-0.89	-0.67	0.34	-0.09	-0.32
		2	**7.30**	0.10	-0.48	0.41	-0.04	-0.35
		3	**7.60**	-0.50	-0.87	0.55	0.60	-0.60
	E2	1	1.40	1.48	-0.77	**6.53**	1.98	**6.27**
		2	**3.06**	1.04	**-1.43**	**6.42**	2.29	**5.77**
		3	-0.59	-2.15	**-4.17**	**2.75**	0.98	**3.95**
	17βT	1	1.72	0.92	**-1.23**	**6.15**	2.12	**4.02**
		2	**2.46**	1.64	**-2.36**	**5.19**	2.00	**5.42**
		3	-1.90	-1.13	**-3.09**	**4.07**	1.44	**6.61**
slices	TCDD	1	**4.96**	-0.18	-0.13	0.03	-0.34	-0.32
		2	**6.10**	0.25	-0.12	0.12	-0.54	-1.04
		3	**5.76**	-0.40	0.30	0.33	-0.33	-0.57
	E2	1	**-4.06**	2.45	1.00	1.96	0.74	1.40
		2	1.36	1.36	-0.44	1.96	-0.40	-0.39
		3	1.19	0.90	-0.02	**2.05**	-0.09	0.55
	17βT	1	0.82	0.42	0.31	**2.22**	-0.14	-0.17
		2	0.49	0.59	-0.26	1.17	-0.23	-0.41
		3	0.11	1.68	0.24	-0.08	-0.05	-1.36
in ovo	TCDD	1	-0.29	0.87	-0.74	0.45	0.45	
		2	**4.76**	-0.06	-0.77	0.69	0.41	
		3	**6.33**	1.72	0.71	0.43	0.20	
	E2	1	-0.74	-1.25	-1.15	-1.40	**-1.68**	
		2	-0.33	-2.00	**-1.53**	-3.02	-1.29	
		3	-0.64	-1.60	**-1.57**	**5.50**	-1.65	
	17βT	1	**-5.69**	0.14	**-1.22**	-1.44	-1.39	
		2	-0.43	1.13	**-1.49**	-1.98	-1.43	
		3	-0.58	-1.74	**-1.23**	-1.83	**-1.84**	

Supplementary Table 4.2: A summary of results of changes in expression of genes from this study and previous studies.

Genes	Chemical	Species/ method	Up/down regulation	Reference	Present study
CYP1A4	TCDD	Chicken embryo	Up	(34)	Up
		Chicken hepatocytes	Up	(35)	
VTG	17βT	Fathead minnow, mosquitofish	Up	(38,59)	Up
	E2	Chicken hepatocytes	Up	(28)	Up
SULT1E1	E2	Rat ovarian cells	Down	(42)	Down
		Fetal mouse prostrate cells	Down	(41)	
SCD	E2	Human breast cancer cell line	Down	(44)	Up
	17α-Ethinyl estradiol	Rat liver	Down	(45)	
LBFABP	E2	Human hepatoma cell lines	Down	(47)	Down
3βHSD1	E2	Rat	Down	(60)	No change
		Human placenta cell line	Up	(61)	
	TCDD	Rat	Down	(62)	No change
		Mouse follicle culture	Up	(63)	

Figure 4.1: Number of publications related to alternative toxicity testing between 1997 and 2017; a substantial increase in publications can be seen after 2007, which was when the NRC report on Toxicity Testing in the 21st Century (TT21C) was released. Search terms were: (environment* AND toxic* AND alternat*). Search results were refined for human/mammal studies: (human* OR rat* OR mice OR mammal*); fish studies: (fish* OR minnow* OR medaka* OR trout* OR perch* OR walleye* OR sturgeon*); avian studies: (bird* OR avian* OR chick* OR quail* OR finch* OR duck* OR mallard*). Black circles = all studies related to alternative toxicity testing; Open diamonds = human/mammalian studies; Grey triangles= fish studies; Black squares = bird studies. Number of publications obtained from Web of Science on March 24th, 2018.

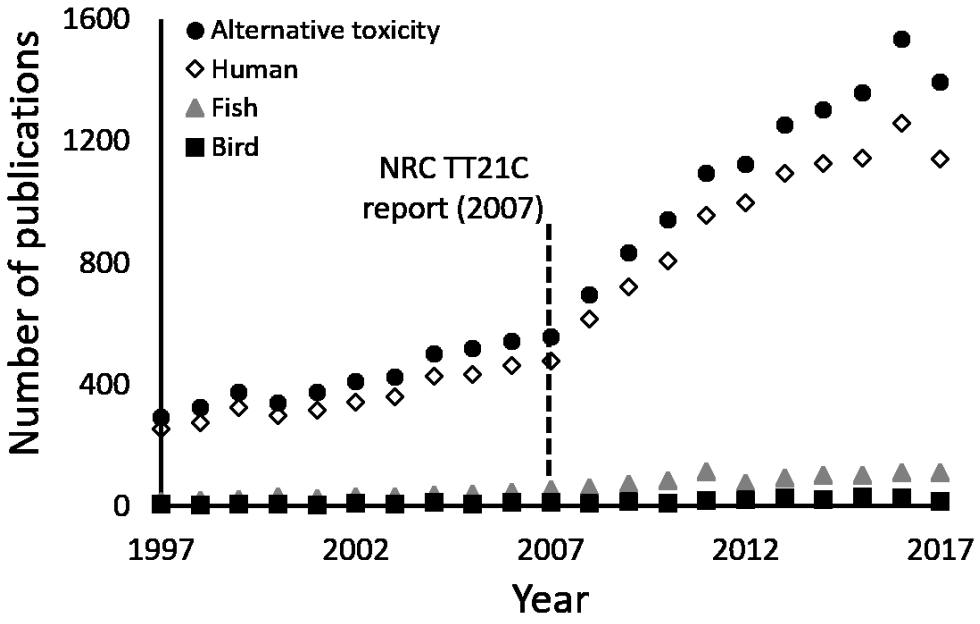
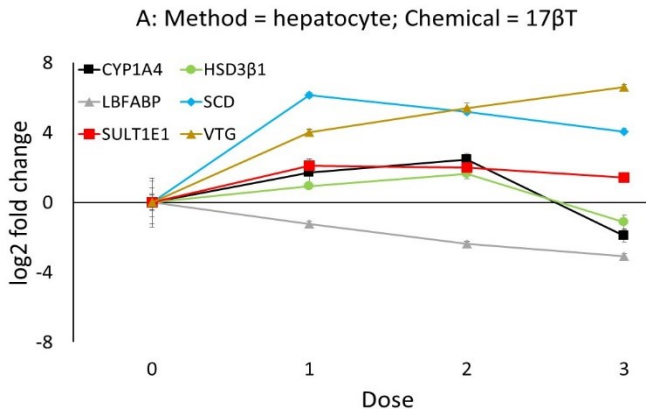
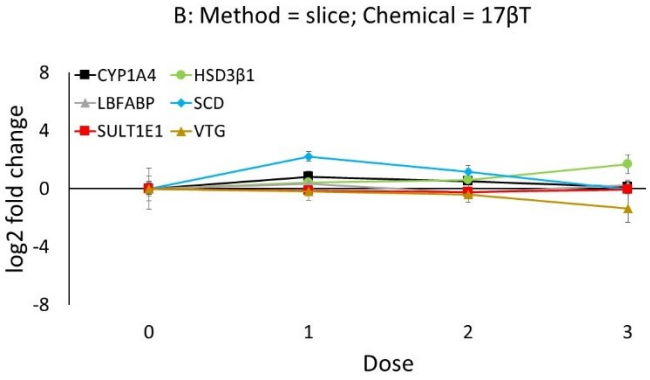
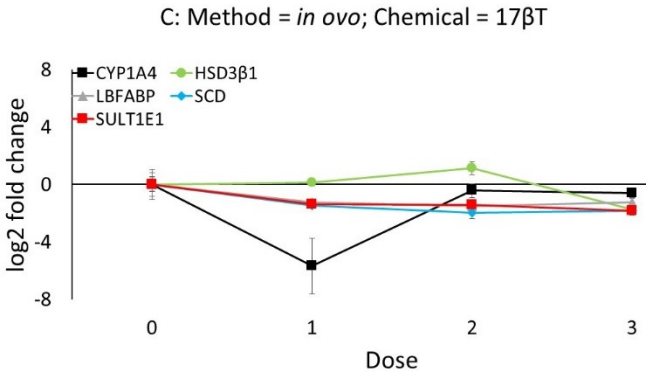


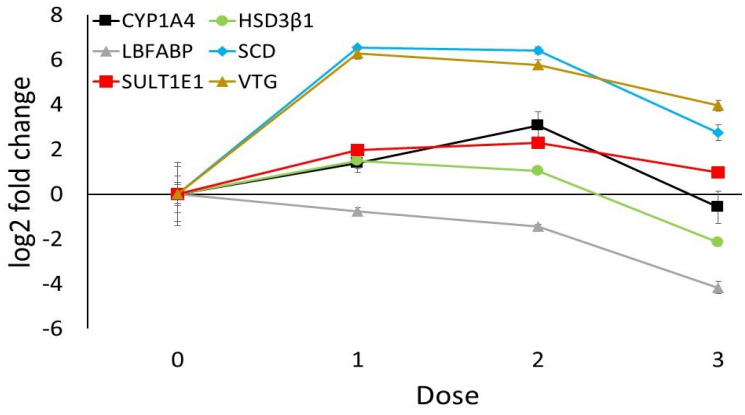
Figure 4.2: Gene expression results for the three methods and chemicals, and all genes. Data are presented as mean log 2 transformed relative normalized fold change in expression; error bars represent standard error of the mean. (A-C) 17βT (3 µM, 15 µM and 60 µM); (D-F) E2 (10 nM, 100 nM and 1000 nM); (G-I) TCDD (0.01 nM, 0.1 nM and 0.5 nM).



