*In ovo E*ffects of Tris(1-chloro-2-propyl) phosphate (TCPP) and Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) Flame Retardants on Chicken Embryo Toxicity and Gene Expression

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Abstract

Tris(1-chloro-2-propyl) phosphate (TCPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are added to polyurethane foams in a variety of industrial and consumer products to prevent flame ignition. The gradual release of these flame retardants (FRs) from such products leads to contamination of various abiotic and biotic media, including wild birds. Recent studies demonstrated endocrine-disrupting effects of TCPP and TDCPP, including alteration of circulating thyroid hormone (TH) levels. The TH-pathway is essential for normal growth and development in birds. There are limited data on the toxicological effects of TCPP and TDCPP in avian species and, prior to this work, no study has examined their effects in avian embryos.

This M.Sc. thesis investigates the developmental, molecular and biochemical effects of TCPP and TDCPP in chicken (*Gallus gallus domesticus*) embryos via egg injection studies. TCPP delayed pipping at doses ≥9.24 µg/g, both TCPP and TDCPP reduced embryo growth at the highest dose (51.6 µg TCPP/g and 45 µg TDCPP/g), and TDCPP decreased free plasma thyroxine and gallbladder size at 7.64 µg/g and 45 µg/g, respectively. Real-time reverse transcription polymerase chain reaction was used to measure changes in mRNA levels of hepatic genes that were responsive to these FRs in a previous *in vitro* study. TCPP dysregulated the expression of TH-responsive genes and xenobiotic metabolizing enzymes (cytochrome P450s; CYPs), whereas TDCPP only affected CYPs. Less than 1% of the administered TCPP or TDCPP was

detected in egg contents following 19 days of incubation, indicating extensive metabolism of the parent compounds.

DNA microarrays were used to perform a global transcriptional analysis on liver samples from embryos that exhibited adverse effects following TDCPP injection. 47 differentially expressed genes were identified at the 45 µg/g dose. Functional analysis revealed that immune function and lipid and steroid metabolism were major targets of TDCPP toxicity and indicated a state of cholestatic liver/biliary fibrosis. Since the TH-pathway is a key regulator of metabolic homeostasis, its disruption early in development is a potential cause of the observed adverse effects. This thesis demonstrates, for the first time, developmental and endocrine-disrupting effects of TCPP and TDCPP in an avian species and attempts to link phenotypic changes to molecular-level disruptions in hopes to improve the understanding of their modes of action.

Résumé

Le tris(1-chloro-2-propyl) phosphate (TCPP) et le tris (1,3-dichloro-2-propyl) phosphate (TDCPP) sont ajoutés à la mousse de polyuréthane dans de nombreux produits industriels et de consommation en vue de les rendre ininflammables. Le rejet graduel de ces ignifugeants de tels produits contamine divers milieux abiotiques et biotiques, notamment l'avifaune. De récentes études ont démontré les effets perturbateurs du système endocrinien du TCPP et du TDCPP, y compris la modification des taux d'hormones thyroïdiennes circulantes. La circulation des hormones thyroïdiennes est essentielle à la croissance et au développement normaux des oiseaux. Il existe très peu de données toxicologiques sur les effets du TCPP et du TDCPP sur les espèces aviaires. De plus, la présente étude est la première à se pencher sur les effets de ces ignifugeants sur les embryons d'oiseaux.

La présente thèse de maîtrise ès sciences examine le développement moléculaire et les effets biochimiques du TCPP et du TDCPP dans les embryons du poulet (*Gallus gallus domesticus*) au moyen de l'étude d'œufs injectés. À des doses supérieures ou égales à 9,24 µg/g, le TCPP retarde le bêchage. Le TCPP et le TDCPP, à leur plus forte dose respective de 51,6 µg/g et de 45 µg/g, réduisent la croissance des embryons. Le TDCPP à une dose de 7,64 µg/g réduit les niveaux de thyroxine libre dans le plasma et, à une dose de 45 µg/g, réduit la taille de la vésicule biliaire. La méthode PCR-CDNA en temps réel a été utilisée pour mesurer les changements dans les niveaux d'ARN messager des gènes hépatiques qui ont réagi à ces ignifugeants lors d'une étude *in vitro* antérieure.

Le TCPP perturbe la régulation de l'expression des gènes sensibles aux hormones thyroïdiennes et des enzymes du métabolisme xénobiotique (cytochrome P450), alors que le TDCPP n'a un effet que sur les cytochromes. Après 19 jours d'incubation, moins de 1 % des doses injectées du TCPP et du TDCPP ont été détectées dans le contenu des œufs, ce qui indique une forte métabolisation des composés d'origine.

Des micropuces à ADN ont été utilisées pour réaliser les analyses transcriptionnelles globales des échantillons de foie des embryons ayant subi des effets nocifs à la suite d'une injection de TDCPP. À une dose de 45 µg/g, 47 gènes exprimés différemment sont identifiés. Les analyses fonctionnelles montrent que les fonctions immunitaires et les métabolismes stéroïdien et lipidique sont particulièrement sensibles à la toxicité du TDCPP et révèlent la présence d'une cholestase par une fibrose hépatique/biliaire. Compte tenu de l'importance de la circulation des hormones thyroïdiennes dans la régulation de l'homéostasie métabolique, il est possible que sa perturbation lors des premiers stades de développement soit la cause des effets nocifs observés. La présente thèse démontre pour la première fois les effets perturbateurs du TCPP et du TDCPP sur le développement et le système endocrinien des espèces aviaires, et tente de lier les changements phénotypiques aux perturbations moléculaires en vue d'améliorer la compréhension de leurs modes d'action.

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List of Abbreviations

ANOVA	analysis of variance
APOE	apolipoprotein E
ATH	aluminum trihydrate
ATP2B2	ATPase, Ca++ transporting plasma membrane 2
BATF3	basic leucine zipper transcription factor, ATF-like 3
BCPP	bis(1-chloro-2-propyl) phosphate
BDCPP	bis(1,3-dichloro-2-propyl) phosphate
BFR	brominated flame retardant
bw	body weight
CATH	cathelicidin
CD36	thrombospondin receptor
cDNA	complimentary DNA
CEH	chicken embryonic hepatocytes
Ct	cycle threshold
CY3	cyanine 3-CTP
CY5	cyanine 5-CTP
CYP	cytochrome P450
D1	type I iodothyronine 5'-deiodinase
D2	type II iodothyronine 5'-deiodinase
D3	type III iodothyronine 5'-deiodinase
D_{27}-TBP	d_{27}-tributyl phosphate
DE	differentially expressed
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
EGR1	early growth response 1
F0	first generation
F1	second generation
FDR	false discovery rate
FR	flame retardant
GAL	gallinacin
GST	glutathione S-transferase
HD	high dose
HNF4A	hepatocyte nuclear factor 4 alpha
HPLC	high performance liquid chromatography
hr	hours
IGF-1	insulin-like growth factor-1
IPA	ingenuity pathway analysis
LC-ESI(+)-MS-MS	liquid chromatography-electrospray ionization (+)-tandem quadrupole mass spectroscopy
LD	low dose
L-FABP	liver fatty acid-binding protein
LOD	limit of detection
LSI	liver somatic index
LXR/RXR	liver X receptor/retinoid X receptor

min	minutes
mRNA	messenger RNA
NTC	no template control
OPFR	organophosphate flame retardant
PFR	phosphorus flame retardant
PBDE	polybrominated diphenyl ether
PPARA	peroxisome proliferator-activated receptor alpha
PUF	polyurethane foam
RBC	red blood cells
Real-time RT-PCR	real-time reverse transcription polymerase chain reaction
SD	standard deviation
SEM	standard error of means
SI	supplementary information
SPE	solid phase extraction
SPP1	secreted phosphoprotein 1
STEAP4	six-transmembrane epithelial antigen of the prostate member 4
T_3	triiodothyronine
T_4	thyroxine
TCPP	tris(1-chloro-2-propyl) phosphate
TDCPP	tris(1,3-dichloro-2-propyl) phosphate
TH	thyroid hormone
TTR	transthyretin
UGT1A9	uridine 5’-diphospho- glucuronosyltransferase 1A9
ww	wet weight
WNT4	wingless-type MMTV integration site family member 4

Statement of Contributions

CHAPTER TWO	**Contributions by:**
Experimental design, results analysis and manuscript preparations	Amani Farhat Doug Crump Sean Kennedy Rob Letcher
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Microarray data pre-processing	Andrew Williams
Microarray data analysis post-processing	Amani Farhat Jason O'Brien
Primer design and real-time RT-PCR validation	Amani Farhat Suzanne Chiu

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CHAPTER ONE

INTRODUCTION

1.1. Flame Retardants

Flame retardants (FRs) are chemicals that are added to a variety of flammable materials to hinder ignition and reduce the spread of flame. Strict fire safety regulation implemented in the 1970s in North America require polymers and textiles such as sleepwear, plastics and upholstery foam to withstand an open flame for a specified duration without igniting (CPS 2010; NAFRA 2013). Global FR production has since been increasing, reaching 1.9 million tonnes in 2011 (The Freedonia Group 2013) and is predicted to continue to rise with ever rising safety standards (Ceresana 2011). Manufacturers meet fire safety standards by chemically binding FRs to polymers or incorporating them as physical additives, and are challenged with doing so while maintaining the desired mechanical properties of the polymer (Weil and Levchik 2009).

There are three major types of flame retardants: endothermic, halogenated (brominated/chlorinated), and phosphorus flame retardants (PFRs). Endothermic FRs, such as aluminum trihydrate (ATH), degrade at high temperatures, absorbing heat that would otherwise fuel the fire and emitting water vapour in the process (Bonsignore and Claassen 1980). ATH is considered safe for use as a FR and is even found in pharmaceutical antacids and cosmetics (Weil and Levchik 2009) making it the most commonly used FR worldwide (Ceresana 2011). However, the relatively large quantity of ATH

required to achieve its desired effect limits its application (Leisewitz *et al.* 2000). Halogenated FRs work through the gas phase by interfering with high energy hydrogen and hydroxide radicals required for flame propagation. The high risk of adverse health effects associated with many halogenated flame retardants has incited much controversy and initiated a movement towards their removal from many applications in North America (McPherson *et al.* 2004; Shaw *et al.* 2010). Although the demand for halogenated FRs is declining in North America and Western Europe, it continues to rise in other regions such as South America, Asia-Pacific and the Middle East (Ceresana 2011). Phosphorus flame retardants (PFRs) are also broadly used to improve fire resistance by forming a physical char barrier between the flame and fuel source due to the carbonization of the polymer by phosphoric acid. The char barrier reduces toxic gas emissions making them a safer alternative to halogenated FRs (McPherson *et al.* 2004).

The unfortunate truth is that most chemicals, including flame retardants, enter the marketplace without undergoing chronic toxicity testing. Over 80 thousand chemicals are produced in the Unites States annually, but basic toxicity data are available for a mere 7% of these compounds (Woodruff 2009). A recent movement in toxicology suggests shifting away from relatively inefficient, animal-based testing towards high-throughput *in vitro* screening methods to make the daunting task of chemical-testing more feasible (National Research Council 2007). However, this paradigm shift remains relatively new and standardized guidelines and regulations for implementation are still under development. In the

meantime, the health risks of numerous compounds in the environment remain largely unknown.

1.1.1 PFRs as replacement flame retardants

Polybrominated diphenyl ethers (PBDEs) are a group of FRs with a wide range of applications that were recently phased out of production due to their persistence in the environment and endocrine-disrupting and neurotoxic properties (Shaw *et al.* 2010). Stringent fire-safety regulations remain however, necessitating their replacement with alternative FRs, but whether replacements should be non-halogenated is a question that is yet to be resolved (Levchik and Weil 2006). A European assessment reported 27 potential replacements for decaBDE in 2007, sixteen of which were halogenated (van der Veen and de Boer 2012). A report by McPherson *et al*. (2004) however warned against the health effects of halogenated flame retardants collectively and urged for their replacement by non-halogenated alternatives. PFR are widely applicable and easy to use and have therefore been suggested as effective replacements for brominated flame retardants (BFRs) (Levchik and Weil 2006; van der Veen and de Boer 2012). Halogenated FRs were deemed unsafe for replacement of BFRs due to their carcinogenic potential among other adverse health effects and three non-halogenated PFR were suggested as non-problematic replacements: resorcinol-bis(diphenylphosphate), bisphenol-A diphenyl phosphate, and melamine polyphosphate (van der Veen and de Boer 2012). These compounds have low bioaccumulative potential and minimal health effects but their toxicity

data are limited and no data exist on their environmental occurrence (van der Veen and de Boer 2012). Melamine polyphosphate is the ideal flame retardant with respect to exposure risk as it is chemically bound to the material and is therefore unlikely to be released into the environment. Reactive FRs can, however, appreciably impair the properties of the polymer to which they are bound limiting their application as broad BFR replacements (Weil and Levchik 2009). In the midst of the debate regarding the most appropriate BFR replacement, tris(1,3-dichloro-2-propyl) phosphate (TDCPP) has become a common replacement for PBDEs (Shaw *et al.* 2010). A recent survey of US household furniture found that TDCPP was the most commonly detected FR in couches purchased post-2005, in contrast to older couches in which pentaBDE was the major FR detected (Stapleton *et al.* 2012). Tris(1-chloro-2-propyl) phosphate (TCPP) had also been suggested as a replacement for BFRs, but its recent increase in production better correlates with the withdrawal of another chlorinated FR, tris(2-chloroethyl) phosphate (TCEP) (European Union 2008a). Effectively, PBDEs and TCEP have been replaced with other halogenated flame retardants that also have the potential to cause adverse health effects, as described in section 1.2.

1.1.2. Chemical structure and properties of TCPP and TDCPP

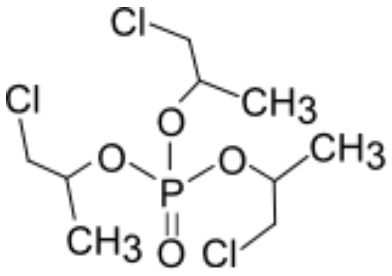

Figure 1.1. Chemical structure of TCPP

Molecular Formula:	$C_9H_{18}Cl_3O_4P$
Molecular Mass:	327.55
CAS registry number:	13674-84-5
Synonyms:	2-propanol, 1-chlorophosphate (3:1); 1-chloro-2-propyl phosphate (1:3); tris(1-chloromethylethyl) phosphate; tris(2-chloroisopropyl) phosphate;
Trade names:	Fyrol PCF; Amgard TMCP; Antiblaze 80; Levagard PP; Tolgard TMCP; TCPP
Common names:	TCPP; TMCP; TCIP
Physical state:	clear, colourless liquid
Melting Point:	-40 °C
Boiling point:	235–248 °C
Flash point:	218 °C
Vapour pressure:	2.02E-5 mmHg at 25 °C
Solubility in water:	1.2 g/L at 25 °C
Octanol-water coefficient (log Kow):	2.59

Sources: (ChemIDplus; IPCS 2004)

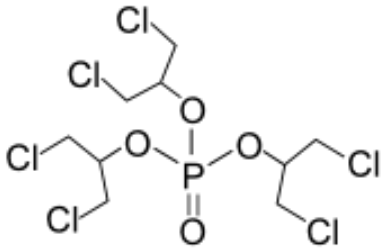

Figure 1.2. Chemical structures of TDCPP

Molecular Formula:	$C_9H_{15}Cl_6O_4P$
Molecular Mass:	430.91
CAS registry number:	13674-87-8
Synonyms:	1,3-dichloro-2-propanol phosphate; 2-propanol, 1,3-dichloro-, phosphate; phosphoric acid tris(1,3-dichloro-2-propyl) ester; tris(1-chloromethyl-2-chloroethyl) phosphate; tris(1,3-dichloroisopropyl) phosphate;
Trade names:	CRP; Firemaster T33P; Fyrol FR 2; PF 38; PF 38/3; Apex Flame Proof Emulsion 197 and 212; Antiblaze 195; Amgard; TDCP
Common names:	TDCPP; TDCP
Physical state:	viscous colourless liquid
Melting point:	27 °C
Boiling point:	236–237 °C at 5 mmHG
Flash point:	252 °C
Vapour pressure:	7.36E-8 at 25 °C
Solubility in water:	7 mg/L at 24°C
Octanol-water coefficient (log Kow):	3.8

Sources: (ChemIDplus; IPCS 2004)

1.1.3. Production, use, stability and occurrence of TCPP and TDCPP

TCPP and TDCPP are high production volume chemicals added to a variety of materials to meet fire safety standards. A short summary of the consumer demand for different regions is listed in Table 1.1. Due to their structural similarity, TCPP and TDCPP are utilized in the same marketplace; however, TCPP is about half the price of TDCPP making it more broadly used across different applications (European Union 2008a). TCPP is mainly used as an additive in rigid PUF for insulation, but is also added to fillers, foaming agents, adhesives, and rubbers. The additional chlorine atoms in TDCPP increase its fire quenching efficiency relative to TCPP, but due to the added expense, it is only used in cases when more stringent fire safety regulations need to be met (European Union 2008b). TDCPP is mainly added to flexible PUF for use in automotive upholstery, but is also commonly added to bedding and household upholstery including baby products (European Union 2008b; Shaw *et al.* 2010). Both compounds are also used to a lesser extent to treat concrete, plastics and textile products (European Union 2008a; European Union 2008b).

TCPP and TDCPP escape into the environment during chemical manufacturing, in the process of product treatment, through the use of the product and at the disposal or recycling stage of the product (IPCS 2004). The high volatility of TCPP allows for 40% of the added chemical to escape into the environment throughout a product's lifetime, whereas only 10% of the added TDCPP is available for release (European Union 2008b). These compounds persist in the environment because they are resistant to abiotic and bacterial

Table 1.1. Global usage of TCPP and TDCPP flame retardants

Location	Production/Consumption (tonnes/year)	Year
TCPP		
Global	40000	1997
Europe	22950	1995
Europe	36000	2000
Norway	42.7	2008
Denmark	177	2008
Finland	16429	2008
Sweden	132	2008
Unites States	24700	2012
TDCPP		
Global	8000	1997
Europe	<10000	2000
Denmark	134.1	2002
Unites States	4500-22500	2012

(European Union 2008a; US EPA 2012; van der Veen and de Boer 2012)

degradation. Hydrolysis of TCPP under acidic and alkaline conditions is slow, with an estimated half-life ≥1 year (European Union 2008a), whereas TDCPP is stable under acidic conditions but is more susceptible to hydrolysis at pH-9 (half-life=14.7 days) (European Union 2008b). Neither TCPP nor TDCPP are readily biodegradable; the reported 28 day degradation in activated sewage sludge ranges from 0-21% and 0-4% for TCPP and TDCPP, respectively (European Union 2008a; European Union 2008b). However, a prolonged closed bottle test of TCPP in sewage sludge under aerobic conditions showed it to be inherently biodegradable (European Union 2008a) and therefore ultimate biodegradation is possible. The stability of TCPP and TDCPP owes to their detection across the globe in various media (Table 1.2) and to concerns about the risks of animal and human exposure.

Table 1.2. Levels of TCPP and TDCPP in the environment

Sample Type	Highest Detected Level	Location	Reference
TCPP			
Indoor Air	1080 ng/m^3	Computer Hall, Sweden	Staaf and Ostman (2005)
Surface Water	379 ng/L	Lake Nidda, Germany	Regnery and Puttmann (2010)
Treated drinking water	50 ng/L	River Ruhr, Germany	Andersen and Bester (2006)
River water	300 ng/L	River Ruhr, Germany	Andersen et al. (2004)
Lake water	17.8 µg/L	Italy	Galassi et al. (1992)
Snow	210 ng/kg	Northern Sweden	Marklund et al. (2005c)
Influent	18 µg/L	Sweden	Marklund et al. (2005b)
Effluent	24 µg/L	Sweden	Marklund et al. (2005b)
Landfill Leachate	66.6 µg/L	United Kingdom	European Union (2008a)
Sediment	24 µg/g	Norway	Green et al. (2008)
Dust	14 µg/g	Not specified	Leiswitz et al. (2000)
Packaged food	9.3 ng/L	United States	Kan-Do Team (1995)
Human breast milk	82 ng/g	Sweden	Sundkvist et al. (2010)
Fish	17 ng/g	Burbot liver, Norway	Leanards et al. (2011)
Birds	10 ng/g wet weight	Blood, Norway	Leonards *et al*. (2011)
TDCPP			
Indoor Air	150 ng/m^3	Hospital Ward, Sweden	Marklund et al. (2005a)
Surface Water	50 ng/L	River Ruhr, Germany	Anderson et al. (2004)
Treated drinking water	17 ng/L	River Ruhr, Germany	Andersen and Bester (2006)
River water	31 µg/L	Japan	Okumura (1994)
Snow	230 ng/kg	Northern Sweden	Marklund et al. (2005c)
Influent	820 ng/L	Norway	Green et al. (2008)
Effluent	740 ng/L	Norway	Green et al. (2008)
Landfill Leachate	5.5 µg/L	Japan	Yasuhara et al. (1999)
Sediment	8.8 µg/g	Sediment Norway	Green et al. (2008)
Dust	326 µg/g	Vehicle, Boston, United States	Carignan et al. (2013)
Pine needles	1319 ng/g wet weight	California	Aston et al. (1996)
Human seminal plasma	50 ppb	North America	Hudec et al. (1981)
Human adipose tissue	32 ng/g	Ontario, Canada	LeBel and Williams (1986)
Human breast milk	5.3 ng/g	Sweden	Sundkvist et al. (2010)
Fish	140 ng/g	Perch, Sweden	Sundkvist et al. (2010)
Birds	1.9 ng/g wet weight	Gull egg, Norway	Leonards *et al*. (2011)

Modified from European Union risk assessment (2008a; 2008b) and Van der Veen and Boer (2012).

Although TCPP and TDCPP are resistant to environmental and bacterial degradation, they are susceptible to metabolic degradation. Minegeshi *et al.* (1988) showed that 97.8% of the TCPP orally administered to rats was eliminated

after seven days and that no bioaccumulation occurred. An unpublished report by Stauffer Chemical Company described that <2% of the TCPP administered orally to rats was excreted as the parent compound, indicating extensive metabolism (European Union 2008a). The main metabolite (>50%) was identified as 0,0-[bis(1-chloro-2-propyl)]-0-(2-proprionic acid) phosphate (Fig. 1.3) and the minor metabolites were bis(1-chloro-2-propyl) phosphate (BCPP) and 1-chloro-2-propanol. Some studies claim that all OPFRs are hydrolysed to form their corresponding dialkyl phosphate as the main metabolite, which would make BCPP the main metabolite of TCPP (Schindler *et al.* 2009), but there is no direct evidence confirming this. TDCPP was also extensively metabolized in rats, showing 96% elimination 5 days post oral administration with <0.1% excreted as TDCPP and 62% excreted as bis(1,3-dichloro-2-propyl) phosphate (BDCPP; Fig. 1.3) (Lynn *et al.* 1981). Minor metabolites identified were 1,3-dichloro-2-propyl phosphate and 1,3-dichloro-2-propanol; an additional metabolite was identified in rat liver microsomes as 3-chloro-1,2-propanediol (Nomeir *et al.* 1981). Even though the literature suggests little potential for bioaccumulation of TCPP and TDCPP, they have been detected in both wildlife and humans worldwide (Table 1.2). For example, monitoring studies in birds indicate that both TCPP and TDCPP are detectable in wild avian species. TCPP was detected in various Norwegian bird species and in Great Lakes herring gull eggs at concentrations of 10 and 4.1 ng/g wet weight (ww), respectively (Chen *et al.* 2012; Leonards *et al.* 2011), and TDCPP concentrations of 1.9 and 0.17 ng/g ww were measured in

Norwegian great black-backed gull eggs and Great Lakes herring gull eggs, respectively (Chen *et al.* 2012; Leonards *et al.* 2011).

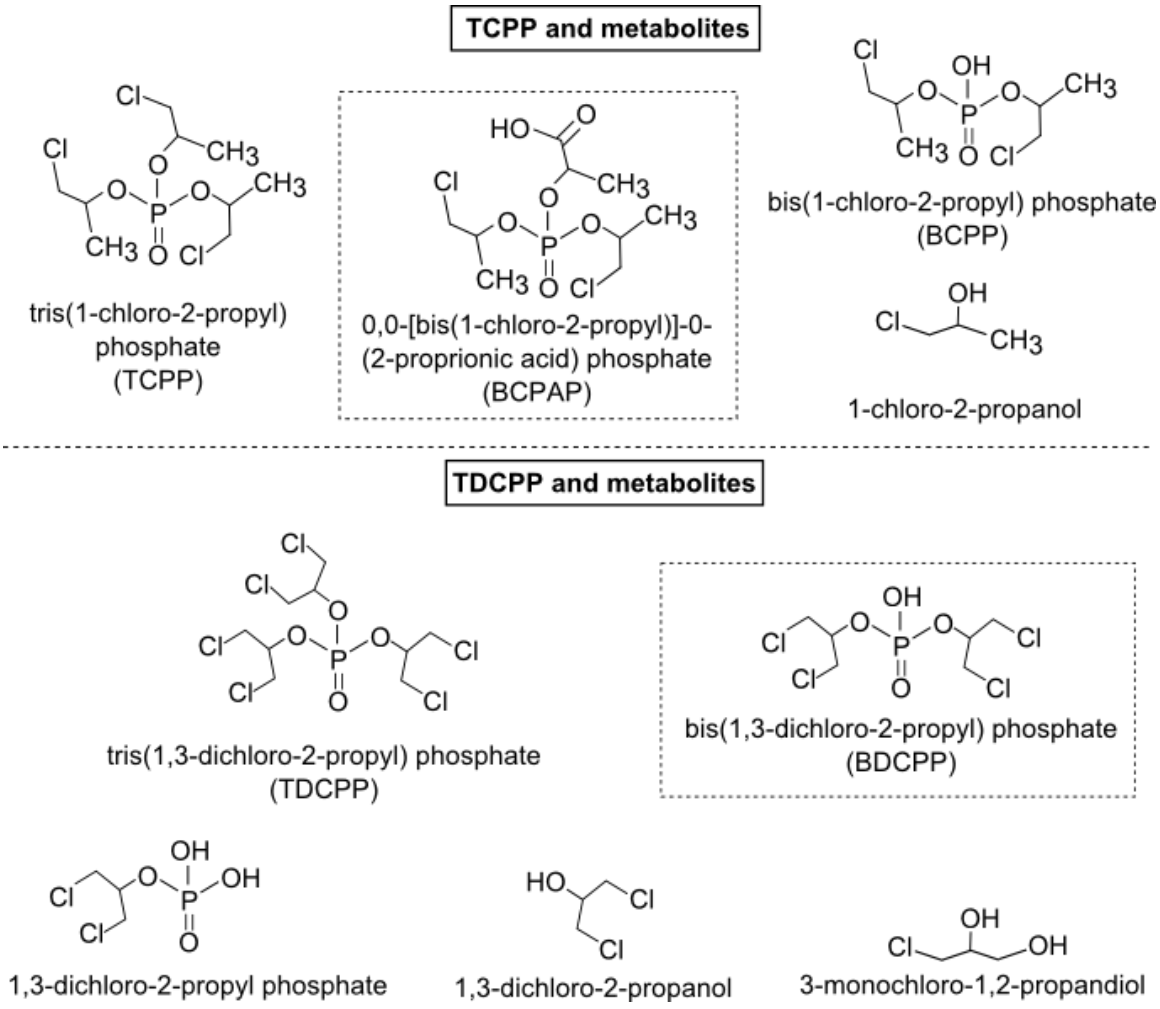

Figure 1.3. Metabolites of TCPP and TDCPP. The major metabolite of each flame retardant is indicated by a dashed box.