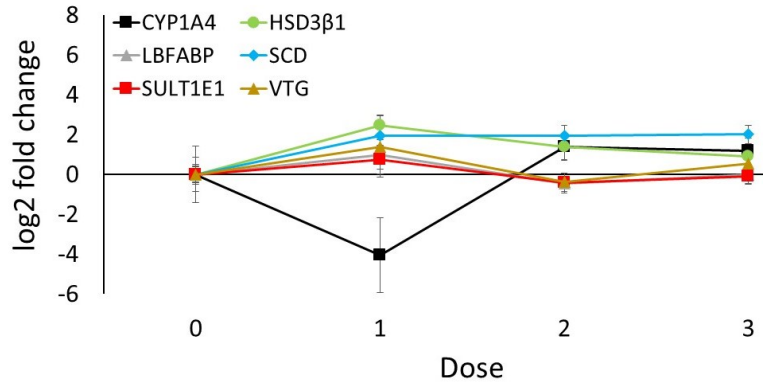




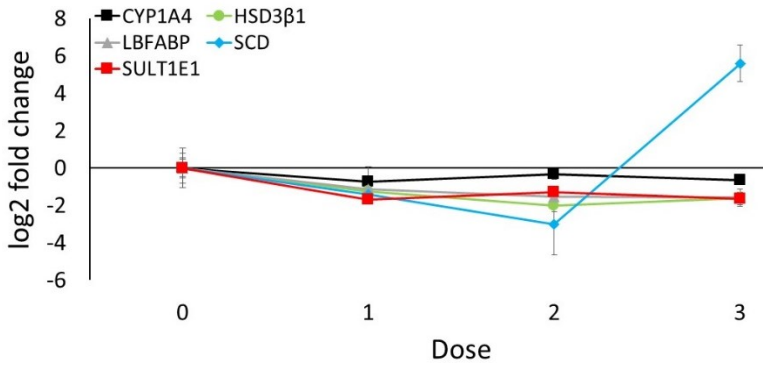
D: Method = hepatocyte; Chemical = E2

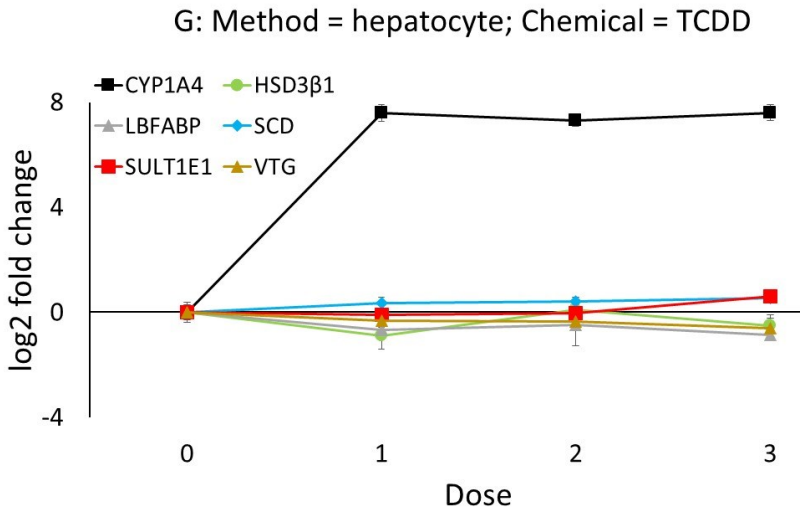


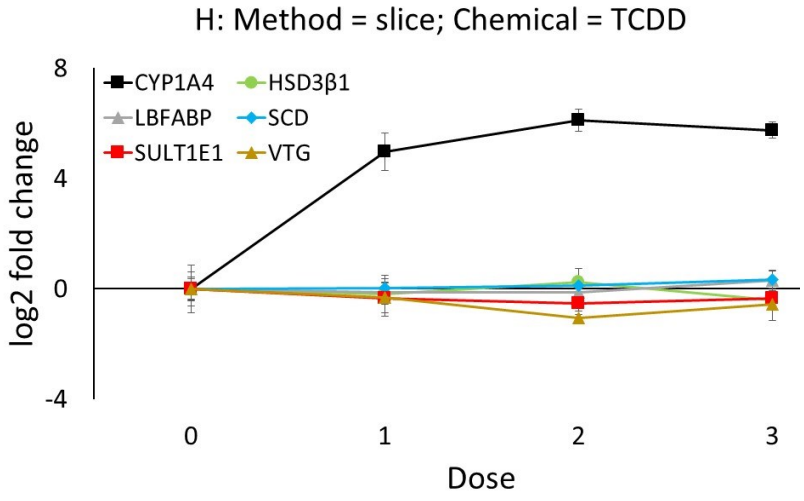
E: Method = slice; Chemical = E2



F: Method = in ovo; Chemical = E2




G: Method = hepatocyte; Chemical = TCDD
CYP1A4
HSD3β1
LBFABP
SCD
SULT1E1
VTG
log2 fold change
8
4
0
-4
0
1
2
3
Dose


H: Method = slice; Chemical = TCDD
CYP1A4
HSD3β1
LBFABP
SCD
SULT1E1
VTG
log2 fold change
8
4
0
-4
0
1
2
3
Dose

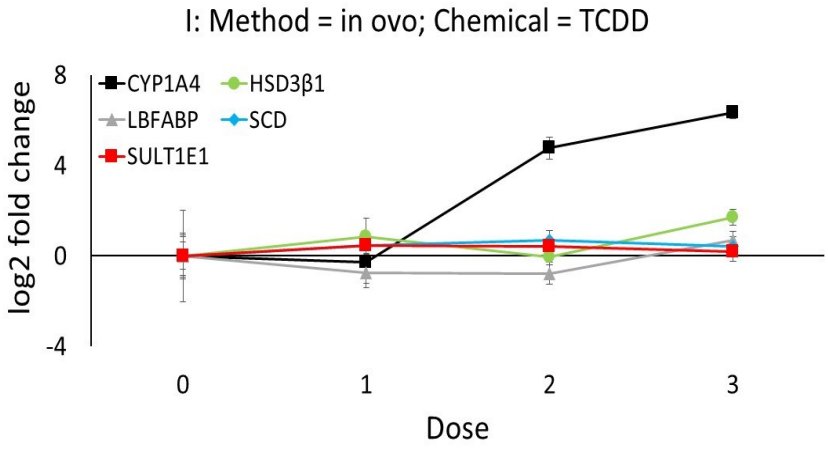
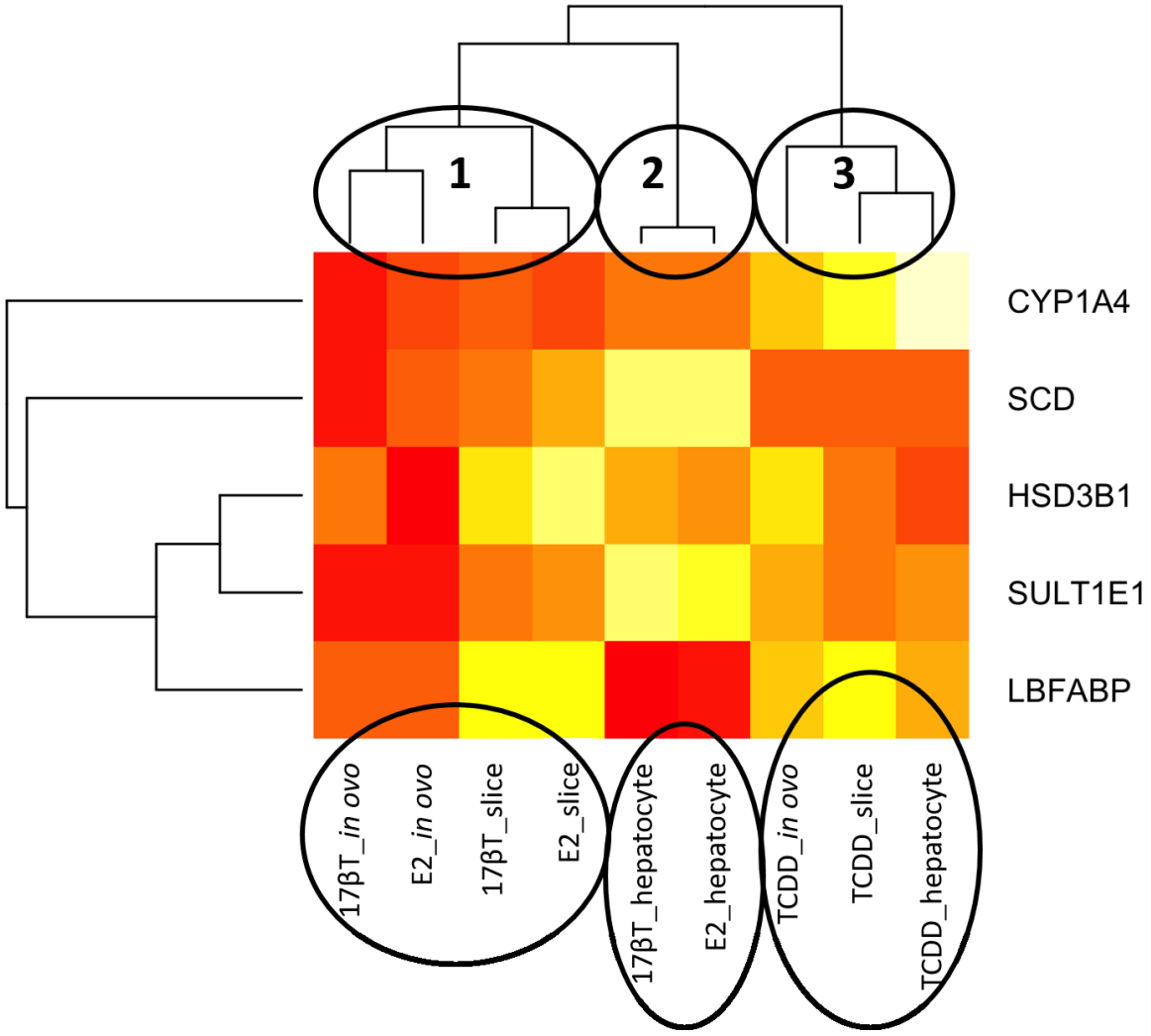

I: Method = in ovo; Chemical = TCDD
CYP1A4
HSD3β1
LBFABP
SCD
SULT1E1
log2 fold change
8
4
0
-4
0
1
2
3
Dose

Figure 4.3: Hierarchical clustering the gene expression data for alternative method*chemical vs genes are presented as a heatmap. Individual blocks in a row represent gene expression data normalized to the mean of the whole row with cells shaded in red and yellow representing lower and higher gene expression values, respectively, relative to this mean. Numbers indicate main clusters.



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Preface to Chapter 5

Chapters 2, 3, and 4 described the study of endocrine disruption in model avian species. While these chapters examined similar endpoints (such as gene expression), chapters 2 and 3 involved traditional *in vivo* methods, and chapter 4 involved alternative testing methods.

The field of toxicity testing is undergoing a paradigm shift towards alternative testing methods; however, numerous challenges still remain in the adoption and integration of alternative methods into regulatory decision making. These methods are considered to be cheaper, faster, and use far fewer animals than *in vivo* methods, however there is a lack of evidence to support these notions. This chapter attempts to study the evolving field of environmental toxicity testing by highlighting landmark events, challenges and opportunities, and synthesizing information from various data-streams on the costs associated with toxicity testing. Finally, this chapter includes select case studies to highlight potential benefits of using alternative methods vs traditional *in vivo* methods.

This chapter is authored by the candidate and coauthored by Dr. Niladri Basu, Doug Crump, and Dr. Markus Hecker. The candidate was responsible for the design of bibliometric searches, compilation and interpretation of information, discussion of results, and preparation of the manuscript and was provided advice on all aspects by the candidate's supervisor Dr. Niladri Basu. Doug Crump (ECCC) and Dr. Markus Hecker (University of Saskatchewan) provided advice and guidance on collection and compilation of information and manuscript preparation. It is planned for submission in June 2018 to the journal Environmental Science and Technology.

Chapter 5

5 Can Alternative Toxicity Testing Methods be more Efficient than Traditional Methods?

5.1 Abstract

The field of toxicity testing is evolving from whole animal or *in vivo* based testing approaches to alternative testing methods. Alternative toxicity testing involves the use of various new approach methods (*in vitro* assays, *in silico* modeling) that are said to be more advantageous than whole animal testing because they are faster, cheaper, and use fewer animals. However, much of the advancement in this field has occurred without empirical evidence to support these notions. The objectives of this article were to A) outline key events, challenges, and opportunities in the field of environmental toxicity testing, B) synthesize available information on the costs associated with toxicity testing in terms of monetary costs, testing times, and the number of animals used, and C) present select case studies that highlight the potential benefits of using alternative methods. Experts in the field were consulted, and deliberate bibliometric searches were carried out for papers and reports detailing the various costs associated with toxicity testing. We observed that landmark events such as seminal publications on toxicity testing in the 21st century (TT21C) and the increasingly global threat of chemical contamination, and international legislations on chemical testing have highlighted the need for a paradigm shift in toxicity testing. Current challenges are manifold: there are around 20,000 to 100,000 chemicals registered in countries worldwide that need toxicity testing; concomitantly, there is an urgent need for methods that are faster and more resource efficient, and that provide the type of data usable for

regulatory decision making. Rapid advancement in technologies have occurred at opportune moments and can help realize the vision of TT21C in ecotoxicology. These include high throughput sequencing, toxicogenomics, bioinformatics and tissue engineering, adoption of the adverse outcome pathway (AOP) framework along with large-scale efforts such as ToxCast and progress on the Endocrine Disruptor Screening Program (EDSP). Comparisons between whole animal methods and alternative methods indicate that the former tend to be more expensive (hundreds of thousands vs thousands), slower (months vs weeks), and use higher numbers of animals (hundreds vs tens). While the inherent differences in experimental design between traditional and alternative methods make it difficult to directly compare costs, select case studies are presented that demonstrate that there is scope for the implementation of alternative methods.

5.2 Introduction and Objectives

Seminal papers in recent years have emphasized the global threat posed by chemical contamination (1,2), with ample evidence suggesting that both intentionally produced chemicals in commerce and unintended chemical by-products are eroding the fabric and resilience of our ecosystems and humanity. Societal concerns are fueled by numerous media accounts of fish with tumors, dwindling bird populations, and contaminant-related deformities in frogs (3). The recent Lancet report on pollution and health estimated a 2% decrease in the gross domestic product (GDP) in low- to middle-income countries due to productivity losses from pollution related diseases (2). Additionally, it also estimated that annual welfare losses due to pollution likely amount to around $4.6 trillion or nearly 6.2% of the global economic output (2). The traditional approach of testing chemicals for toxicity using live animals and characterizing apical measures (e.g., survival, growth, development) that has been the mainstay since the 1920s, is in the midst

of transforming to an approach founded on a diverse range of new approach methods (NAMs) such as *in silico* modeling, *in vitro* bioassays, and toxicogenomics. "Toxicity Testing in the 21st Century – a Vision and Strategy" is a landmark 2007 U.S. National Research Council (NRC) report advocating a need to transform toxicity testing into an approach that is more predictive and resource efficient, and ultimately one that can better satisfy regulatory and societal needs (4). Since the publication of this report 10 years ago we have witnessed a rapid change in the field towards alternative toxicity testing methods (Figure 5.1). While actors in key stakeholder groups (academia, government, NGO, industry) believe that this transformation holds promise in terms of tremendous scientific, ethical, regulatory, and economic benefits, there is surprisingly little empirical evidence to support these notions.

Accordingly, the purpose of this article was to A) briefly outline key challenges, opportunities, and events in the field of environmental toxicity testing, B) collect and synthesize reports that quantify costs associated with money, time, and number of animals used in traditional and alternative approaches, and C) present select case studies that highlight the potential benefits of using alternative methods. This piece focused on ecological risk assessment but drew from the human health community as necessary. Most information was obtained through deliberate bibliometric searches that consisted of specific search terms (Table 5.1). From the papers and reports collected, a snowball approach was taken to follow referenced papers. We also consulted widely with key experts in the field. Such an introspective evaluation is timely and necessary so as to examine the validity of the idea that alternative toxicity testing can be more resource efficient than traditional methods and also highlight the key challenges and barriers of the move towards alternative toxicity testing methods.

5.3 Chemicals Challenging Regulatory Processes

International legislations mandate the assessment and reduction of risk for thousands of chemical substances used by society and released into the environment. Large-scale regulatory efforts such as the U.S. EPA's ToxCast program under the *Toxic Substances Control Act* (1976), Chemicals Management Plan (CMP) in Canada mandated by the *Canadian Environmental Protection Act* (1999), the European Union's (EU) Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) program (2007), Japan's *Chemical Substance Control Law* (1974) under the Existing and New Chemical Substances Inventory, Korea's *Toxic Chemicals Control Act* (1991) and Act of Registration and Evaluation of Chemicals or Korea-REACH (K-REACH, 2015), and China's *New Chemical Substance Notification* which was updated to China REACH (2010), have been implemented and amended in recent years to address legislative obligations to identify, prioritize and take action on chemicals found to be harmful. In addition, regulations such as Canada's Environmental Effects Monitoring program (Sect 36, *Fisheries Act*) and the EU's Water Framework Directive (2000/60/EC) mandate the risk characterization and testing of complex environmental samples (for example, municipal and industrial, effluents and sediments) for compliance with regard to their potential impacts on surface waters and fish populations and habitat. However, the evaluation of treatment and remediation efforts under these legislations and programs represent unresolved and huge challenges for stakeholders including regulators and industry. Foremost are the thousands of chemicals that are registered in commerce in various countries, and this number continues to grow annually by about 500 – 1000 (5) (Table 5.2).

Several Organization for Economic Cooperation and Development (OECD) and technical reports have estimated that countries spend billions of dollars on environmental protection expenditures pertaining to pollution abatement and control (6–8) (Table 5.2). Major concerns

associated with current testing strategies under these legislations are that they rely on extensive animal testing and on extrapolation from standard laboratory model species to native species of relevance in local ecosystems. In addition to significant ethical concerns, these practices result in prohibitive time and monetary costs, and inaccurate estimations of risk to native species. These realities represent major barriers to fulfilling legal obligations to manage chemicals and anthropogenic activities. As a result, regulatory agencies and industry are highly interested in the development and adoption of alternative, mechanistic approaches to support chemical safety evaluations and ecological risk assessments to meet new legislative mandates while reducing animal use, costs, and time required for testing.

5.4 "Toxicity Testing in the 21st Century": The First Decade

It has now been a decade since the release of the landmark TT21C paper in 2007. Fueled by challenges and events outlined above, we are starting to witness a surge of new approach methodologies (NAMs) in toxicity testing such as *in silic*o approaches, *in vitro* bioassays, and high throughput screening methods (Figure 5.2A). Table 5.3 provides a glossary of terms relevant to alternative toxicity testing approaches and programs. While much of the transformation has occurred within the human health domain (Figure 5.2B), motivated by the 2007 U.S. NRC report, an expert group of natural scientists and regulators convened a pivotal workshop in 2009 to discuss strategies to apply alternative methods to advance ecological and chemical risk assessment (9). Since then several key meetings have been held (Figure 1) and the approach that evolved from these workshops was the adverse outcome pathway (AOP). An AOP is a conceptual framework that aims to link, and ultimately predict, an apical outcome of relevance to risk assessors (e.g. disruption of reproduction or development) to a specific

molecular perturbation pattern within a biological system (10). It is believed that these transformations will vastly improve the ability of scientists and regulators to better prioritize chemicals based on their mode of action using molecular-based assays that predict apical outcomes. Adverse outcome pathways rely on mechanistic 'omics data, and biochemical endpoints downstream from key molecular initiating events (MIE) to characterize toxicity pathways. The development of standardized methods such as an exchange format for AOPs (AOP-XML) along with an OECD-endorsed regulatory reporting format for results from alternative testing methods (OHT 201) can potentially increase confidence in an AOP and thus facilitate their adoption into regulatory processes (11).

The 21st century toxicity testing framework is envisioned as a tiered testing system whereby chemicals would first be screened via suites of mechanistically based alternative assays followed by prioritizing those that showed toxic potential for follow up animal tests (4,12). Thomas et al. (13) expanded on this tiered approach and estimated that 3-15% of chemicals would be passed on for intensive animal testing. In 2010, the Euroecotox network was established to advance the development and validation of such alternative methods and promote their regulatory acceptance in ecotoxicology (14). In addition to the regulatory sector, there has been great interest in the private sector in the adoption of alternative methods that would be capable of screening the thousands of existing and emerging chemicals. For example, companies such as L'Oreal partnered with the US EPA and spent $1.2 million to validate the applicability of ToxCast to screen chemicals used in cosmetics; since 2004, Unilever's Safety and Environmental Assurance Centre (SEAC) has been investing around $3 million annually on the development and integration of animal-free testing approaches to assess consumer safety of ingredients in their products. Collectively, these programs and initiatives represent some of the

key opportunities surrounding the development and integration of alternative methods into toxicity testing.

5.5 Cost Comparisons between Traditional and Alternative Methods

In this section we display the cost differences with respect to money, number of animals used, and time needed for traditional and alternative toxicity testing. All costs mentioned are in USD. See Table 5.4 for a snapshot of the monetary cost, animals and time needed for a representative traditional and alternative test in fish (fathead minnow or zebrafish) and bird (Japanese quail). For details of cost estimates, assumptions and references see Supplementary Table 5.1.

5.5.1 Monetary Costs of Toxicity Testing

Testing the hazard potential of chemicals using animal bioassays is costly. It has been estimated that worldwide, $2.8 billion is spent annually on animal experimentation for toxicological research (15) and that it cost around $2 billion to obtain toxicity data on 300 chemicals using animal testing (16). Rovida and Hartung (17) estimated that $13.6 billion would be needed to test ~100,000 chemicals based on the REACH requirements in the EU alone, of which ~70% would be spent on reproductive toxicity testing. Vliet et al. (18) estimated that ~$3 billion is spent worldwide per year on testing chemicals for carcinogenicity; Carney et al. (19) estimated that it costs upwards of $100,000 for a chemical to undergo just developmental toxicity testing, and some reproductive toxicity assays in rodents can cost around $600,000 per chemical. Figure 5.3 compares costs of common toxicity tests including *in vivo* and alternative methods. Within the

ecological risk assessment community, costs of standard animal bioassays with standardized guidelines range from $6,000 to $411,000. In comparison cost of alternative screening assays range from $1,000 to $5,000 and some mammalian *in vitro* assays cost up to $40,000.

In addition to the costs of toxicity testing there is the tremendous commercial value of *in vitro* toxicity testing. A professional market analysis of the global *in vitro* toxicity testing market estimated it to reach $8.74 billion by 2022 (20). This presents a lucrative area for companies including Qiagen, Bio-Rad Laboratories, and Gene Logic Inc. that are some of the major players in the technological side of alternative testing, and numerous contract research organizations worldwide that are regularly hired to perform *in vitro* toxicity testing tests to screen chemicals.

5.5.2 Animal Numbers in Toxicity Testing

In 1959, Russell and Burch introduced the principles of the 3R's with respect to animal experimentation – Reduce the number of animals, Refine currently employed tests with better strategies, and Replace animals with alternative testing methods wherever possible (21). Alternative testing methods that follow the 3Rs are not necessarily expected to eliminate animal testing entirely, rather screen and prioritize chemicals for animal testing. Taylor et al. (22) estimated that the total use of animals for experimental purposes in 2005 (not including genetically modified studies or animals for tissue use) was 58.3 million from 179 countries. While this demonstrates the large numbers of animals used in testing, Rovida and Hartung (17) estimated that 54 million vertebrates would be needed to test the ~100,000 chemicals according to the REACH requirements in the EU alone.

The numbers of animals being used in toxicity testing are much greater when considering environmental monitoring and compliance needs. For example, numbers from the Canadian

Council on Animal Care (CCAC) show that 273,764 animals, including fish, mice, birds, and amphibians were used for regulatory testing alone, in 2016 (23). Specifically, about 84,000 trout are tested annually in relation to two key Canadian regulations (Metal Mining Effluent Regulations and Pulp and Paper Effluent Regulations). The compliance rate for these two regulations is 97%, essentially indicating that only 3% of these effluents displayed adverse effects in the fish. In the private sector, Shell reported that they used 88,000 fish for regulatory testing in 2014.