1.2. Toxicity of TCPP and TDCPP

A great deal of toxicity data are available regarding the effects of TCPP and TDCPP in mammalian, and to a lesser extent, aquatic species; however, data are scarce on the effects of these flame retardants in avian species. In fact,

only three *in vivo* avian (chicken) studies have been reported (European Union 2008a; Sprague *et al.* 1981; Ulsamer *et al.* 1980), all of which were based on post-hatch exposures and investigated neurotoxic potential. Therefore, not only are toxicity data limited for TCPP and TDCPP in avian species, but absolutely no data exist on their effects in avian embryos. The presence of TCPP and TDCPP in wild avian birds and eggs (Section 1.1.3) warrants investigation into the toxicological effects of *in ovo* and post-hatch exposures in avian species.

1.2.1. Acute and sub-chronic toxicity

TCPP has low to moderate acute toxicity in aquatic and terrestrial species. The LC_{50} of TCPP in aquatic vertebrates ranges from 30 mg/L in guppies (*Poecilia reticulata*) to 84 mg/L in bluegill sunfish (*Lepomis macrochirus*) and the LD_{50} in rats ranges from 632 to 3300 mg/kg (European Union 2008a). Four-day exposures to TCPP in rats, whether orally or by nasal inhalation, result in similar signs of systemic toxicity including ataxia (lack of coordination of muscle movements), lethargy, laboured respiration, increased salivation, depressed body weight, body tremors, convulsions and partially closed eyes (European Union 2008a). A 13-week oral exposure in rats at doses ranging from 5 to 1745 mg/kg/day resulted in zero mortalities but reduced body weight, increased absolute and relative liver weight at the high dose and mild thyroid follicular cell hyperplasia at all doses in males (European Union 2008a). No changes in clinical chemistry or haematology were observed. The lowest observed adverse effect level (LOAEL) was 52 mg/kg/day, which is 2 orders of magnitude greater

than the maximum expected human occupational exposure to TCPP (0.104 mg/kg/day) (European Union 2008a).

The aquatic vertebrate LC_{50} for TDCPP ranges from 1.1 mg/L in rainbow trout (*Oncorhynchus mykiss*) to 5.1 mg/L in goldfish (*Carassius auratus*) (European Union 2008b). TDCPP has low acute toxicity in mammals with reported LD_{50} values above 2000 mg/kg in mice and rats (IPCS 2004; Kamata *et al.* 1989) and 6800 mg/kg in rabbits (European Union 2008b). Common clinical signs of toxicity following TDCPP exposure were laboured respiration and ataxia. In all cases, necropsy showed abnormalities in the lungs, liver and stomach or intestine. A three-month oral exposure study in mice showed that high dose TDCPP exposure (1792/1973 mg/kg for males/females) caused extreme weight loss and tremors and resulted in 100% mortality. Hemoglobin concentrations were decreased at 576/598 mg/kg and relative kidney weights increased at concentrations ≥ 171/214 mg/kg. The LOAEL was determined to be 171 mg/kg for males and 62 mg/kg for females (Kamata *et al.* 1989), which is much greater than the estimated maximum human exposure of 6.99×10^{-4} mg/kg/day (European Union 2008b).

1.2.2. Reproductive toxicity

A single reproductive toxicity study of TCPP in rats demonstrated effects on fertility and embryonic development, leading to follow-up reproductive studies being requested by the European Commission (European Union 2008a). TCPP treatment resulted in a lengthened oestrus cycle in first (F0) and second (F1)

generation females at all doses and an increase in the mean number of cycles per animal at the high dose (1000 mg/kg/day). A decrease in uterus weight was observed in F0 females at all doses and in high dose-treated F1 females. Furthermore, an increase in the number of runts was observed at all dose groups of the F0 generation. Based on these findings, a LOAEL of 99 mg/kg was derived for the effects of TCPP on fertility and developmental toxicity.

TDCPP showed minimal reproductive effects in a 2-year oral exposure study in male rats, but no information is available on the effects of TDCPP on female fertility (European Union 2008b). TDCPP caused developmental effects in rats exposed to 400 mg/kg/day from days 6-15 of gestation. There was an increased rate of resorptions leading to significantly reduced foetal viability and surviving embryos showed retarded skeletal development. Maternal weight was significantly reduced throughout gestation and clinical signs of toxicity such as hunched posture, loss of hair and salivation were observed. The no observed adverse effects level was reported to be 100 mg/kg/day for maternal toxicity and foetal development.

1.2.3. Mutagenicity and carcinogenicity

The mutagenic potential of TCPP has been well investigated *in vitro*. Evidence from several mutagenicity studies shows that TCPP is neither a bacterial nor a fungal mutagen (European Union 2008a). TCPP was negative for unscheduled DNA synthesis (UDS) in male rat liver primary cell cultures (European Union 2008a) and in a rat hepatocyte DNA-repair assay (Williams *et*

al. 1989). An *in vitro* Comet assay in a Chinese hamster cell-line in the presence and absence of metabolic activation was also negative (Follmann and Wober 2006). A study by Covance Laboratories Ltd., however, demonstrated the potential clastogenic activity of TCPP in a mouse lymphoma cell-line in the presence of metabolic activation (European Union 2008a). Three unpublished industrial studies reported in a European Union risk assessment (2008a) were performed to investigate the clastogenic potential of TCPP *in vivo.* TCPP was negative for DNA damage in a mouse bone marrow micronucleus test and a rat bone marrow cytogenetics assay. Furthermore, a Comet assay determined that TCPP did not induce DNA damage in the liver of rats treated with either 750 or 1500 mg/kg TCPP. Therefore, TCPP is not considered to be genotoxic *in vitro* or *in vivo.* The carcinogenic potential of TCPP has not yet been determined, but a two-year oral exposure carcinogenicity study is currently underway under the US National Toxicology Program (CPSC 2012).

In contrast to TCPP, TDCPP is considered to be genotoxic as it caused mutations in multiple *Salmonella* strains (OEHHA 2011b). *In vitro* mammalian assays have given both positive and negative results. TDCPP induced mutations in mouse lymphoma cells in a study by Inveresk Research International but not when performed by Brusick *et al.* (1980) (OEHHA 2011b) and also failed to induce mutations in a Chinese hamster cell line (Soderlund *et al.* 1985). TDCPP increased chromosomal aberrations in mouse lymphoma cells and Chinese hamster fibroblast cells (Brusick *et al.* 1980; Ishidate 1983) and weakly induced sister chromatid exchange in mouse lymphoma cells (Brusick *et al.* 1980).

Although *in vivo* genotoxicity studies have largely been negative, TDCPP has been shown to increase the occurrence of tumours in multiple organs in rats (Males: liver, kidney, testes; Females: liver, kidney, adrenal gland) and has therefore been classified as a cancer causing agent by the California Environmental Protection Agency (OEHHA 2011b).

1.2.4. Neurotoxic potential

Organophosphate esters can be potent inhibitors of acetylcholinesterase causing delayed polyneuropathy, which is an irreversible neuro-degenerative disease (Lotti and Moretto 2005). However, TCPP and TDCPP were only shown to induce mild neurotoxicity at very high doses in hens. Two oral doses of 13 g TCPP/kg administered 21 days apart resulted in reduced body weight, impaired walking behaviour and cessation of egg production, but minimal axonal degradation was observed (Sprague *et al.* 1981). Chickens exposed orally to TDCPP at doses of 0.6, 1.2, 2.4 or 4.8 g/kg/day for 5 days exhibited leg and wing weakness at doses ≥1.2 g/kg and 100% mortality at 4.8 g/kg (Ulsamer *et al.* 1980). The lack of potent neurotoxic response may be due to the fact that these compounds are quite rapidly metabolized as previously described (Section 1.1.3). A recent study in PC12 cells, a standard *in vitro* model for neuronal development, demonstrated the neurotoxic potential of TCPP and TDCPP (Dishaw *et al.* 2011). In undifferentiated PC12 cells, TCPP promoted differentiation into the cholinergic neurotransmitter phenotype but failed to promote differentiation into the dopaminergic phenotype, whereas TDCPP promoted both neuronal phenotypes

but favoured differentiation into the dopaminergic type. Both TCPP and TDCPP reduced cell numbers and TDCPP reduced DNA synthesis at concentrations equivalent to the neurotoxic pesticide chlorpyrifos. These results demonstrate the ability of TCPP and TDCPP to hinder neural cell replication and to shift neuronal fate by promoting differentiation into one neurotransmitter phenotype at the expense of another. Although neither FR was observed to be cytotoxic in PC12 cells, TDCPP did reduce the viability of chicken embryonic neuronal cells (Crump *et al.* 2012), further supporting the adverse effect of TDCPP on neural development.

1.2.5. Endocrine-disrupting potential

The endocrine-disrupting potential of TDCPP has recently become evident in multiple test systems and species. TCPP is less potent in this regard and recent studies suggest it has a weak endocrine-disrupting effect. TCPP affected the expression of steroid metabolizing enzymes in a human adrenocortical carcinoma cell-line (H295R) (Liu *et al.* 2012) and thyroid hormone (TH)-responsive genes in chicken embryonic hepatocytes (CEH) (Crump *et al.* 2012). Steroid hormones regulate a range of processes including sex characterization, pregnancy, electrolyte balance, and stress response (Hiller-Sturmhofel and Bartke 1998). THs regulate metabolism in tissues to maintain homeostatic processes such as thermoregulation but are also essential for growth, maturation and tissue differentiation including that of the central nervous system (Hiller-Sturmhofel and Bartke 1998). The disruption of these hormones can effect

fertility and development as was observed in TCPP-treated rats (European Union 2008a). TDCPP also disrupted the expression of steroid metabolizing enzymes in H295R cells (Liu *et al.* 2012) and TH-responsive genes in CEH (Crump *et al.* 2012). Additionally, it reduced whole body thyroxine (T_4), increased whole body triiodothyronine (T_3) (Wang *et al.* 2013), dysregulated expression of thyroid hormone-responsive genes (Liu *et al.* 2013) and altered sex hormone concentrations in zebrafish (Liu *et al.* 2012). Furthermore, TDCPP showed strong antagonistic activity towards the androgen receptor and weak activity towards estrogen and progesterone receptors in human osteosarcoma cells (Suzuki *et al.* 2013). Moreover, TDCPP indoor dust concentrations have been correlated with decreased circulating T_4 and increased circulating prolactin levels in men (Meeker and Stapleton 2010). These studies demonstrate that TDCPP exposure affects endocrine endpoints, which may help to explain the increased incidence of malformations (McGee *et al.* 2012) and reduced survival in zebrafish embryos (Wang *et al.* 2013) and stunted development in TDCPP-exposed rats (European Union 2008b).

1.3. Thesis Overview

1.3.1. Rationale

TCPP and TDCPP have been in use since the 1970s to meet fire safety regulations, but as more halogenated flame retardants are withdrawn from the market, the demand for these flame retardants increases. The majority of toxicity data for these compounds come from mammalian studies, and the few avian

studies available are based on post-hatch exposures. Although TCPP and TDCPP have been detected in wild avian eggs, there has been no attempt to study their developmental effects in the avian embryo. Recent findings have illuminated the endocrine-disrupting potential of these chemicals, which could have dramatic effects on avian embryonic development. To fill this data gap, this M.Sc. thesis focuses on the developmental, biochemical and molecular effects of TCPP and TDCPP in white leghorn chicken (*Gallus gallus domesticus)* embryos following *in ovo* exposure.

The chicken was chosen as a model organism for avian toxicity because it is quite sensitive to contaminant exposure, develops rapidly and is relatively inexpensive. Furthermore, the stages of chicken embryonic development are well established and the chicken genome has been fully sequenced. This makes it possible to detect subtle effects on embryonic development and to determine effects on gene expression in order to link potential phenotypic effects to their underlying molecular changes. This approach will help us to better understand the modes of action potentially leading to TCPP and TDCPP toxicity.