Based on a recent economic assessment, it was estimated that testing needs using live animals could be reduced by up to 70% by the adoption of intelligent testing strategies including *in vitro* testing and read-across techniques (24). Figure 5.4 provides estimates of the number of animals or embryos needed for *in vivo* and alternative toxicity testing. Depending on the test, *in vivo* methods, in general use between 42-350 animals (these estimates are for fish, birds, and frogs) per chemical (25). Cell-based methods (primary cell culture, tissue slice culture) or cell-free methods use around 4 to 20 animals or 12 to 320 embryos. Since embryos are nonself-feeding organisms, they are considered to be alternatives. *In silico* modeling methods, and *in vitro* cell-line based assays which have been developed for some species (HepG2, Hep3B, HepaRG, ZF4, LMH, DT40, DT95) do not use animals. While cell culture and cell-free methods may represent an over-simplified version of biological interactions in an organism and the integrity of organ tissue and biological interactions between organs are not maintained, they provide pathway based mechanistic information. Alternative methods such as tissue slice cultures can offer a better representation of whole tissue than cell cultures (26,27). In general, it is apparent that *in vitro* assays use much fewer animals than conventional *in vivo* assays. (See case studies for more information).

5.5.3 Testing Times in Toxicology

Continuing to follow the status quo in toxicity testing is simply not practical due to the backlog of chemicals that still need to be tested, and the hundreds of new chemicals that are introduced every year. It was estimated that it took 30 years to obtain toxicity data on 300 chemicals using animal tests, in comparison the ToxCast program generated data on 300 chemicals across 600 mechanistic endpoints in around five years (16). A comparison of alternative and conventional toxicity testing approaches by Settivari et al. (28) discussed that it takes months to years to test chemicals through animal-based methods, whereas days to weeks to screen chemicals through *in silico* and *in vitro* approaches. It can take ~6 months to test one chemical for developmental toxicity and 2 years for a rodent cancer bioassay (29,30). Figure 5.5 gives approximations on the time needed for standardized toxicity studies, and alternative tests. For example, the Fish Sexual Development Test (OECD 234) takes a minimum of 12 weeks; the Avian Reproduction Test (OECD 206) takes a minimum of 24 weeks while the Avian Multi-Generation Study (US EPA 890.2100) takes at least 38 weeks. In comparison, personal communication from contract research organizations, research conducted in our labs, and the *in vitro* assays within T1 of the EDSP show that it takes 2-4 weeks for a cell-based or cell-free assay for chemical screening (See case studies for more information).

5.6 Case studies

Here we briefly describe 3 case studies in which we outline costs (in terms of money, time, and animals) of some new approach methods, and aim to relate these to the status quo.

5.6.1 Endocrine Disruptor Screening Program (EDSP)

The EDSP is a tiered testing program that is also based on chemical screening and prioritization but focuses on chemicals that have the potential to affect the estrogen, androgen or the thyroid pathways. The first tier (T1) focuses on screening chemicals through a battery of *in vitro* and *in vivo* assays. Chemicals prioritized in T1 are then subject to a second tier (T2) of further animal based testing (31). The OECD (32,33) estimated that the cost of *in vitro* studies in tier 1 (T1) of the EDSP ranged from $10,000 - 42,000 per chemical. For example, the estrogen transcriptional activation assay costs on average $10,150, the androgen receptor binding assay $42,000. In comparison, the cost of the wildlife *in vivo* assays was estimated to range from $87,000 to $104,922, and the cost of the mammalian *in vivo* assays from $39,440 to 369,228 per chemical (32,33). Willett et al. estimated that it costs in between $355,100 to 964,250, takes a minimum of 520 animals and around 2 years to conduct all 11 EDSP T1 tests for a single chemical (34). While these numbers are estimates, it is clear that *in vivo* assays tend to be more resource expensive than *in vitro* assays.

Rotroff et al. (35) compared data from the ToxCast high-throughput screening (HTS) estrogen and androgen pathway data to the EDSP T1 data and found that the HTS data predicted EDSP results with 0.91 and 0.92 ($p < 0.001$) accuracy. Browne et al. (36) also examined the ToxCast estrogen pathway data and concluded that contingent on the availability of ToxCast *in vitro* data for the estrogen pathway, no EDSP T1 tests would be needed to assess the estrogenic potential of a chemical.

5.6.2 Avian ToxChip array

Recently, a custom qPCR array (ToxChip) was developed through an industrial collaboration between Environment and Climate Change Canada and Qiagen/SABiosciences. Using this chicken ToxChip, transcriptomic signatures of >16 priority flame retardants were compared in order to assist in prioritization for follow-up *in vivo* evaluations (37)**.** Primary chicken embryonic hepatocytes were dosed with these flame retardants, and expression of 32 key genes (including controls), associated with toxicologically-relevant pathways, were analyzed. Hierarchical clustering assisted in identifying those flame retardants that should be prioritized for whole animal evaluation. Livers dissected from 30, 19-day old chicken embryos yield a sufficient amount of primary hepatocytes to screen 16 organic flame retardants at 2 concentrations for biochemical, cytotoxicity and toxicogenomic end points. The data for this study were obtained within 3 weeks and cost $16,000, i.e. ~$1,000 per chemical. In comparison, performing egg injection studies for all 16 chemicals (even if exposures were performed for two chemicals at a time) would have taken around 8 months to complete and cost ~$40,000.

5.6.3 Comparison of Alternative Methods

A study conducted in our lab compared changes in gene expression in response to three chemicals at three concentrations in chicken embryos using three alternative methods: primary hepatocyte culture, tissue slice culture, *in ovo* injected embryonic liver. Twenty-four eggs were incubated for the hepatocyte culture, 12 eggs for the liver slice culture method and 98 eggs for the *in ovo* method. With respect to hepatocytes, livers dissected from 18 embryos yielded sufficient hepatocytes to screen more than three chemicals at three concentrations; with respect to tissue slices, livers dissected from four, 19-day old chicken embryo yielded sufficient liver

slices to assay three chemicals at three concentrations. In this study 12 genes were examined using custom designed qPCR arrays manufactured by Qiagen. Each method cost around $3,000 and took 4 weeks to complete; thus ~ $1,000 per chemical. For the same study design, extrapolating to 32 genes, for each chemical at three concentrations, it would cost $1,600, 96 genes would cost $3,200, and 384 genes would cost $8,500 per method.

In addition, we recently performed a time-course *in vivo* study to examine molecular and biochemical changes in Japanese quail exposed to 17βT at two concentrations. This study cost over $60,000, needed 221 birds and about 40 weeks to execute. While we were able to obtain information on gene expression, plasma hormone levels, egg production and morphometric measurements, this was expensive, and rather time and labor intensive as compared to alternative methods.

5.7 Summary

This report aimed to synthesize information from numerous data-streams to provide a glimpse of the evolving field of toxicity testing and the various costs associated with traditional and alternative toxicity testing. Globally, the existing number of chemicals that need toxicity testing ranges from 20,000 to 100,000, with around 500-1000 new chemicals introduced annually, and it is estimated that countries spend anywhere between USD 7 – 24 billion annually for pollution abatement and control. Large-scale efforts such as ToxCast, which cost ~USD 30 million, have been able to screen hundreds of chemicals through hundreds of *in vitro* assays. While most of the advances in toxicity testing has been seen in the human health domain, the development of frameworks such as the AOP and standardized reporting methods can increase their potential to be integrated into regulatory processes. Examining costs related to *in vivo* testing indicates that

the median cost of a single *in vivo* toxicity study is $118,000, it requires around 130 animals and around 20 weeks, per chemical. In comparison, triangulating from available cost estimates related to screening assays, the median cost of an alternative assay is $2,500, it would require around 20 animals or 40 embryos, and 2 weeks to test up to 400 chemicals, since typically several chemicals are tested per batch. Thomas et al. (13), recently, estimated that based on the types of alternative screening assays and the threshold for toxicity established, of all the chemicals that undergo a screening process, 3-15% would be prioritized for further *in vivo* testing. Thus, while it is quite unrealistic to think about a scenario where every existing chemical undergoes rigorous animal testing, envisioning screening assays for thousands of chemicals followed by prioritizing a subset (3-15%) of chemicals that would progress towards advanced stages of animal testing could be practical.

It is apparent that the field is evolving, however, regulatory decisions are still primarily made based on whole animal tests. To put things in perspective, one can look at the advances in the field of gene sequencing since the start of the Human Genome Project (HGP). The National Institute of Health (NIH) estimated that during the initial phases of the HGP in the late 1990s, it cost roughly $100 million to sequence one genome; two decades later, now, it costs around $1,000 to sequence a genome (38). This represents drastic advances in technology and a huge reduction in cost that one might not have thought possible at the start. However, to realize the true potential of alternative methods, further research needs to be conducted to identify and validate appropriate batteries of alternative assays for the screening process. This is truly the only way they could replace or reduce the number of whole animal tests. This doesn't mean they all have to be OECD test guidelines (as that will end up being onerous and time consuming), but

the validation piece will have to be convincing enough to get regulators and other end users on board.

5.8 Acknowledgements

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5.9 Tables and Figures

Table 5.1: List of bibliometric search terms for publications related to environmental toxicology overall, and for specific areas within environmental toxicology, in Web of Science.

Key term	Search terms
Environmental toxicology	environment* AND toxic*
Human/mammalian studies	human* OR mammal* OR rat* OR mice OR mouse OR rabbit
Fish/aquatic studies	aquatic OR fish* OR medaka OR fathead* OR trout* OR perch* OR sturgeon*
Avian/bird studies	avian OR eagle* OR gull* OR heron* OR duck* OR quail* OR chicken* OR finch* OR bird* OR turkey*
Alternative toxicity testing	alternat*
Toxicogenomics	toxicogenomic*
IVIVE	(IVIVE* OR (in vitro to in vivo extrapolation) OR ("in vitro" AND "in vivo" AND extrapolation"))
in vivo	"in vivo"
in vitro	"in vitro"
in ovo	"in ovo"
in silico	"in silico"
predictive toxicology	predictive toxicology OR "predictive toxicity" OR "predictive ecotoxicology"
AOP	AOP* OR "adverse outcome pathway" OR "adverse outcome pathways"
3R	3R* OR "Three Rs" OR "Three R"

Table 5.2: List of national chemical legislations and recent amendments, number of chemicals in inventory and annual expenditure on pollution abatement and control in US dollars (USD) and as a percentage of the national Gross Domestic Product (GDP) in 6 countries. References (6–8); Abbreviations: EU = European Union; REACH = Registration, Evaluation, Authorization and Restriction of Chemicals;

Country	Chemical Legislation	Year	Chemical Inventory	Annual expenditure on pollution abatement and control (billion USD)	% GDP
USA	Toxic Substances Control Act; ToxCast	1976; 2007	84,000	24.6 (2014)	0.14
Canada	Canadian Environmental Protection Act; Chemicals Management Plan	1999; 2006	23,000	7 (2014)	0.39
EU	REACH	2007	107,000	391 (2015)	2.39
Japan	Chemical Substance Control Law	1974; amended in 2011	27,000	12.8 (2014)	0.26
Korea	Toxic Chemicals Control Act; K-REACH	1991; 2015	42,600	4 (2014)	0.28
China	New Chemical Substance Notification; China REACH	2003; 2010	45,600	134 (2016)	1.2