1.3.2. Research objectives and hypotheses

A number of studies were performed to investigate the toxicological and molecular effects of TCPP and TDCPP exposure in chicken embryos. These have been organized into two chapters, for which the individual objectives and hypotheses were as follows:

CHAPTER TWO

Objectives:

1. Determine the embryo-lethality and adverse effects of TCPP and TDCPP in chicken embryos following *in ovo* exposure. This includes measurements of pipping success, embryonic development, tissue accumulation, changes in gene expression levels and circulating and glandular thyroid hormone levels.
2. Monitor the concentrations of TCPP and TDCPP in chicken egg content homogenate throughout incubation to determine whether the low residual tissue concentrations of TCPP and TDCPP that resulted from objective one were due to metabolism or accumulation in un-tested tissues.

Hypotheses:

A. TDCPP reduced viability in zebrafish and rat embryos and is therefore predicted to reduce the pipping success of treated chicken embryos. TCPP is less toxic to aquatic organisms and did not affect rat embryo viability; therefore, it is not expected to reduce chicken embryo viability.

B. Circulating T_4 levels are expected to decrease following TDCPP exposure based on recent endocrine disruption studies, which should lead to a decrease in embryo growth. Changes in the mRNA expression levels of TH-responsive genes are also expected.

C. TCPP and TDCPP concentrations in chicken egg homogenate will be similar to injected concentrations shortly after injection and will decrease to baseline levels by the end of incubation.

CHAPTER THREE

Objectives:

1. Perform a full genome microarray mRNA expression analysis on the liver tissue of TDCPP-treated embryos at doses that exhibited adverse effects in chapter two. Use Ingenuity Pathway Analysis to identify biological pathways and regulatory molecules most affected by TDCPP treatment.

Hypotheses:

D. TDCPP reduced circulating T_4 levels and growth in chicken embryos (Chapter 2, objective 1); therefore, a significant dysregulation of TH-responsive genes is expected.

E. The reduced gallbladder size in TDCPP-treated chicken embryos (Chapter 2, objective 1) is likely due to a reduction in bile acid synthesis; therefore, disruption of the cholesterol metabolism is expected.

F. Disruption of steroid hormone metabolism is also expected based on recent zebra fish studies.

CHAPTER TWO

IN OVO EFFECTS OF TCPP AND TDCPP ON PIPPING SUCCESS, DEVELOPMENT, mRNA EXPRESSION AND THYROID HORMONE LEVELS IN CHICKEN EMBRYOS

Modified from Farhat, A., Crump, D., Chiu, S., Williams, K.L., Letcher, R.J., Gauthier, L.T., and Kennedy, S.W. (2013) *In ovo* effects of two organophosphate flame retardants, TCPP and TDCPP, on pipping success, development, mRNA expression and thyroid hormone levels in chicken embryos. *Toxicol Sci.* **134**(1), 92-102.

2.1. Abstract

Tris(1-chloro-2-propyl) phosphate (TCPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are organic flame retardants (FRs) detected in the environment and biota for which avian toxicological data are limited. In this study, domestic chicken eggs were injected with TCPP or TDCPP (maximum dose=51600 and 45000 ng/g egg, respectively) to determine dose-dependent effects on pipping success, development, hepatic messenger RNA (mRNA) expression levels of genes associated with xenobiotic metabolism and the thyroid hormone (TH) pathway, and TH levels following 20-22 days of incubation. Neither compound reduced pipping success; however, TCPP significantly delayed pipping at 9240 and 51600 ng/g and reduced tarsus length at 51600 ng/g. TDCPP exposure resulted in significant decreases in head plus bill length, embryo mass and gallbladder size at 45000 ng/g and reduced plasma free thyroxine (T_4) levels at 7640 ng/g. Type I deiodinase (D1), liver fatty-acid binding protein (L-FABP) and cytochrome P450 (CYP) 3A37 mRNA levels were significantly induced by TCPP, while TDCPP induced CYP3A37 and CYP2H1. Chemical analysis of egg contents at incubation days 0, 5, 11, 18, and 19

revealed that >92% of the injected TCPP or TDCPP concentration was detectable up to day 5; however, <1% was detected by day 19. The observed phenotypic responses to TCPP and TDCPP exposure may be associated with disruption of the TH-axis, which is critical for normal growth and development in birds. The effects of TDCPP on the gallbladder indicate that the disturbance of lipid metabolism is a likely mechanism of toxicity.

2.2. Introduction

TCPP and TDCPP are high production volume organophosphate flame retardants (OPFRs) added to a variety of consumer products to prevent flame ignition. TCPP is used primarily in rigid polyurethane foam (PUF) for construction, while TDCPP is mainly used in flexible PUF for automotive upholstery (Shaw *et al.* 2010). In 2000, European production of TCPP reached 36000 tonnes/yr (European Union 2008a), while TDCPP production was about 10000 tonnes/yr (European Union 2008b); production of these FRs has since grown as they have replaced other commonly used flame retardants, such as tris(chloroethyl) phosphate and polybrominated diphenyl ethers, due to health concerns (IPCS 2004; Stapleton *et al.* 2009). Because these FRs are not chemically bound to the product to which they are added, they can leach into the environment at ambient conditions; 40% of the added TCPP may be released throughout a product's lifetime, while 10% of TDCPP is available for release (European Union 2008b). Because neither compound is readily degraded in water or soil (European Union 2008a; European Union 2008b) they tend to

persist and accumulate in the environment, posing potential exposure risks for humans and wildlife.

The rapid metabolism of TCPP and TDCPP in fish and rats suggests that they have little potential for bioaccumulation (European Union 2008a; European Union 2008b); however, both compounds have been detected across the globe in various environmental media, including biota. Significant sources of wildlife exposure to these FRs include surface water, river water, sediment and landfill leachate (European Union 2008a; European Union 2008b). Aquatic wildlife, including mussels and fishes, had concentrations of TCPP and TDCPP up to 1300 and 140 ng/g lipid weight, respectively (Sundkvist *et al.* 2010; van der Veen and de Boer 2012). TCPP was detected in various Norwegian bird species and in Great Lakes herring gull eggs at concentrations of 10 and 4.1 ng/g wet weight (ww), respectively (Chen *et al.* 2012; Leonards *et al.* 2011). TDCPP concentrations of 1.9 and 0.17 ng/g ww were measured in Norwegian great black-backed gull eggs and Great Lakes herring gull eggs, respectively (Chen *et al.* 2012; Leonards *et al.* 2011).

Although TCPP is produced in greater volumes and is often detected at higher environmental concentrations relative to TDCPP, there are fewer studies characterizing its potential toxicity. TCPP has been suggested to cause reproductive toxicity in female mice (European Union 2008b); however, data are limited on its potential carcinogenicity, embryotoxicity, teratogenicity and immunotoxicity. TDCPP was recently classified as a chemical carcinogen (OEHHA 2011a) and has neurotoxic properties in PC12 cells (Dishaw *et al.*

2011). In addition, overt neurotoxic effects of TDCPP in chickens, such as leg/wing weakness, have been observed at doses >1.2 mg/g (Ulsamer *et al.* 1980). TDCPP was cytotoxic in chicken embryonic hepatocytes (CEH) and human adrenal cells (Crump *et al.* 2012; Liu *et al.* 2012) and both TCPP and TDCPP up-regulated phase I and II xenobiotic metabolizing enzymes in CEH and zebrafish (Crump *et al.* 2012; Liu *et al.* 2012). Furthermore, TDCPP is suspected of being an endocrine disrupting chemical as it has been correlated with decreased circulating TH levels in humans (Meeker and Stapleton 2010), disrupted sex hormone levels in zebrafish (Liu *et al.* 2012) and altered mRNA levels of TH-responsive genes in CEH (Crump *et al.* 2012).

In the present study, we injected fertilized chicken (*Gallus gallus domesticus*) eggs with TCPP or TDCPP to determine their effects on 1) pipping success, 2) embryonic growth and development, 3) chemical accumulation in whole egg contents and specific tissues, 4) hepatic mRNA expression levels of genes associated with xenobiotic metabolism, the TH pathway, lipid metabolism and growth, and 5) T_4 and triiodothyronine (T_3) levels in plasma and thyroid glands. This study assessed numerous levels of biological organization with the goal of linking potential molecular/biochemical changes to overt phenotypic effects.

2.3. Materials and Methods

2.3.1 Chemicals and solutions

TCPP (CAS#13674-84-5; 97% purity) was purchased from Pfaltz and Bauer (Waterbury, CT) and TDCPP (CAS#13674-87-8; >95% purity) was purchased from TCI America (Portland, OR). Stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON). Working solutions were prepared to yield nominal doses of 10, 100, 1000, 10000 and 50000 ng TCPP/g egg and 10, 10000 and 50000 ng TDCPP/g egg. Chemical analysis of the working solutions (described below) indicated that the actual dosing concentrations were close to the desired concentrations; they ranged from 12 to 51600 ng TCPP/g egg and 9 to 45000 ng TDCPP/g egg (Table 2.2).

The internal standard for liquid chromatography-electrospray ionization (+)-tandem quadrupole mass spectroscopy (LC-ESI(+)-MS-MS), d_{27}-tributyl phosphate (d_{27}-TBP), was purchased from Cambridge Isotope Laboratories (Andover, Cambridge, MA). Dichloromethane and n-hexane were purchased from Caledon Laboratories (Georgetown, ON) and HPLC grade methanol was purchased from Sigma-Aldrich.

2.3.2 Egg injection and tissue collection

Three egg injection studies were conducted following protocols approved by the Animal Care Committee at the National Wildlife Research Centre (Environment Canada) using fertilized, unincubated, white Leghorn chicken eggs

obtained from the Canadian Food Inspection Agency (Ottawa, ON). Studies **1** and **2** determined the concentration-dependent effects of TCPP and TDCPP on pipping success, embryonic development, tissue-specific accumulation, hepatic mRNA expression and TH levels. Study **1** included the following groups: DMSO vehicle control (*n*=23), 12 ng TCPP/g egg; (*n*=21), 90 ng TCPP/g egg (*n*=21), 928 ng TCPP/g egg (*n*=21), 9240 ng TCPP/g egg (*n*=21) and 51600 ng TCPP/g egg (*n*=21). Treatment groups for study **2** were DMSO vehicle control (*n*=20), 9 ng TDCPP/g egg (*n*=17), 7640 ng TDCPP/g egg (*n*=19) and 45000 ng TDCPP/g egg (*n*=41). Study **3** was conducted to determine the concentration of TCPP and TDCPP in whole egg contents throughout incubation following injection of either 51600 ng TCPP/g egg (*n*=5) or 50222 ng TDCPP/g egg (*n*=5); the contents of a random egg from each treatment group were collected on days 0 (unincubated), 5, 11, 18 and 19 post-injection and stored at -20°C until chemical analysis.

Egg injections were performed as previously described (Cassone *et al.* 2012). For studies 1 and 2, embryos that pipped (day 20-22 of incubation) were euthanized by decapitation and the following measurements were recorded: time to pip, embryo mass, yolk sac mass, liver mass, tarsus length, gallbladder length and head plus bill length (head+bill: back of head to tip of bill). Pipping success was calculated by dividing the number of embryos that pipped (by day 22) by the total number of fertile eggs. Embryos that did not make a pipping star by the end of day 22 were considered unfit to hatch and were included in the pool of dead embryos, which were excluded from all downstream analyses. Significant differences in morphological/developmental endpoints among groups were

determined using a one-way analysis of variance (ANOVA) followed by a Bonferroni's *t*-test for multiple comparisons versus the vehicle control (GraphPad Prism v. 5.02). Changes were considered statistically significant if $p<0.05$.

Blood samples for studies 1 and 2 were collected from every individual and mixed with 10 µL of heparin (0.2 mg/ml) and centrifuged (14000 rpm, 10 mins) to separate plasma from red blood cells. Plasma samples were stored at -20°C until subsequent free T_4 and T_3 determination. The left lobe of the liver was collected from the first eight individuals to pip in each group, flash-frozen in liquid nitrogen and stored at -80°C for RNA extraction. The yolk sac, right lobe of the liver and left cerebral hemisphere were collected from the same eight individuals and stored at 4°C; yolk sacs and subsamples of liver and cerebral hemisphere were pooled for chemical analysis of TCPP and TDCPP. Finally, both thyroid glands were collected from the same eight individuals and stored at -20°C until subsequent TH determination.

2.3.3. Determination of TCPP and TDCPP concentrations

The method for quantification of organophosphate triesters described by Chen and colleagues (2012) was used to determine tissue concentrations of TCPP and TDCPP. In brief, approximately 0.5 g of tissue was homogenized with diatomaceous earth (J.T. Baker, NJ) and spiked with 5 ng of internal standard d_{27}-TBP. TCPP and TDCPP were extracted from the sample (Dionex ASE 200, Sunnyvale, CA) with 50:50 dichloromethane:hexane. The extract was cleaned and separated on a 1 g ISOLUTE aminopropyl silica gel SPE column (Biotage,

Charlotte, NC) packed into a 6 mL Supelclean^{TM} glass cartridge (Sigma–Aldrich). The eluant was dried, suspended in 200 µL methanol, and filtered through a centrifugal filter (0.2 µm Nylon membrane, 500 µL; VWR, Mississauga, ON). A 10 µL aliquot of the resulting filtrate was used for instrumental analysis. The resulting filtrates for samples from study **3** were diluted 100-250 times prior to chemical quantification of TCPP and TDCPP.

TCPP and TDCPP were determined in sample fractions using a Waters 2695 high performance liquid chromatography (HPLC) system coupled to a Waters QuattroUltima tandem quadrupole mass spectrometer (Milford, MA) and equipped with a Waters Xterra® C_{18} column (2.1 mm ×100 mm, 3.5 µm particle size). The mobile phases consisted of water (A) and methanol (B), both spiked with 0.1% formic acid (v/v). The elution gradient was as follows: 5% B increased linearly to 70% B over 3 min then to 80% B over 12 min, followed by a linear increase to 95% B over 3 min and held for 12 min. Finally, it was reduced to 5% B within 1 min and held for 15 min. The mass spectroscopy system was equipped with an electrospray ionization probe operated in positive mode. The detection and quantification of analytes was performed in selected reaction monitoring mode using the most abundant parent and daughter ions for each flame retardant.

Procedural blanks containing only diatomaceous earth spiked with d_{27}-TBP (n = 3) were included to monitor for background contamination. TCPP and TDCPP levels in the blanks were subtracted from tissue sample fractions to yield the reported tissue concentrations. The method limits of quantification in egg

samples were previously determined to be 0.20 ng/g ww for TCPP and 0.06 ng/g ww for TDCPP (Chen *et al.* 2012).

2.3.4. RNA isolation and cDNA synthesis

Total RNA was isolated from ~30 mg of the left lobe of the liver (*n* =8 per treatment group) using Qiagen RNeasy mini kits according to the manufacturer's instructions. The concentration and purity of extracted RNA was quantified by determining the A260/A280 absorbance ratio with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Approximately 2 µg of total RNA was DNase treated using DNA-free kits as per the manufacturer's instructions (Ambion, Austin, TX). Samples with an A260/A280 absorbance ratio above 1.7 were reverse transcribed to complimentary DNA (cDNA) using SuperScript II and random hexamer primers as described by the manufacturer (Invitrogen, Burlington, ON). A 1:5 dilution of cDNA was prepared with diethylpyrocarbonate (DEPC)-treated water and stored at -80°C for subsequent real-time reverse transcription PCR (real-time RT-PCR).

2.3.5. Real-Time RT-PCR

All real-time RT-PCR assays were performed using Brilliant Multiplex QPCR Mastermix kits (Agilent Technologies, #600553) and run on a Stratagene Mx3000P PCR instrument (La Jolla, CA). Primer pairs (Invitrogen) and TaqMan fluorogenic probes (Biosearch, Novato, CA) were previously designed and optimized for real-time RT-PCR (Table 2.1). Each reaction contained 1X Brilliant

Multiplex Mastermix, 30 nM ROX reference dye, forward and reverse primers, 200 nM of each probe, 5 µL of diluted cDNA (1:5) and DEPC water to reach a reaction volume of 25 µL. The thermocycle program consisted of an enzyme activation step at 95°C for 10 mins followed by 40 cycles of 95°C for 30 seconds, and 60°C for 1 min. All reactions were performed in duplicate for eight individual liver samples per treatment group. No template controls (NTC) and no reverse transcriptase controls were run to monitor contamination of reagents and RNA, respectively.

Table 2.1. List of transcripts assessed by real-time RT-PCR in liver tissue of chicken embryos exposed to TCPP and TDCPP *in ovo.*

Gene	Accession number	Reference
β-actin	X00182	Hickey *et al.* (2009)
Type I iodothyronine 5'-deiodinase (D1)	NM_001097614	Crump *et al.* (2008)
Type II iodothyronine 5'-deiodinase (D2)	NM_204114	Crump *et al.* (2010)
Type III iodothyronine 5'-deiodinase (D3)	NM_001122648	Vongphachan *et al.* (2011)
Cytochrome P450 2H1 (CYP2H1)	NM_001001616	Crump *et al.* (2008)
Cytochrome P450 3A37 (CYP3A37)	NM_001001751	
Uridine 5'-diphospho-glucuronosyltransferase 1A9 (UGT1A9)	XM_421883	
Transthyretin (TTR)	NM_205335	
Liver fatty acid-binding protein (L-FABP)	AF380998	
Insulin-like growth factor-1 (IGF-1)	NM_001004384	Crump *et al.* (2010)

All transcripts, except for transthyretin (TTR), were assessed using triplex assays that included β-actin as the invariable control gene as follows: CYP3A37 + type II iodothyronine deiodinase (D2), CYP2H1+ type III iodothyronine

deiodinase (D3), UDP glucuronosyltransferase 1A9 (UGT1A9) + insulin-like growth factor-1 (IGF-1), and L-FABP + D1. TTR was run in duplex with β-actin. A standard curve was generated for all assays from a 1:2 dilution series of cDNA to ensure that reaction efficiencies of all genes were similar to that of β-actin.

β-actin expression was consistent across all treatment groups and was therefore an effective control gene to which all cycle threshold (Ct) data were normalized. The fold change in target gene mRNA expression for treatment groups compared to the DMSO control was calculated using the $2^{-\Delta Ct}$ equation (Schmittgen and Livak 2008). Significant differences in mRNA expression were determined using a one-way ANOVA of $2^{-\Delta Ct}$-transformed data followed by a Bonferroni correction for multiple comparisons to identify concentrations that elicited a significant effect relative to the control ($p<0.05$). In cases where the assumption of equal variance was violated (Bartlett's test $p<0.05$; CYP3A37, CYP2H1 and L-FABP), a Kruskal-Wallis test followed by a Dunn's multiple comparisons test was performed instead of the ANOVA (GraphPad Prism v. 5.02).

2.3.6. Determination of thyroid hormone concentrations

Plasma free T_4 levels were determined using AccuBind Free T_4 kits (Monobind Inc., Lake Forest, CA) as per the manufacturer's instructions. The same 8 individuals per group that were analyzed by real-time RT-PCR were selected for free T_4 determination; any further additions to the sample size were chosen at random. The TCPP dose groups included: DMSO control ($n=9$), 12

ng/g egg (*n*=9), 90 ng/g egg (*n*=9), 928 ng/g egg (*n*=10), 9240 ng/g egg (*n*=9) and 51600 ng/g egg (*n*=10). The TDCPP dose groups assessed were: DMSO control (*n*=15), 9 ng/g egg (*n*=13), 7640 ng/g egg (*n*=12) and 45000 ng/g egg (*n*=28). Free T_4 concentrations (ng/dl) were calculated from a standard curve generated using the 6 reference samples (fit with a 4 parameter variable slope model; GraphPad Prism v. 5.02). The limit of detection (LOD) was 0.05 ng/dl; values equal or less than this were assigned a value of 0.035 (LOD/ $\sqrt{2}$). A one-way ANOVA followed by a Bonferroni correction was used to determine significant differences in free T_4 concentrations between the treatment groups ($p<0.05$).

Free T_3 levels in plasma were determined using AccuBind Free T_3 kits (Monobind Inc., Lake Forest, CA) as per the manufacturer's instructions, and analysed as described above for T_4. The dose groups for TCPP were: DMSO control (*n*=9), 12 ng/g egg (*n*=8), 90 ng/g egg (*n*=8), 928 (*n*=8), 9240 ng/g egg (*n*=7) and 51600 ng/g (*n*=10). For TDCPP, the following groups were included for T_3 determination: DMSO control (*n*=8), 9 ng/g egg (*n*=7), 7640 ng/g egg (*n*=6), and 45000 ng/g (*n*=8).

Extraction of THs from thyroid glands was performed using a method modified from McNabb *et al.* (2004). Briefly, thyroid glands were homogenized (Retsch MM301 Mixer Mill, Newtown, PA) in 100 µL of digestion medium (0.605 g Tris base, 40 g urea, 1 mL Triton X-100 and 100 mL H_2O titrated to pH 8.0) containing 7 mg of Pronase E (Sigma-Aldrich, St. Louis, MO) per gram of thyroid, and incubated at 37°C for 24hr. Extraction was continued by adding 1 mL of

absolute ethanol and incubating at -20°C for 24 hr. Samples were then centrifuged and the supernatant was collected. Presumably all bound T_4 became free so TH measurement was of total T_4 in thyroid. Prior to total T_4 determination (Accubind Free T_4 kit; described above), samples were diluted 10× to 50× depending on the thyroid mass. A total of eight individuals were included for all dose groups with the exception of the DMSO (*n*=7) and 12 ng/g (*n*=7) dose groups from study **1**.

2.4. Results

2.4.1. Pipping Success

Neither TCPP nor TDCPP affected the pipping success of chicken embryos up to the highest administered dose (HD = 51600 ng TCPP/g or 45000 ng TDCPP/g; Table 2.2). The minimum pipping success in the HD TDCPP group was 78%, which falls within the range of values previously observed for DMSO-treated chicken embryos (Cassone *et al.* 2012; Crump *et al.* 2011; O'Brien *et al.* 2009). TCPP significantly delayed pipping time at the 2 highest doses tested. The average DMSO-treated embryo required 495 hr (20.6 days) to pip, whereas embryos exposed to 9240 or 51600 ng TCPP/g required an additional 13 or 17 hr, respectively (Fig. 2.1). TDCPP-exposed embryos also showed a trend towards delayed pipping with increasing treatment, with a maximum 10 hr delay at 7640 ng/g; however, this delay was not statistically significant (Fig. 2.1).

Table 2.2. Concentrations of TCPP and TDCPP in liver, cerebral hemisphere and yolk sac sampled from chicken embryos 20-22 days post-injection and the effects of exposure on pipping success.

Flame retardant (FR)	Injected [FR] (ng/g egg)	Liver [FR] (ng/g ww)	Cerebral Hemisphere [FR] (ng/g ww)	Yolk sac [FR] (ng/g ww)	Pipping Success	
					Ratio	%
TCPP	<0.2	<0.2	2.3	<0.2	20/23	87
	12	<0.2	5.8	2.6	19/21	90
	90	1.1	3.5	10	17/18	94
	928	2.8	1.5	8.7	18/20	90
	9240	1.1	1.5	6.2	16/20	80
	51600	4.8	0.7	3.6	16/19	84
TDCPP	<0.06	<0.06	<0.06	0.9	17/19	89
	9	0.6	0.6	1.0	15/17	88
	7640	1.4	8.1	54	14/17	82
	45000	2.0	15	100	31/40	78

Note. Tissue samples were pooled from eight individuals prior to chemical analysis. Concentrations of working solutions and tissue concentrations were determined by HPLC-MS/MS. Pipping success is the number of embryos that pipped by day 22 of incubation divided by the total number of fertile embryos.

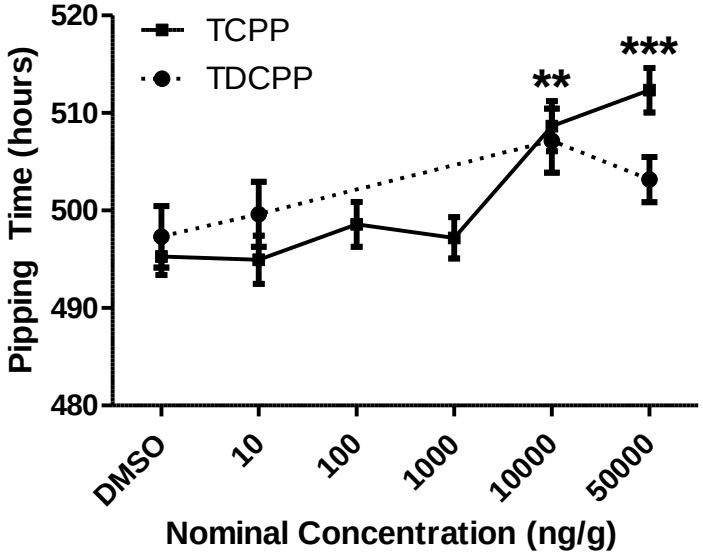

Figure 2.1. Effects of *in ovo* TCPP and TDCPP exposure on pipping time of chicken embryos. Pipping time is the number of hours of incubation required by the embryo to form a pipping star. Significant delays are indicated for TCPP relative to the DMSO control. Error bars represent the standard error of the mean (SEM; n=16-20; **p<0.01, ***p<0.001).

2.4.2. Embryonic Development

Both TCPP and TDCPP had an effect on at least one of the morphological endpoints measured. HD TCPP caused a significant reduction in tarsus length (24.2 vs. 25.4 mm) and a dose-dependent increase in liver somatic index (LSI) (Fig. 2.2 A and B). Embryos exposed to HD TDCPP had a significantly reduced mass (26.8 vs. 28.7 g) and shorter head+bill (26.3 vs. 27.6 mm) compared to the DMSO group (Fig. 2.2 C and D).