Table 5.3: Glossary

Terms	Definition	Links
In vivo toxicology	Studies in which the effects of chemicals are tested on whole organisms including humans, animals and plants.	(39)
3Rs	The principles of Replacing, Reducing and/or Refining toxicity testing studies to encourage ethical uses of animals in testing	(21)
Alternative toxicity testing	Methods that follow the 3Rs principle and can replace, or reduce the use animals for testing, or refine an existing study to make it less painful or stressful for the animals.	(4)
New Approach Methodologies (NAMs)	Broadly include in silico approaches, in chemico and in vitro assays, high-throughput screening, and high-content methods e.g. genomics, proteomics, metabolomics as well as inclusion of information from the exposure of chemicals in the context of hazard assessment. They can also include some “conventional” methods that aim to improve understanding of toxic effects, either through improving toxicokinetic or toxicodynamic knowledge for substances.	(40)
In vitro toxicology	Studies in which the effects of chemicals are tested on extracted biological components from an organism including cell-free assays, cell lines, cell culture, tissue slices, organ cultures	(39)
In silico toxicology	The integration of modern computing and information technology with molecular biology to improve agency prioritization of data requirements and risk assessment of chemicals	(41)
In vitro to *in vivo* extrapolation (IVIVE)	Transposing *in vitro* effects to *in vivo* responses	(42)
Adverse Outcome Pathway (AOP)	A conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment	(10)
‘omics, toxicogenomics	Technologies that explore roles, relationships, and actions of various types of cellular molecules (genes, mRNA, proteins, or small metabolites) have been named by appending the suffix "-omics" such as genomics, transcriptomics, proteomics,	(43)

	etc. Toxicogenomics is the comparison of genes that are significantly affected in organisms that have been exposed to a drug, chemical, or toxin to those of unexposed organism	
Chemical screening and prioritization	Assessing a candidate chemical against certain toxicity criteria or considerations to classify it as a low or high-priority chemical	(44)
Computational toxicology	Branch of toxicology concerned with the development and use of computer-based models to understand and predict the interactions of biological organisms (at population, individual, cellular, and molecular levels) with pollutants in the air, water, soil and food, and their adverse health effects that they may cause.	(39)
Intelligent testing strategies	Integrated approaches comprising multiple elements aimed at speeding up the risk assessment process while reducing costs and animal tests	(24)
Predictive toxicology	Involves identifying significant perturbations of biological pathways at a molecular level through to the cellular or organ level to predict outcomes.	(45)
ToxCast	Using high-throughput screening methods and computational toxicology approaches to rank and prioritize chemicals.	(46)
Endocrine Disruptor Screening Program (EDSP)	Uses a two tiered approach to screen pesticides, chemicals, and environmental contaminants for their potential effect on estrogen, androgen and thyroid hormone systems.	(31)
Quantitative Structure Activity Relationships (QSARs)	Simplified mathematical representations of complex chemical-biological interactions that can be used to predict the physicochemical and biological properties of molecules.	(39)

Table 5.4: Comparison of cost, animals needed and duration of a fish and bird, *in vivo* and alternative toxicity test, per chemical. OECD = Organization for Economic C-operation and Development; TG = Test Guideline; ECCC = Environment and Climate Change Canada

Species	Tests	Money (USD)	# animals	Time (weeks)
Fish (Fathead minnow or Japanese medaka)	OECD TG 229 (*in vivo*)	104922	72	7
	OECD TG 210 (alternative)	5,000	240 eggs	5
Japanese quail	OECD TG 223 (*in vivo*)	120000	70	7
	ECCC *in ovo* injections (alternative)	1,200	60 eggs	4

Supplementary Table 5.1: Monetary cost (in USD), number of animals needed and durations (in weeks) of *in vivo* and alternative tests.

Trad = Traditional; Alt = Alternative; OECD = Organization for Economic Cooperation and Development;

Method	Species/ method	Assay	Guideline	Median ($)	Minimum ($)	Maximum ($)	Animals	Time (weeks)	Reference/ Assumptions
Trad	Fish	Short-term reproduction	OECD 229	104,922	46,400	150,800	72	8	32, 34
Trad	Fish	Twenty one-day assay	OECD TG 230	63,800	34,800	96,280	150	5	32
Trad	Fish	Sexual development	OECD TG 234	161,820	127,600	185,600	350	12	32
Trad	Fish	Lifecycle toxicity	EPA 850.1500	291,450	112,984	447,760	320	8	32
Trad	Fish	Multi-generation	OECD 240/EPA 890.2200	411,800	295,800	580,000	324	20	32
Trad	Fish	Short-term acute toxicity	OECD TG 203	6,000	6,000	6,000	42	2	25
Trad	Fish	Bioaccumulation	OECD TG 305	50,000	50,000	50,000	70	6	25
Trad	Avian	Reproduction	OECD TG 206	116,000	116,000	116,000	120	24	32
Trad	Avian	Multi-generation	EPA 890.21	319,000	319,000	319,000	240	38	32
Trad	Avian	Acute oral toxicity	OECD TG 223	120,000	120,000	120,000	70	6	17
Trad	Amphibian	Metamorphosis	USEPA 890.1100/OECD TG 231	87,000	58,000	111,360	320	5	32, 34
Trad	Amphibian	Growth and development	OECD TG 241	250,560	58,000	443,120	80	20	32
Alt	Cell-free	Receptor binding/ enzyme activity	400 chemicals X 1 endpoint	2,500	2,200	3,000	20	2	20 chemicals/96-well plate; 20 plates X 15$ = $300; Isotope = $500; Personnel = ~$1,500; Misc = $200.
Alt	Cell-line	Hepato/cardio/cytotoxicity	Per endpoint	2,500	1,200	4,000	0	3	Personal communiation with CROs
Alt	Fish	Early-life stage toxicity	OECD 210	5,000	4,000	6,500	320	4	Estimates based on guidelines
Alt	Fish	Embryo acute toxicity	OECD 236	3,000	2,000	4,000	320	4	Estimates based on guidelines
Alt	Avian	Primary hepatocyte culture	3 conc X 27 genes	1,000	700	1,200	12	4	For 16 chemicals; ToxChip = $6,000; SYBR = $3,000; RNA isolation and RT = $2,100; Personnel = ~$4,500; Misc = $500
Alt	Avian	Liver slice culture	3 conc X 7 genes	1,000	900	1,300	4	2	For 3 chemicals; ToxChip = $600; SYBR = $300; RNA isolation and RT = $700; Personnel = ~$1,400; Misc = $200.
Alt	Avian	Early-life stage toxicity	3 conc X 7 genes	1,200	1,000	1,500	40	4	For 3 chemicals, ToxChip = $600; SYBR = $300; RNA isolation and RT = $700; Personnel = ~$1,800 ; Misc = $200.

Figure 5.1: Timeline of toxicity testing from the 1920s to the present. Abbreviations - ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods; EDSP: Endocrine Disruptor Screening Program; NRC: National Research Council; TT21C: Toxicity Testing in the 21st Century; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals; EU: European Union; SETAC: Society of Environmental Toxicology and Chemistry; AOP: Adverse Outcome Pathway; OECD: Organisation for Economic Co-operation and Development.

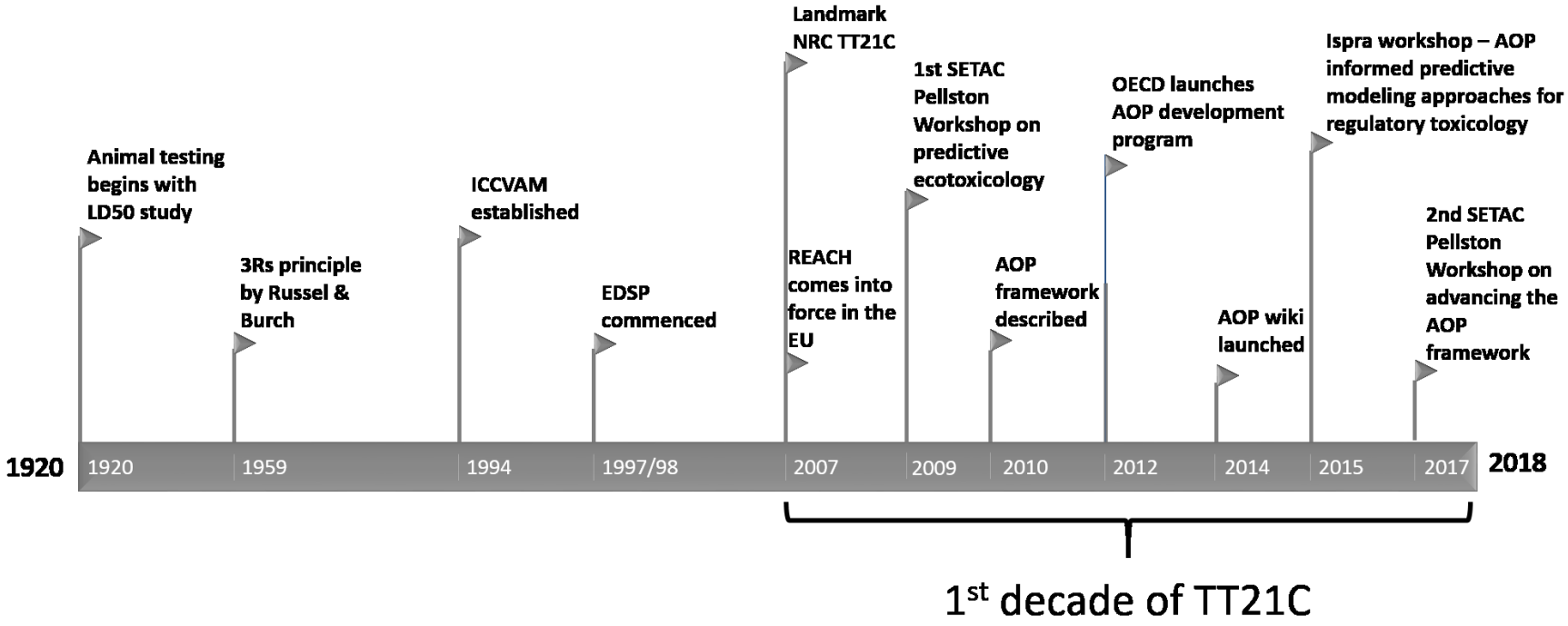
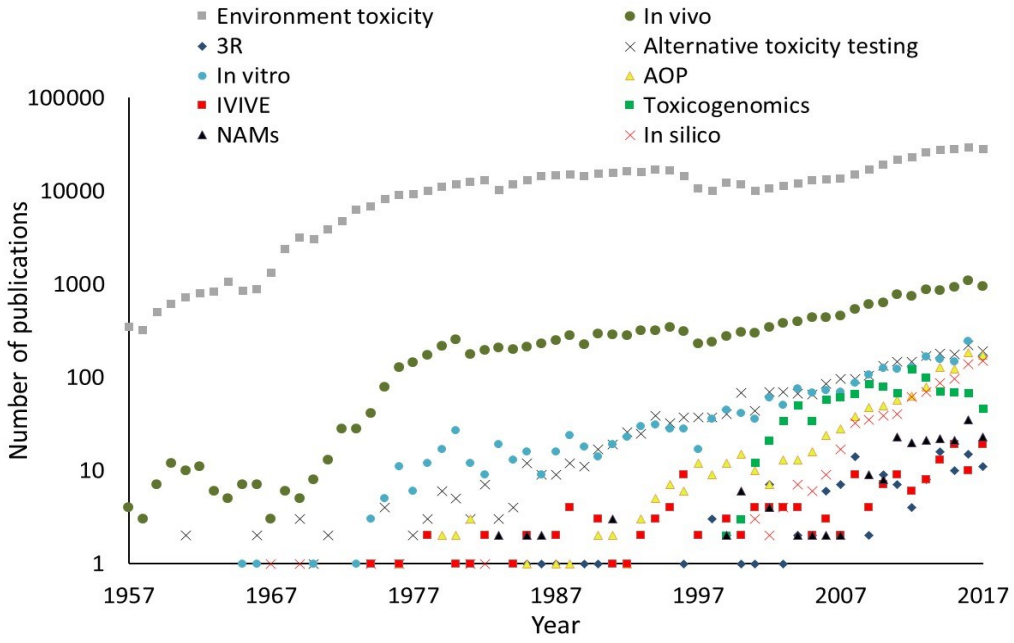


Figure 5.2: Graphical representation of bibliometric search in Web of Science using search words detailed in Table 5.1. A search was performed for the overall field of environmental toxicology. Results within this search were further refined with key words related to A) the toxicity testing field, and B) human/mammalian, fish or avian species.