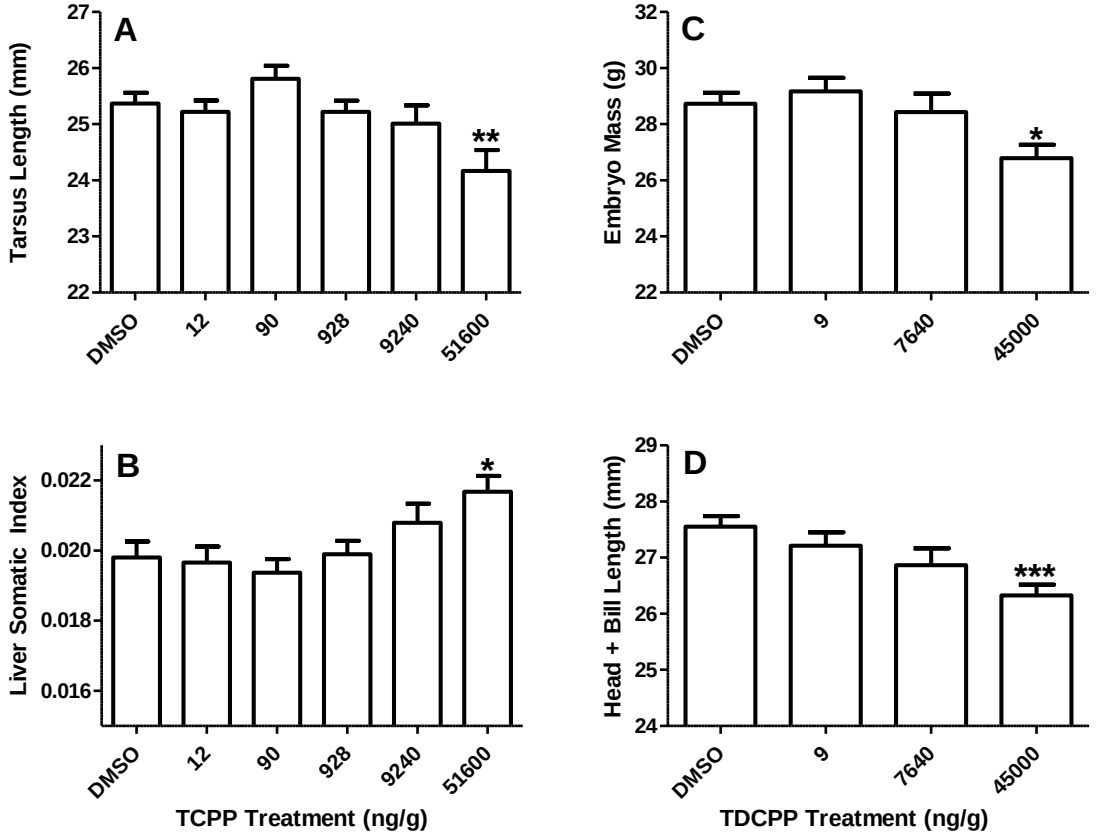

Figure 2.2. Morphometric effects of *in ovo* TCPP exposure on (A) tarsus length and (B) liver somatic index (LSI) and TDCPP exposure on (C) embryo mass and (D) head plus bill length in chicken embryos. Endpoints were determined for all embryos that pipped. LSI = liver mass/embryo mass. Error bars represent the SEM and significant changes are indicated relative to the DMSO control (n=14-31; *p<0.05, **p<0.01, ***p<0.001).

There was also a significant reduction in gallbladder size of embryos treated with HD TDCPP (Fig. 2.3 A); 4/31 embryos in the HD group did not have a gallbladder (determined by visual inspection). Where measurements could be made, the gallbladder of a HD TDCPP-treated embryo was, on average, 42% of the size of a gallbladder from the DMSO group. There was a significant positive correlation between gallbladder size and embryo mass (Fig. 2.3 B; Pearson correlation $p<0.05$).

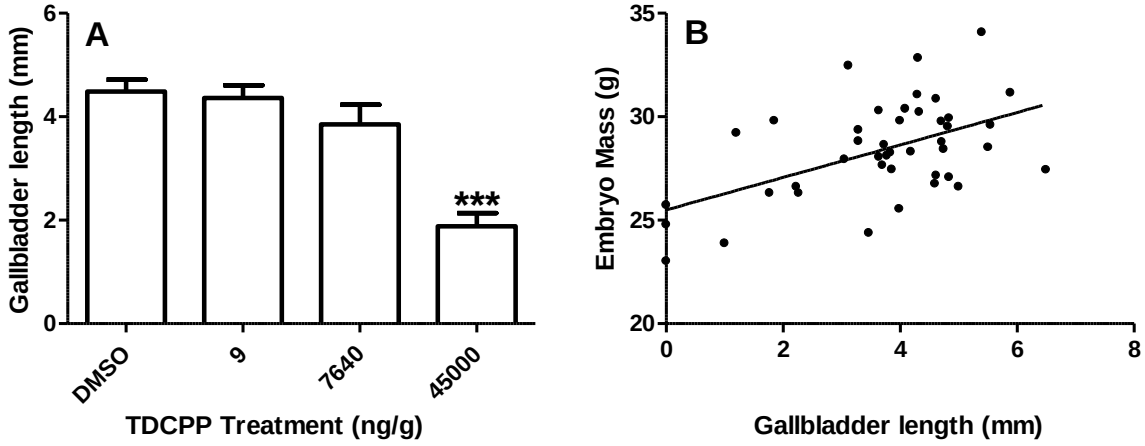

Figure 2.3. Effects of TDCPP exposure on the gallbladder size of developing chicken embryos. (A) The reduction in gallbladder size with increasing TDCPP treatment. Error bars represent the SEM (n=8-11; ***$p<0.001$). (B) The correlation between gallbladder size and embryo mass (significant Pearson correlation; $p<0.05$). The solid line depicts a linear regression with an R-square=0.27.

2.4.3. TCPP and TDCPP concentrations

*i) Liver, cerebral hemisphere, and yolk sac (studies **1** and **2**)*

TCPP was detected in the liver, cerebral hemisphere and yolk sac for all dose groups except the 12 ng/g group; however, tissue concentrations were not correlated with the injected concentrations (Table 2.2). There was a positive

correlation between injected TDCPP concentrations and tissue concentrations (Table 2.2), but this was not statistically significant; concentrations increased in a dose-dependent manner for all tissues reaching a maximum of 2.0, 15 and 100 ng/g ww for the liver, cerebral hemisphere and yolk sac, respectively, in the highest dose group. These values are 22500, 3000, and 450 times lower than the injected dose of 45000 ng/g.

ii) *Egg contents (study **3**)*

The contents of chicken eggs that were injected with 51600 ng TCPP/g egg or 50222 ng TDCPP/g egg contained 64876 ng TCPP/g or 46589 ng TDCPP/g on day 0 (>92% of the injected concentrations; Fig. 2.4). TCPP and TDCPP concentrations decreased to 26713 ng/g and 18951 ng/g by day 11, and were further reduced to 1 ng/g and 5 ng/g by day 19 (<1% of the injected concentrations).

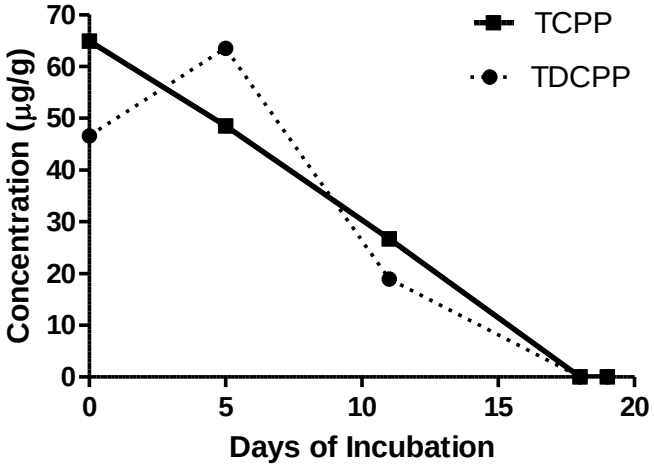

Figure 2.4. Effects of incubation time on concentration of TCPP and TDCPP in entire chicken egg contents following injection of 51.6 µg TCPP/g egg or 50.2 µg TDCPP/g egg into the air cell at day zero. Day zero eggs were sampled 3 hours post-injection without incubation; the remaining eggs were incubated until day 5, 11, 18 or 19 (*n*=1/day).

2.4.4. Hepatic mRNA expression

Of the nine mRNA transcripts assessed, only three were significantly affected by TCPP treatment (Fig. 2.5). There was a dose-dependent increase in D1 mRNA expression following TCPP exposure, with a significant two-fold induction at the HD. Hepatic L-FABP mRNA expression was significantly induced 2.7-fold and CYP3A37 was up-regulated 4.5-fold in the TCPP HD treatment group; no significant effects were observed at the lower treatment concentrations. Two phase I metabolizing enzymes, CYP3A37 and CYP2H1, were significantly induced 7.9-fold and 2.1-fold, respectively, in embryos treated with HD TDCPP (Fig. 2.6). No significant changes in hepatic mRNA expression were observed for the other transcripts (D2, D3, TTR and UGT1A9) in chicken embryos exposed to TCPP or TDCPP (data not shown).

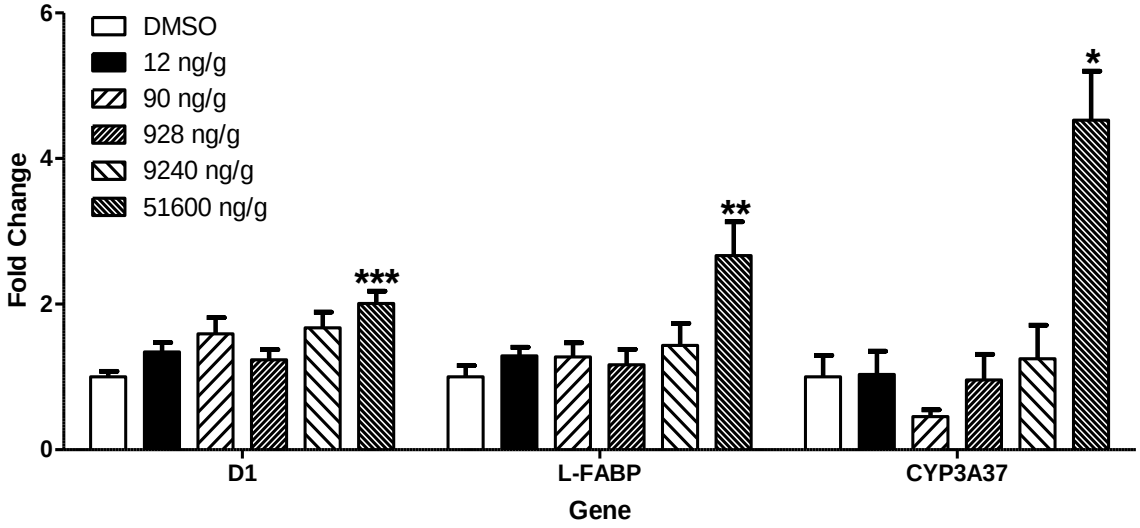

Figure 2.5. Hepatic mRNA expression of thyroid hormone-responsive genes (D1, L-FABP) and a xenobiotic metabolizing enzyme (CYP3A37) in chicken embryos exposed to TCPP. Injected concentrations are in ng TCPP/g egg. Fold changes are presented relative to the DMSO vehicle control, as are significant changes in expression. Error bars represent SEM (n=8; *p<0.05, **p<0.01, ***p<0.001).

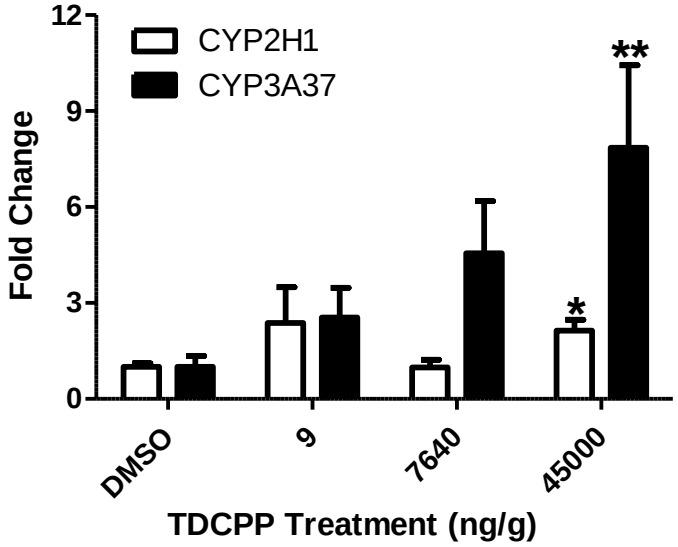

Figure 2.6. Effect of TDCPP exposure on hepatic mRNA expression of two xenobiotic metabolizing enzymes, CYP2H1 and CYP3A37, in chicken embryos. Fold changes are presented relative to the DMSO vehicle control, as are significant changes in expression. Error bars represent SEM (n=8, *p<0.05, **p <0.01).

2.4.5. Thyroid Hormone Status

TDCPP-exposed chicken embryos had lower plasma free T_4 levels relative to controls at all concentrations tested; however, only the decrease at the 7640 ng/g dose was statistically significant (Fig. 2.7). There were no significant effects

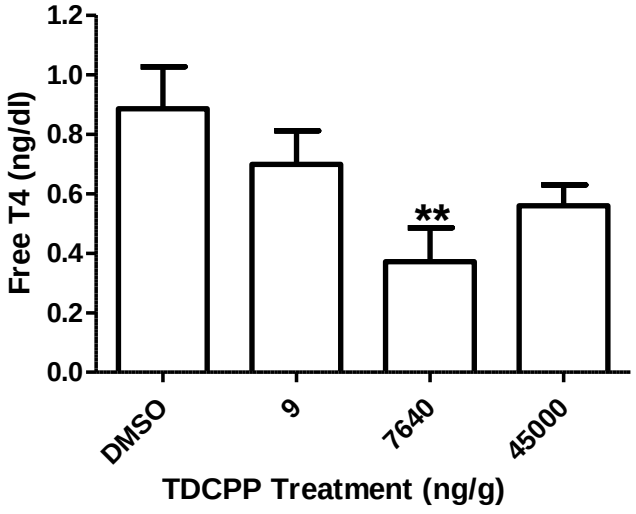

Figure 2.7. Free plasma thyroxine (T_4) levels of chicken embryos exposed to increasing concentrations of TDCPP. Significant changes are indicated relative to the DMSO control. Error bars represent the SEM (n=12-28, **p<0.01).

on plasma free T_4 levels of TCPP-exposed embryos. Neither flame retardant caused significant change in plasma free T_3 nor thyroid gland total T_4 concentrations in any of the dose groups assessed (data not shown).

2.5. Discussion

To our knowledge, this is the first study to investigate the effects of embryonic exposure to TCPP or TDCPP in an avian species. We determined the effects of *in ovo* exposure on pipping success, morphological growth parameters, hepatic mRNA expression and thyroid hormone levels in chicken embryos at a wide range of doses; the lowest dose was similar to concentrations detected in wild avian species (Chen *et al.* 2012; Leonards *et al.* 2011). Due to the rise in production volume of these OPFRs (Stapleton *et al.* 2009; van der Veen and de Boer 2012) and their persistence in the environment and biota, it is important to understand the biological and toxicological implications of exposure.

Neither TCPP nor TDCPP elicited a lethal response to treatment following injections up to 51.6 µg/g egg and 45 µg/g egg, respectively. These maximal injected concentrations were lower than the minimum acute LD_{50} values previously determined in rats: 1017 µg TCPP/g body weight (bw) and 2250 µg TDCPP/g bw (IPCS 2004). The only avian studies available for these FRs investigated neurotoxic potential and were based on post-hatch exposures (IPCS 2004). Exposure of hens to two separate doses of 13200 µg TCPP/ g bw was not lethal (Sprague *et al.* 1981) whereas a five-day oral exposure to 4800 µg TDCPP/g/day bw in chickens caused 100% mortality (Ulsamer *et al.* 1980). No

mortality was observed, however, after a 90-day oral exposure up to 100 µg TDCPP/g/day (IPCS 2004).

In the present study, delays in pipping up to 17 and 10 hours were observed following TCPP and TDCPP exposure, respectively. This finding could have variable consequences based on the parent-young relationship at hatching. Precocial birds, such as chickens, tend to hatch synchronously and leave the nest soon after hatching (Nice 1962). Delayed pipping could result in a late hatchling being (a) abandoned and/or (b) unable to compete for resources due to its smaller size. It is well established that THs peak in the perihatch period, stimulating metabolic and developmental processes necessary for pipping (McNabb 2007), and numerous studies have demonstrated the correlation between hypothyroidism and delayed hatching (Balaban and Hill 1971; Decuypere and Kûhn 1988; Haba *et al.* 2011).

Both TCPP and TDCPP affected at least one of the morphological endpoints measured. The tarsus length of embryos exposed to HD TCPP was significantly reduced relative to controls. King and May (1984) emphasized the importance of THs in the final four days of incubation on chicken embryo growth; exposure to goitrogens late in incubation caused a 35% decrease in leg growth (King and Delfiner 1974). HD TCPP also increased LSI, which is a general indicator of metabolic energy demands that is sensitive to environmental contamination (Adams *et al.* 1993) and is viewed as an adaptive response to increase the detoxification capacity of the liver (Goede and Barton 1990). TDCPP treatment led to a significant reduction of head+bill length and embryo

mass. Comparable results have been observed for TDCPP in other species; prenatal exposure in rats caused an increase in fetal death and a decrease in maternal body weight at 400 µg/g/day (Tanaka *et al.* 1981), and exposure to ≥50 µg/L decreased body weight in zebrafish (Wang *et al.* 2013).

The most pronounced effect of TDCPP exposure was the reduction in gallbladder size of HD-treated embryos. Four out of thirty-one embryos did not develop a gallbladder, and those that had gallbladders large enough to be measured were approximately 42% the size of those from the DMSO control. Only one incidence of gallbladder agenesis has been reported in an avian species and was caused by the potent carcinogen diethylnitrosamine (Williams *et al.* 2011). Studies on mice did not report any effect of TDCPP on the gallbladder at doses well above those tested in this study (Kamata *et al.* 1989). Bile acids, which are stored and concentrated in the gallbladder, aid in the digestion and absorption of fatty acids (Schmidt and Ivy 1937). A reduction in bile flow could help explain the depressed growth in TDCPP-treated chicken embryos; the inability to utilise the available lipid resources in the yolk efficiently would reduce the embryo's energy supply, thereby hindering its growth. Gallbladder size was positively correlated with embryo mass in this study (Fig. 2.3) and embryos without gallbladders had some of the lowest body weights. This finding is consistent with a previous study that associated reductions in chicken embryo weight and tarsus length with a reduced yolk lipid uptake (Feast *et al.* 1998). Further research on the mechanism(s) of gallbladder development in birds is

warranted in order to understand the nature of the disruption caused by TDCPP exposure.

Concentrations of TCPP and TDCPP in liver, cerebral hemisphere and yolk sac were surprisingly low relative to injected concentrations. The lack of correlation between TCPP tissue concentrations and injected concentrations can be attributed, in part, to TCPP contamination associated with sample preparation. For example, the concentration of TCPP in the DMSO dosing solution was below the limit of detection whereas the concentration in cerebral hemispheres from the DMSO group was 2.3 ng/g (Table 2.2). This indicates that the TCPP detected in cerebral tissue did not originate from the DMSO dosing solution but through the sample preparation (e.g. solvents used during extraction). Furthermore, because residual tissue concentrations of TCPP were so low, they effectively fell within the background levels of TCPP, further obscuring the relationship between injected concentrations and tissue concentrations. A time course study (study **3**) was carried out to determine whether the low tissue concentrations at pipping were due to (a) rapid metabolism of the parent compounds during development, and/or (b) preferential accumulation in tissues that were not examined. More than 92% of the injected FR was detected in the egg content on day zero of injection; however, by day 19 less than 1% of the parent compound was detected. The metabolic function of the liver is established in chicken embryos by day seven of incubation (Sandstrom and Westman 1971). This supports the likelihood that enzyme-mediated metabolism of TCPP and TDCPP occurred between days 5 to 19 of incubation leading to extremely low tissue residue concentrations relative to

the injected doses in studies **1** and **2**. It is remarkable that the developing chicken embryo was able to metabolise/eliminate almost 2.5 mg of FR (50000 ng FR/g egg; average egg ≈ 50g), especially considering that TCPP and TDCPP have been detected in wild avian eggs (Chen *et al.* 2012; Leonards *et al.* 2011) that were likely exposed to far lower quantities through maternal transfer. It is possible that the enzymes necessary for complete biotransformation are only activated above a certain exposure threshold that is not reached at current environmental levels. The rapid metabolism of these FRs in chicken embryos agrees with studies performed in rats in which >95% of TCPP or TDCPP was eliminated within 7 days of treatment (Lynn *et al.* 1981; Minegishi *et al.* 1988).

In addition to the determination of phenotypic alterations caused by TCPP or TDCPP exposure, we assessed effects on hepatic mRNA levels of genes associated with xenobiotic metabolism, TH homeostasis and lipid metabolism; these genes have previously been identified as responsive to flame retardant exposure (Crump *et al.* 2008; Crump *et al.* 2010; Crump *et al.* 2011). Furthermore, developmental effects observed in this study and endocrine effects, observed in previous studies on TDCPP (Liu *et al.* 2012; Meeker and Stapleton 2010; Wang *et al.* 2013), supported the determination of TH levels in plasma and thyroid gland.

CYP3A37 and CYP2H1 are phase I xenobiotic metabolizing enzymes regulated by the chicken xenobiotic receptor (Podvinec *et al.* 2002). The mRNA levels of CYP3A37 and CYP2H1 were increased after TDCPP exposure, whereas TCPP only induced CYP3A37. Our findings are consistent with a recent

avian *in vitro* study, which demonstrated that CYP3A37 was the most responsive gene to TCPP and TDCPP exposure in CEH (Crump *et al.* 2012). Induction of these CYPs is associated with enhanced biotransformation of xenobiotic compounds (Goriya *et al.* 2005) and is likely what led to the almost complete elimination of TCPP and TDCPP. Ideally, CYP3A37 enzyme activity would be monitored throughout incubation to confirm this hypothesis, but an avian CYP3A37 activity assay has not yet been developed in our laboratory. An increase in LSI has also been associated with activation of CYPs (Huuskonen and George 1995), an observation that is consistent with TCPP-exposed embryos in this study.

D1, one of three deiodinase enzymes assessed, was up-regulated in response to TCPP treatment. Induction of hepatic D1 often indicates an increased conversion of T_4 to T_3 to maintain circulating TH levels (Darras *et al.* 2006). In rats, a hypothyroid state is associated with decreased hepatic D1 activity (Santini *et al.* 1993). The TH-deiodinase relationship in birds however, is less clear; some have observed transient increases in expression of D1 with reduced plasma TH levels (Beck *et al.* 2006), while others have seen a marked decrease in D1 expression (Gould *et al.* 1999). Finally, L-FABP, a lipid-binding protein involved in fatty acid transport, uptake and metabolism (Wang *et al.* 2006), was induced by TCPP treatment. L-FABP mRNA levels were responsive to T_3 administration in hypothyroid rats (Iwen *et al.* 2001) and were higher in hyperthyroid vs. hypothyroid chickens (Cogburn *et al.* 2003). The disruption in expression of TH-responsive genes and genes involved in metabolism may

explain the observed delay in pipping of TCPP-exposed embryos; Willemsen *et al.* (2011) associated changes in metabolic rate during late incubation to delays in hatching.

The TH pathway serves numerous functions, including metabolic maintenance and pipping initiation, and is essential for normal growth and development in birds (McNabb 2007). TCPP did not affect plasma or glandular THs at pipping in this study; however, disruption in TH levels is not always apparent at external pipping (first break in the shell). For example, chicken embryos exposed to polychlorinated biphenyl-77 had reduced plasma TH levels and were delayed in pipping by 12 hr (Roelens *et al.* 2005); plasma T_3 levels were significantly reduced at internal pipping (penetration of the inner membrane), but were restored to normal by external pipping (12 to 24 hr later). It is possible that the internal pipping stage of TCPP-exposed embryos was prolonged due to a disruption of the TH pathway; however, TH levels were only measured at external pipping thereby potentially overlooking earlier disruption. Conversely, a significant reduction in circulating free T_4 levels was observed in TDCPP-treated embryos, albeit only at 7640 ng/g. The slight increase at 45000 ng/g, compared to 7640 ng/g, might be considered non-monotonic, which would not be unusual among endocrine disrupting chemicals (Vandenberg *et al.* 2012); however, the T_4 level at the HD remains well below the DMSO-control and does not likely reflect a reverse in trend. The depleted T_4 levels may have been associated with the observed reduction in mass and head+bill length, and because numerous organ systems depend on THs for tissue-specific

differentiation (McNabb 2007), it may have also hindered gallbladder development. Furthermore, studies have shown hypothyroidism to alter bile composition due to effects on lipid metabolism (Andreini *et al.* 1994; Day *et al.* 1989) and reduce bile flow in mammals (Laukkarinen *et al.* 2002; Laukkarinen *et al.* 2003), which could have led to the reduced gallbladder size. The endocrine disrupting potential of TDCPP has previously been observed in humans and in fish. Meeker *et al.* (2010) associated TDCPP concentrations in dust with an increase in serum prolactin levels and a decrease in free plasma T_4 of the household residents. A recent study on zebrafish (Liu *et al.* 2012) found that TDCPP disrupted the balance of sex hormones such as 17β-estradiol and testosterone and affected the expression of vitellogenin. Our observations of reduced growth and free T_4 levels in TDCPP-exposed chicken embryos support the existing pool of evidence that marks TDCPP as a potential endocrine disrupting chemical.

In conclusion, no adverse morphological or developmental effects were observed at environmentally-relevant doses of TCPP (9 ng/g egg) or TDCPP (12 ng/g egg). However, at injected concentrations 3 orders of magnitude higher, TCPP increased LSI, delayed pipping time, reduced tarsus length and altered genes associated with xenobiotic metabolism, the TH-axis and lipid metabolism. TDCPP exposure impaired embryo growth, gallbladder development and plasma T_4 levels and affected the mRNA levels of phase I metabolizing enzymes. Furthermore, we showed that even at the highest administered concentration, TCPP and TDCPP were almost completely depleted *in ovo* by day 19 of

incubation. Although the present study suggests that current environmental levels are unlikely to cause adverse effects to avian embryo development, the endocrine disrupting potential of these compounds warrants further investigation. To further understand the link between the observed molecular/biochemical disturbances and adverse phenotypic outcomes, a genome-wide microarray expression analysis was performed; the results of which are discussed in chapter three.

CHAPTER THREE

TRIS(1,3-DICHLORO-2-PROPYL) PHOSPHATE PERTURBS THE EXPRESSION OF GENES INVOLVED IN IMMUNE RESPONSE AND LIPID AND STEROID METABOLISM IN CHICKEN EMBRYOS

Modified from Farhat, A., Buick, J.K., Williams, A., Yauk, C.L., O'Brien, J.M., Crump, D., Chiu, S., and Kennedy, S.W. (2013) Tris(1,3-dichloro-2-propyl) phosphate perturbs the expression of genes involved in immune response and lipid and steroid metabolism in chicken embryos. Submitted to *Toxicology and Applied Pharmacology* for review.