A)



B)

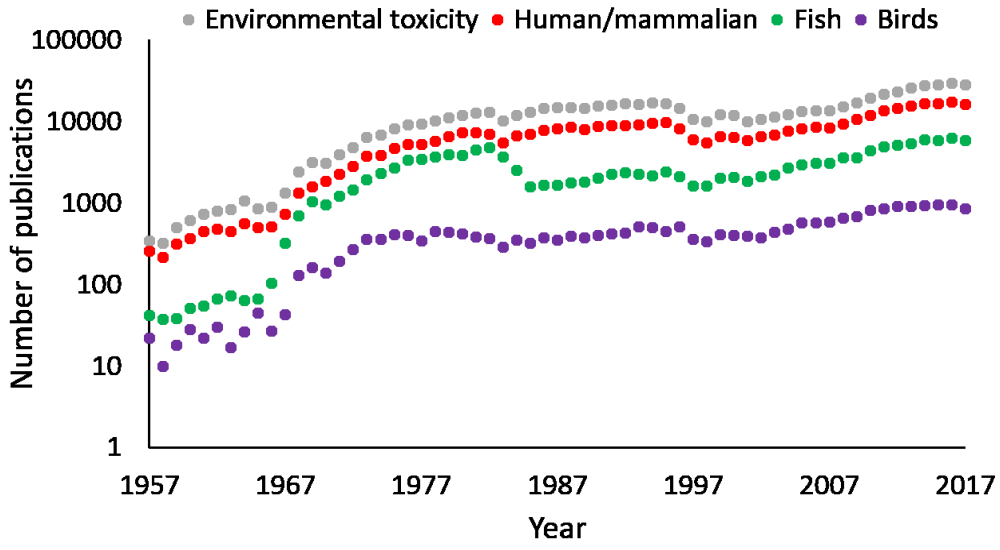


Figure 5.3: Monetary costs associated with traditional and alternative toxicity tests. Where possible, data are presented as median and the range. Dash = median; left vertical bar = minimum; right vertical bar = maximum. For further details on the tests, costs and references please see Supplementary Table 5.1.

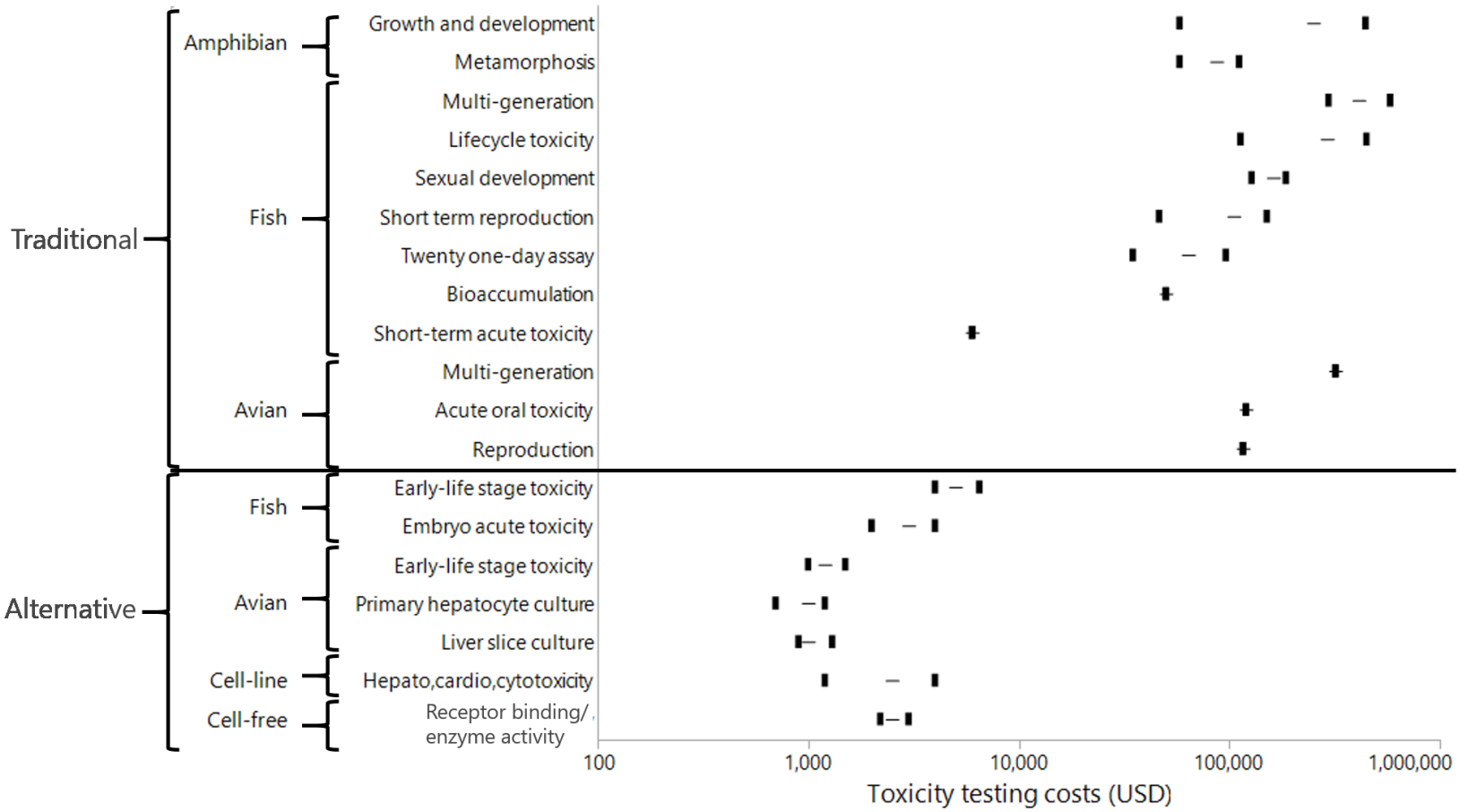


Figure 5.4: Number of adults or embryos needed for traditional and alternative toxicity testing. For further details on the tests and references please see Supplementary Table 5.1.

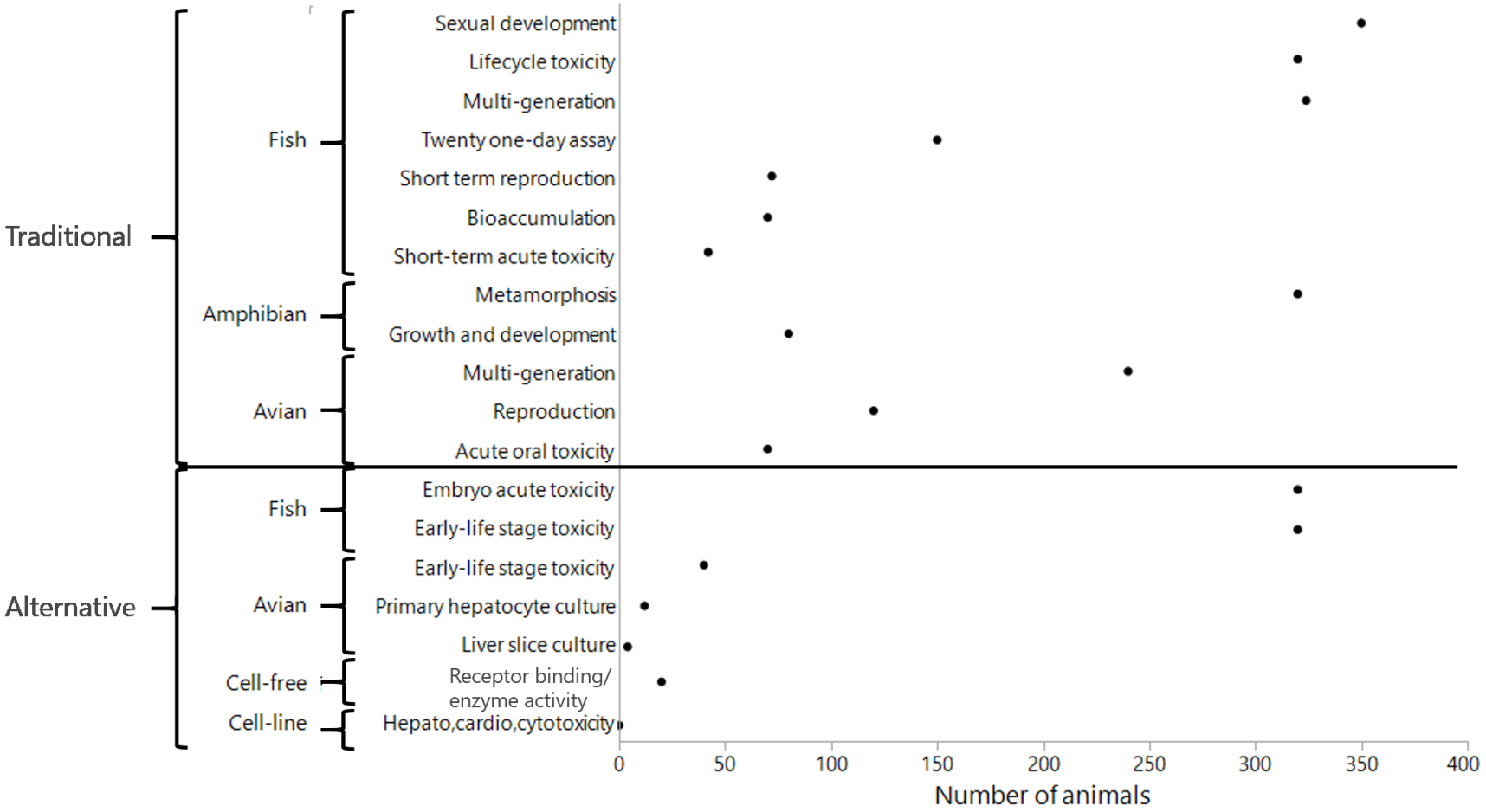
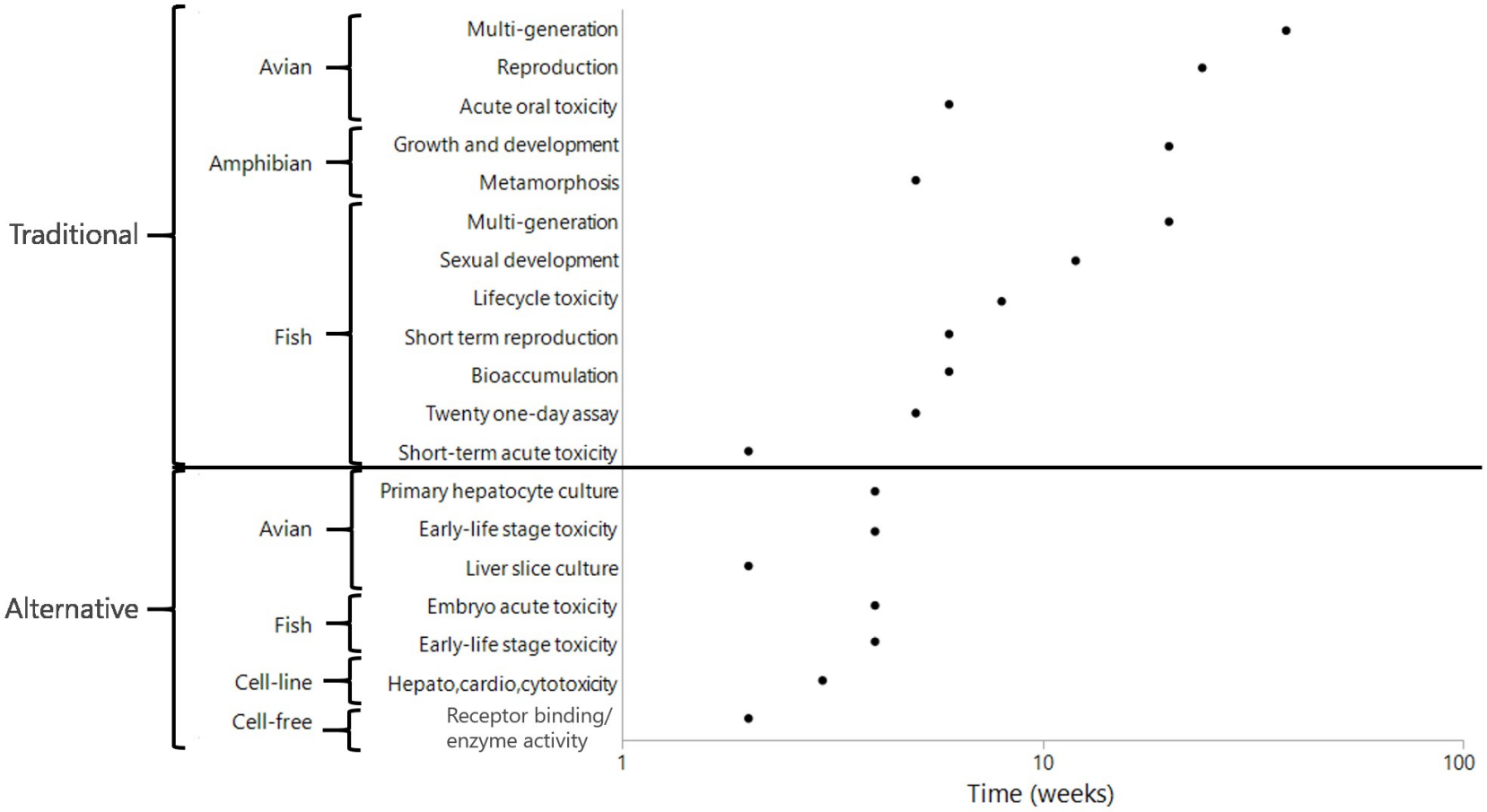


Figure 5.5: Time needed for traditional and alternative toxicity testing. For further details on the tests and references please see Supplementary Table 5.1.



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Chapter 6

6. Summary and Conclusions

The past few decades have witnessed increasing societal awareness that widespread environmental contaminants can exert sub-lethal effects, including deleterious impacts on the endocrine system, in birds, fish, and wildlife. Chemical toxicity testing is an integral aspect of environmental hazard determination and risk assessment. Among the chemicals known to cause adverse effects are endocrine disrupting chemicals (EDCs) that can affect the endocrine system and biological processes such as reproduction and development thus ultimately having population level impacts. The results outlined in this thesis examine various methods involved in toxicity testing and their applicability in studying molecular (gene expression) and biochemical (plasma hormone levels) endpoints in model avian species as a result of exposure to a model EDC.

6.1 Contribution to Knowledge

In this thesis, I aimed to use traditional *in vivo* toxicity testing methods to advance knowledge on the adverse effects of 17β-trenbolone (17βT), an androgenic EDC, on molecular and biochemical endpoints in target tissues and egg production. I examined this in a deeper manner than what is called for in standard toxicity tests, i.e., across sex and developmental stage, from a long-term exposure (Chapter 2) and over the course of a short-term exposure (Chapter 3) in Japanese quail (JQ). Additionally, I compared three alternative methods of toxicity testing to better understand

the differences between these methods (Chapter 4) and examine whether alternative testing can be more advantageous in terms of resources used than traditional testing (Chapter 5).

Overall, this thesis fills knowledge gaps in the use of alternative methods and endocrine disruption with regards to avian toxicology. To the best of our knowledge, this is the first study to examine sex- and developmental stage- related differences in the global hepatic transcriptome of avian species exposed to a model EDC. Additionally, this thesis identified significantly enriched biological pathways that could be affected by differentially expressed genes. This is also the first time-course study to examine negative impacts of short-term exposure of 17βT on the JQ endocrine system. We also demonstrated the use a liver slice culture in avian toxicology and performed a comparison of various alternative methods in an avian species. Furthermore, this study provided a much-needed examination of the field of environmental toxicology and comparison on the resource associated costs and time needed for toxicity testing.

6.2 Summary of Results

In chapter 2, we examined global hepatic gene expression using RNA Sequencing in JQ exposed to *in ovo* and dietary (17 weeks) 17βT and looked at differences related to sex (male vs female) and developmental stage (embryo vs adult). The number of SDE genes in the male embryo (724) was higher than in the female embryo (373); the number of genes in the male embryo (724) was higher than in the male adult (85). Developing embryos can be more vulnerable than adults, possibly explaining why more genes were differentially expressed in that stage. Examining the SDE genes across males and females revealed a few sexually dimorphic genes, suggesting that aside from the differences in the genes themselves, the genes that overlap between the sexes might also be affected differentially. Vitellogenin (VTG) and apolipoprotein were found to be

differentially expressed only in the adult males. Pathway enrichment analyses showed that many of the pathways in female embryos were related to lipid and carbohydrate metabolism and muscle contraction. In the male embryos, many of the pathways seemed to be related to cell proliferation, cell differentiation and signal transduction. These findings are similar to results from previous studies examining 17βT exposure in fish and cattle. The function of 17βT as a growth promoter and our observations suggest the involvement of pathways beyond reproductive functions, such as cell proliferation, muscle development, tissue structure and function.

Chapter 3 greatly expands upon previous studies on the effects of 17βT on the avian endocrine system not only by investigating effects at multiple time-points but also by investigating effects from a short-term exposure – during the first 3 weeks of exposure. We observed differing changes in plasma concentrations in male and female JQ that were similar to results in previous time-course studies in fathead minnow (FHM). The main effect in males was the increase in plasma levels of E2, while in the females changes in plasma T, E2 and Zn (as a proxy for VTG) fluctuated over the course of the exposure; initial effects seen may have been in direct response to the exogenous androgenic stressor while latter changes may be a compensatory response. Analyses of the expression of genes related to these biochemical endpoints revealed no changes in the 17βT treated birds compared to the controls. The interactions of steroids and steroid mimics have been shown to extend beyond AR and ER, to numerous signaling pathways, non-nuclear receptors and steroid metabolizing enzymes. Thus, it is possible that changes in individual gene expression alone may not be sufficient to explain the differences observed in biochemical endpoints.

In chapter 4, we compared changes in gene expression in three alternative methods of toxicity testing (hepatocyte culture, liver slice culture and the *in ovo* exposed liver) using a

model avian species upon exposure to three chemicals. . Hierarchical clustering of the gene expression results indicated that the slice cultures and *in ovo* results were grouped to be more similar to each other and that hepatocytes were more different from the *in ovo* liver. This could be explained in that liver slices have been demonstrated to be more representative of the intact organ than cell culture, and this similarity has also been demonstrated with rat liver tissue in previous studies. However, no clear pattern could be observed across the three methods for all the genes analyzed. The differing results in gene expression emphasize the challenges posed by factors such as chemical toxicokinetics, duration of exposure, species, and toxicity pathways in the use of alternative testing methods. Additionally, while we were able to study changes in expression of some relevant genes, these cover a limited set of biological functions.

In chapter 5, we aimed to examine the evolving field of toxicity testing by highlighting events, challenges and opportunities surrounding the paradigm shift from animal based testing to alternative testing. This chapter also aimed to synthesize information from various data-streams on the monetary costs, testing times and number of animals associated with conventional and alternative toxicity testing, and presents select case studies to show potential benefits of alternative methods. Globally, the thousands of existing and emerging chemicals lacking toxicity information in addition to amended regulatory requirements to introduce these chemicals in commerce present some of the challenges associated with toxicity testing. Large-scale efforts such as ToxCast, have been able to screen thousands of chemicals through hundreds of *in vitro* assays. Development of frameworks such as the adverse outcome pathway (AOP) and standardized reporting methods can aid in their integration into regulatory processes. Examining costs related to *in vivo* testing indicates that the median cost of a single *in vivo* toxicity study is $118,000, it requires around 130 animals (estimates are based on bird, fish and frog studies) and

around 20 weeks, per chemical. It is quite unrealistic to think about a scenario where every existing chemical undergoes rigorous animal testing. In comparison, triangulating from available estimates, the median cost of an alternative assay is $2,500, it would require around 10 animals or 40 embryos, and 2 weeks for around 20 chemicals. Thus, based on estimates that 3-15% of all the chemicals that undergo a screening process would be prioritized for further *in vivo* testing, envisioning screening assays for thousands of chemicals followed by prioritizing a subset of chemicals that would progress towards advanced stages of animal testing could be practical.

6.3 Discussion

6.3.1 17β-trenbolone

The main sources of 17β-trenbolone (17βT) in the environment are solid dung in livestock farms where the cattle have been prescribed 17βT implants, feedlot runoffs, and soil samples from fields receiving liquid manure as fertilizer [1]. There are no accurate numbers on trenbolone usage published, but in USA, it was estimated that annual production is around 5000 kg, and 60-90% of all cattle receive trenbolone implants [2]. While there are no reports on the body burden of 17βT in wild birds, previous studies measuring 17βT concentrations in dung and soil found that levels ranged from $1*10^{-6}$ ppm to 0.0043 ppm [3]. For mechanistic laboratory studies, concentrations of test chemicals used are often higher than what is considered to be environmentally relevant, to elicit measurable responses. The levels tested in this thesis ranged from 1 ppm to 20 ppm, to allow a deeper understanding of mechanisms of action in birds, and aid in developing alternative methods. For Chapter 2, 1 and 10 ppm were chosen to represent a low and high dose. For Chapter 3, we considered that 1 ppm might be too low and upon deliberation decided to use 5 and 20 ppm as the low and high doses; these concentrations were

chosen from the previous multi-generation study that tested concentrations ranging from 1 ppm to 50 ppm [4].

17β-trenbolone is a model androgenic chemical that is structurally similar to testosterone and has a high affinity for the AR, and does not get aromatized to estrogenic metabolites [5]. The studies conducted in this thesis showed that 17βT can exert molecular and biochemical level effects in birds. Changes were observed at the transcriptomic level that do not appear to be directly related to AR signaling. Additionally, plasma levels of relevant hormones were also affected form short term exposure, however, the selected genes known to be directly involved in these hormonal pathways did not exhibit significant changes in expression. While there have only been a small number of studies in birds, extensive research has been conducted on the disruptive effects of 17βT in other species, such as fish and cattle [6]. These studies showed similar results in that pathways that were affected at the molecular level could not be directly connected to AR signaling [7–9]. Collectively, these observations indicate that disruption at the biochemical level may be occurring due to transcriptomic changes that are not directly related to endocrine function, rather to processes such as metabolism, homeostasis and transport.

Whether changes seen in this thesis will lead to effects at an organismal level is not yet clear. The development of frameworks such as the AOP provide a basis for linking molecular events to changes in the individual and population [10,11]. Since the pathways being affected appeared to be involved in key functions such as metabolism, cell proliferation and steroidogenesis, it is possible that these could result in adverse effects on growth and reproduction, which could thus have population level impacts. Thus, our results imply that at the concentrations used in this thesis birds may be at risk of endocrine disruption by 17βT, however,

to determine potential effects at environmentally relevant concentrations, further research on wild bird exposure may be needed.

6.3.2 Birds Under Threat

A 1997 paper estimated that there were about 200-400 billion birds in the world [12], however, in the last few decades we have witnessed a substantial decrease in many bird populations. In Canada alone, breeding bird populations have been estimated to have declined by around 12% since 1970 [13]. The 2018 State of the World's Birds report provides further numbers on the global decline of bird populations [14]. While the exact reasons for these declines are often unknown, various factors have been considered as causes for population declines including decrease in insect numbers, loss in habitat, climate change, and chemical use [14].

The need to examine the effects of chemical contamination on avian species is underscored by their importance in the environment. In addition to their ecological relevance, birds are also key bioindicator species; past reports on the effects of pesticides on avian populations were instrumental in bringing the topic of endocrine disruption to the forefront [15,16]. However, results from bibliometric searches have shown that much of the endocrine disruption research in recent years has focused on human health and aquatic species, perhaps due to the increased importance and awareness associated with human health, and the commercial value of fisheries. Thus, technological advances and tools that have been developed for these species are lacking in avian toxicology. Collectively, changes in the endocrine system observed in this thesis due to 17βT exposure, the global decline in bird population, and the value of avian species in detecting future cases of chemical contamination imply that further research on toxicity testing in avian species, is warranted.

6.3.3 Toxicity Testing Methods

Long-established strategies of animal-based toxicity testing that have been in place since the 1920s are giving way for the development of numerous alternative strategies based on the 3R principles of replacing, reducing, and refining methods [17]. This shift was precipitated by the publication of the seminal 2007 NRC report (Toxicity testing in the 21st century: A vision and strategy), and the realization that animal-based methods are inefficient, logistically unfeasible, and often don't account for developmental stage and duration of exposure [18]. Additionally, animal-based methods may examine apical outcomes without examining mechanistic endpoints that could anchor these effects [19].

The *in vivo* studies in this thesis indicated the flaws in current toxicity testing methods by highlighting the importance of accounting for factors such as sex and developmental stage that could influence results observed due to chemical exposure [20–22]. Results from chapter 2 showed that early-life stages may be more vulnerable to effects of exogenous stress than adults. Ankley and Villeneuve [23] discussed temporal changes in molecular and biochemical endpoints and the uncertainty associated while studying endocrine disruption, and underscored the need to include multiple time-points during early stages of exposure. The fluctuations in plasma hormone levels observed with short-term exposure in chapter 3 demonstrate the dynamic nature of biomarkers such as hormone production. Furthermore, differences seen in results from short-term exposures found in this thesis and previous studies investigating long-term exposures indicate that mechanistic effects may be better observed from short-term exposures, while apical endpoints such as egg production maybe better examined from long-term exposures. Thus, the findings from this thesis show that standard toxicity tests that examine effects in a single gender

or at a single time-point after an arbitrary period of exposure could preclude us from obtaining relevant information needed to better understand the mechanisms of action.

This thesis also compared changes in gene expression among three alternative toxicity testing methods. Including an *in ovo* injection method enabled us to compare *in vitro* methods to the whole organism, while still employing alternative methods. The *in ovo* injection method could itself prove to be valuable as an alternative to *in vivo* methods as it both represents the whole organism and is more complex than isolated cells and tissues. The equivalent in aquatic toxicology which is the zebrafish early life stage (ELS) and the fish embryo acute toxicity test (FET) have been established as methods with standardized testing guidelines [24,25]. While the egg injection method is becoming more common, it has not been adopted as a standardized test.

Among the *in vitro* methods, higher similarity between liver slices and liver from *in ovo* injected embryo displayed the potential of this *in vitro* method as an alternative to animal-based toxicity testing. This similarity between slices and tissues from whole animals has also been demonstrated in biomedical studies using rat liver [26,27]. Thus, we were able to develop liver slice cultures for avian species for the first time and show that they may be of great use in toxicity studies. Interestingly, we were unable to determine a consistent pattern across the three methods for all the genes. It is possible that the number of genes chosen here were not high enough to draw a complete comparison among the three methods. Rather, comparing the transcriptomic profile or a comprehensive set of genes across the methods might enable us to better understand the differences. Our results indicate that in the absence of a best alternative method, perhaps, no single method is likely to be effective as an alternative to *in vivo* studies, rather a carefully validated suite of methods maybe a better approach [28].

The use of toxicogenomics in chemical screening is increasingly becoming attractive due to the possibility of determining transcriptomic signatures that could be linked to toxicity pathways [29]. These could be examined during early stages of exposure rather than having to examining apical outcomes from long term exposure studies, thus reducing stress on the animals [30]. The chapters in this thesis looked at toxicogenomics as a main endpoint using different methods – qPCR for a specific set of genes either individually or with arrays, or RNA-seq to examine the entire transcriptomic profile. RNA-seq may not always be economically feasible, and the intensive data analysis pipeline requires advanced computational and bioinformatics skill sets that could be prohibitive [31]. Studies using qPCR, while not requiring intensive bioinformatics, typically measure only a few select genes. Custom qPCR arrays, while also measuring select genes, can make use of high throughput screening methods and be designed to include a large number of genes [32]. Our use of RNA-seq, qPCR for individual genes, and a custom designed qPCR array, suggest that a good balance could be to use qPCR arrays for a comprehensive set of genes that have been carefully chosen to cover a wide network of toxicity pathways. Indeed, custom designed qPCR arrays can be ordered based on specific pathways of interest. However, these are mostly catered towards biomedical species, such as mice, rats, and rabbits. Currently, research is underway to design 384-well qPCR arrays covering relevant biological pathways in a model, and ecologically relevant amphibian, aquatic and avian species.

In addition to the laboratory studies, this thesis examined the feasibility of traditional vs alternative toxicity testing methods in terms of the resources needed. The information collected indicated that the current toxicity testing methods would not be able to sustain the ever-increasing demands of chemical testing [33,34]. Based on estimates of the percentage of chemicals that may have the potential to cause adverse effects [35], a more realistic scenario is to

exploit technological advances to design and validate alternative strategies. The advantages of these strategies will likely be realized in the efficient utilization of the available resources to screen and prioritize the chemicals for further animal testing.

6.3.4 Biological Understanding and Regulatory Acceptance

Alternative toxicity testing methods developed to aid in chemical screening and prioritization such as those that were examined in this thesis, need to provide biologically relevant results to be incorporated into regulatory decision making [36,37].

Among the major challenges associated with the biological relevance of *in vitro* and toxicogenomic methods, one is determining what the changes seen in cells and tissues mean in terms of the whole organism [38]. Liver slices are considered to be more representative of intact tissue, since they can retain cellular interactions better than isolated hepatocytes [39,40]. Accordingly, our results demonstrated higher similarity between the slices and *in ovo* liver. However, despite these similarities, drawing direct comparisons between results from various methods may not be accurate [41]. Differences in toxicokinetics likely plays a major factor in the differing effects observed upon chemical exposure; since *in vitro* methods do not account for metabolism, exposure in the target tissues in these methods is not the same as that in target tissues in a whole organism [42]. This precludes us from drawing direct gene-to-gene comparisons and thus further complicates extrapolating *in vitro* results to the whole organism [26]. Improvements in toxicokinetic models that can more accurately estimate the exposure in *in vitro* methods can aid in extrapolating these results in a more biologically relevant manner.

Second, gene expression is a key endpoint of alternative testing methods, however, changes in gene expression may not always correspond to changes in protein expression, and

downstream effects [30]. In this thesis, changes seen in plasma hormone concentrations were not explained by significant changes in gene expression; however, only a few select genes were examined. The highly transitory nature of gene expression further emphasizes the need to perform transcriptome wide analyses. Additionally, in pathways where there are no prominently defined effects on specific genes, including other molecular and biochemical endpoints could increase our biological understanding of mechanistic effects [28]. For example, cell-based assays such as radiolabeled receptor binding assays for estrogen and androgen receptor, and enzyme activity assays to measure aromatase activity could also be incorporated.

In addition to the challenges associated with incorporating alternative methods into regulatory decision making, obtaining good quality data that is reproducible across laboratories is important [43]. Various quality control measures were included in the experiments performed in this thesis. For example, the control and treated feed provided to quails were tested to determine 17βT levels. For the molecular biology techniques such as RNA isolation, reverse transcription and qPCR, standard steps such as RNA gels to check for quality, primer validation using BLAST, standard curves for primer efficiency, genomic DNA control, positive PCR control, melt curves, and reference genes were included. Among the several differential gene expression analysis packages for RNA-Seq data, a study determined that EdgeR had the highest sensitivity and specificity, and advised using individual RNA samples rather than pooling [44]. In chapter 2, since we used the EdgeR package and individual samples, and examined the transcriptomic profile in JQ liver to look at sex- and developmental stage-related differences, qPCR analysis was not included to validate the RNA-seq data at present. However, future qPCR analysis will be performed on hepatic genes in samples from this study. Additionally, aside from the analysis performed here using NetworkAnalyst, preliminary analysis of the RNA-seq data

were independently performed in R and obtained similar results. Thus, ensuring that the reproducible and high quality can enable comparisons of studies across laboratories and aid in validating results from different methods.

The present toxicity testing system needs to be improved such that we can make better use of the resulting toxicity information [45]. Frameworks such as the AOP can be useful here and toxicogenomic data may play a vital role in identifying molecular initiating events [46]. Future research could perform transcriptomic analysis of the target tissues from these studies and include additional end points such as receptor binding and enzyme activity. This could further aid in developing AOPs and better understanding the data in a biological context. In a discussion on *in vivo* methods and the applicability of *in vitro* methods for regulatory purposes, Hartung & Daston [47] stated, "neither approach is useless, but only fully useful in the right regulatory framework". They concluded that for alternative testing to be used effectively, it is not sufficient to develop them as individual tests, rather, to identify and incorporate relevant endpoints into integrated testing strategies. The collective results from the various exposure methods and endpoints measured in this thesis serve well to reiterate these points and provide ample justification for future large-scale collaborative research into the development of effective tools that could be incorporated into regulatory decision making.

6.4 Concluding Remarks

This doctoral thesis performed laboratory studies to advance knowledge on the effects of various factors such as sex, developmental stage and duration of exposure in traditional, and on differences across alternative methods by examining effects on endpoints associated with the

endocrine system, in model avian species. Ultimately it aimed to improve our understanding on traditional and alternative methods and on negative impacts of 17βT, a model EDC, on the avian endocrine system. The environmental load of chemicals has been increasing concomitant with widespread awareness of the potential of several chemicals to cause adverse effects on the ecosystem. This has led to a call for toxicity testing strategies that make use of technological advancements and available resources in an efficient manner.

Given the widespread nature of EDCs and the ability to affect processes beyond reproductive function, further large-scale efforts examining the use of alternative strategies to screen chemicals for endocrine disrupting potential are justified. In conclusion, studies such as those outlined in this thesis can aid in improving the status of global chemical contamination and thus overall ecosystem health.

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