3.1. Abstract

We previously demonstrated that *in ovo* exposure to the flame retardant tris(1,3-dichloro-2-propyl) phosphate (TDCPP) decreased plasma thyroxine levels, reduced growth parameters and decreased gallbladder size in chicken embryos. In the current study we used DNA microarrays to evaluate global messenger RNA (mRNA) expression in liver tissue of male chicken embryos that exhibited the above mentioned effects. Injected doses were dimethyl sulfoxide vehicle control, 7.6 or 45 µg TDCPP/g egg. TDCPP caused significant changes in the expression of five genes at the low dose and 47 genes at the high dose (False Discovery Rate $p \leq 0.1$, fold change≥ 1.5). The gene expression analysis suggested a compromised immune function, a state of cholestatic liver/biliary fibrosis, and disrupted lipid and steroid metabolism. The onset of cholestasis is suggested as being a cause for the observed reduction in gallbladder size. Interactome analyses identified apolipoprotein E (APOE), hepatocyte nuclear factor 4 alpha (HNF4A), and peroxisome proliferator-activated receptor alpha (PPARA) as key regulatory molecules involved in the effects of TDCPP. Given the observed changes in thyroxine (T_4) levels and the known importance of

thyroid hormones (THs) in regulating immune response and lipid metabolism, we speculate that disruption of TH levels may be a key mediator of the observed changes in gene expression. Our results demonstrate a targeted effect of TDCPP toxicity on cholesterol metabolism that helps explain the aforementioned phenotypic effects, as chicken embryos are highly dependant on yolk lipids for growth and maintenance throughout development. Finally, our results are in concordance with the literature that describes TDCPP as a cancer causing agent, since the majority of dysregulated genes were involved in cancer-pathways.

3.2. Introduction

Organophosphate flame retardants (OPFRs) are added to a variety of materials to hinder flame ignition and have been proposed as replacements for brominated flame retardants (van der Veen and de Boer 2012). Although the carbon char formed upon burning of phosphorous-based flame retardants reduces toxic emissions, there are still health concerns regarding the use of some halogenated OPFRs (McPherson *et al.* 2004). TDCPP is a halogenated, high production volume OPFR mainly used to treat flexible polyurethane foam in automotive upholstery, and is also found in household furniture and children's foam products (Dishaw *et al.* 2011). TDCPP has been in use since the 1970s, however, detection frequency and concentration of TDCPP in household furniture has risen since the phase-out of polybrominated diphenyl ethers in 2005 (Stapleton *et al.* 2012). Stapleton *et al.* (2012) showed TDCPP to be the most frequently detected flame retardant in upholstered furniture in the United States,

reaching concentrations of 110.2 mg TDCPP/g foam. TDCPP is not chemically bound to the foam and is semi-volatile, resulting in spontaneous release of the flame retardant from treated products (European Union 2008b). TDCPP accumulates in dust and is the most abundant OPFR detected in indoor dust samples (van der Veen and de Boer 2012), with concentrations reaching 72 µg/g and 326 µg/g in office and vehicle dust, respectively (Carignan *et al.* 2013).

TDCPP is rapidly metabolized in fish, mammals and chicken embryos (European Union 2008b; Farhat *et al.* 2013), but has been detected in a variety of wild biota. For example, levels of TDCPP in herring, perch, mussels, eelpout, and salmon from Swedish lakes and coastal areas ranged from <1.1 to140 ng/g lipid weight (Sundkvist *et al.* 2010). Furthermore, TDCPP concentrations of 1.9 and 0.17 ng/g wet weight (ww) were measured in Norwegian great black-backed gull eggs and Great Lakes herring gull eggs, respectively (Chen *et al.* 2012; Leonards *et al.* 2011).

A risk assessment by the European Commission classified TDCPP as safe for its intended use (European Union 2008b). However, the US Consumer Product Safety Commission estimated that daily exposure to TDCPP exceeds the acceptable daily intake for non-cancer toxicity by two to five times (Babich 2006). Furthermore, TDCPP was classified as a cancer causing agent by the California Environmental Protection Agency (OEHHA 2011a) and is predicted to increase cancer risk at current exposure levels (Babich 2006). TDCPP increased developmental abnormalities in zebrafish embryos (McGee *et al.* 2012), showed neurotoxic properties in cultured neuroendocrine (PC12) cells (Dishaw *et al.*

2011) and caused leg and wing weakness in chickens (Ulsamer *et al.* 1980). Recent studies suggest that TDCPP has endocrine-disrupting potential; it reduced T_4 levels in chicken embryos and zebrafish (Farhat *et al.* 2013; Wang *et al.* 2013), disrupted sex hormone levels in zebrafish (Liu *et al.* 2012) and dysregulated TH-responsive genes in zebrafish (Wang *et al.* 2013) and chicken embryo hepatocytes (Crump *et al.* 2012). It has also been correlated with reduce circulating T_4 and increased prolactin levels in human males (Meeker and Stapleton 2010).

We previously reported that TDCPP exposure in chicken embryos decreased circulating T_4 levels, reduced head plus bill length, embryo mass, and gallbladder size and induced transcription of xenobiotic metabolizing enzymes in the liver (Farhat *et al.* 2013). In the present study we examined global gene expression changes in liver tissue of TDCPP-treated chicken embryos from dose groups that resulted in the aforementioned adverse effects. Our goal was to contribute knowledge on the molecular mechanisms underlying some of the adverse phenotypic effects caused by TDCPP and we hypothesize that disruption of the TH pathway and lipid metabolism are likely mechanisms of TDCPP toxicity.

3.3. Methods

3.3.1. Chemicals and Solutions

TDCPP (CAS#13674-87-8; >95% purity) was purchased from TCI America (Portland, OR) and working solutions were prepared in dimethyl

sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON) to yield concentrations of 7.6 and 45 µg/µL. The concentrations of the dosing solutions were confirmed by high performance liquid chromatography-tandem mass spectrometry as described previously (Farhat *et al.* 2013).

3.3.2. Egg Injection and Tissue Collection

Liver tissue for microarray mRNA expression analysis was collected from a TDCPP egg injection study conducted previously (Farhat *et al.* 2013). In brief, fertilized, unincubated, white Leghorn chicken (*Gallus gallus domesticus)* eggs were injected with either a DMSO vehicle control (*n*=20), 7.6 (*n*=19; low dose=LD) or 45 µg TDCPP/g egg (*n*=41; high dose=HD) via a hole drilled through the eggshell above the air cell. The hole was then sealed with AirPore^{TM} Tape (Qiagen, Mississauga, ON) and eggs were incubated (Petersime, Model XI; Gettysburg, OH) at 37.5°C and 58% humidity until pipping (Day 20-21). Embryos that pipped successfully were euthanized by decapitation. Blood samples were collected in heparin-coated centrifuge tubes, centrifuged to separate plasma from red blood cells (RBC) and stored at -20°C until sex determination. The left lobe of the liver was collected, flash-frozen in liquid nitrogen and stored at -80°C for RNA extraction.

3.3.3 Genetic Sex Determination

An alkaline lysis procedure was used to extract genomic DNA from chicken RBC. RBC (25-50 µL) were added to 500 µL of 1X TE buffer (10 mM

Tris-HCl; 1 mM EDTA [pH 8.0]), centrifuged at 13226 xg for 10 min and the supernatant was discarded. NaOH (100 µL; 0.2 M) was added to the RBC pellet and incubated at 95°C for 10 min with occasional vortexing. Extraction was terminated with the addition of 80 µL of 1 M Tris-Cl (pH 8.0) and tubes were incubated at room temperature for 10 min with occasional vortexing. Finally, the mixture was centrifuged at 13226 xg for 2 min and a 5 µL aliquot was diluted 20X for subsequent PCR analysis using the 1237L/1272H primer set as described elsewhere (Shizuka and Lyon 2008).

3.3.4. RNA Isolation

Total RNA was isolated from ~30 mg of the left lobe of the liver of male chicken embryos (*n*=5 per treatment group) using Qiagen RNeasy mini kits according to the manufacturer's instructions. Approximately 2 µg of total RNA was DNase treated using DNA-free kits as per the manufacturer's instructions (Ambion, Austin, TX). RNA concentration and purity was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and sample integrity was determined with a Bioanalyzer 2100 (Agilent Technologies, Mississauga, ON). Only samples with an A260/A280 ratio >1.8 and a RNA Integrity Number >8 were used for downstream applications. Aliquots of 200 ng RNA were diluted to a total volume of 8.3 µL in RNase-free water. A reference pool of RNA for microarray hybridizations was prepared from equal parts of all samples. Samples were stored at -80°C until microarray hybridization.

3.3.5. Microarray hybridization

Five independent male liver samples were used for each treatment group in the microarray experiments, for a total of 15 microarray hybridizations. We focussed on males to reduce possible gender-related variation of differentially expressed (DE) genes. Experimental RNA samples were labelled with Cyanine 5-CTP (Cy5) and reference RNA was labelled with Cyanine 3-CTP (Cy3) using the Quick Amp Labeling Kit (Agilent Technologies, Mississauga, ON) according to manufacturer's instructions. Briefly, double-stranded complimentary DNA (cDNA) was synthesized from 200 ng total RNA using MMLV-RT with T7 promoter primer. T7 RNA polymerase was then used to transcribe labelled cRNA and samples were purified using the RNeasy mini kit (Qiagen). The cRNA (825ng) was then fragmented at 60°C for 30 min with fragmentation solution. Cy3-reference cRNA and Cy5-sample cRNA were co-hybridized to Agilent 4X44K chicken gene expression microarrays (containing 43603 60-mer oligonucleotide probes; Array ID 026441) at 65°C for 17 hours with Agilent hybridization solution and washed according to the manufacturer's instructions. Arrays were scanned using an Agilent G2505BC scanner at 3 μm resolution. Data were acquired with Agilent Feature Extraction software, version 11.0.1.1.

3.3.6. Microarray Data Analysis

A reference design (Kerr 2003) was used to generate gene expression data, which were then pre-processed using R software (http://www.R-project.org). Median signal intensities were normalized using the global locally

weighted scatterplot smoothing method (Yang *et al.* 2002) with the transform.madata function in the microarray analysis of variance (MAANOVA) library (Wu *et al.* 2003). Probes with fluorescent intensity signals significantly greater than the local mean background plus 3 standard deviations of background intensity were identified as being expressed. Ratio intensity plots were generated for the raw and normalized data to identify any outliers or arrays of poor quality. DE probes were identified using the MAANOVA library. The analysis of variance (ANOVA) included the main effect of treatment and the block effect of the array. The Fs statistic (Cui *et al.* 2005), a shrinkage estimator, was used for the probe-specific variance components and the associated *p*-values for all statistical tests were estimated using the permutation method (30,000 permutations with residual shuffling); *p*-values were then adjusted for multiple comparisons using the false discovery rate (FDR) approach (Benjamini and Hochberg 1995). The least-squares means (Searle *et al.* 1980) were used to estimate the fold changes for each pair-wise comparison. Probes were considered DE if they had absolute fold changes ≥1.5 relative to controls and an FDR-corrected $p \leq 0.1$.

Hierarchical cluster analysis was performed using GeneSpring GX ver. 12.5 (Agilent Technologies). Clustering was performed on probes and treatments using the Euclidian distance metric and the centroid linkage rule.

Probes spanning the same gene were collapsed into a single value using the maximum fold change among collapsed probes to produce the DE gene list. Functional and canonical pathway analyses were performed using Ingenuity®

Pathway Analysis (IPA) on the DE gene list. The LD was excluded from pathway analysis because it contained too few DE genes. DE genes from the HD chicken liver dataset that were recognized as human, mouse or rat orthologues and were associated with biological functions and/or diseases in Ingenuity's Knowledgebase were used to identify biological functions and pathways that were most significantly (p-value ≤0.05; right-tailed Fisher's exact test) affected by TDCPP exposure. IPA was also used to identify direct and indirect interactions between potential regulatory molecules and the DE genes in the HD group that have been documented in Ingenuity's Knowledgebase.

3.3.7. Real-time RT-PCR

Aliquots of the RNA used for microarray analysis were used for real-time reverse transcription PCR (real-time RT-PCR) validation. Three up-regulated genes (lanosterol synthase, basic leucine zipper transcription factor, ATF-like 3 (BATF3) and glutamine synthetase) and four down-regulated genes (thrombospondin receptor (CD36), GDNF family receptor alpha 1, leukocyte cell-derived chemotaxin 2 and tescalcin) were selected at random; the up-regulation of CYP3A37 was previously verified by real-time RT-PCR (Farhat *et al.* 2013). Primers (Supplementary information (SI) Table 1) were designed to yield a single product that was verified by a single melting curve peak and a single band on an agarose gel of the appropriate size (155-198 bp). Total RNA (~400 ng) was transcribed to cDNA using SuperScript II and random hexamer primers as described by the manufacturer (Invitrogen, Burlington, ON). A 1:20 dilution of

cDNA was prepared with diethylpyrocarbonate (DEPC)-treated water and stored at -80°C for subsequent real-time RT-PCR. Real-time RT-PCR was performed with a Stratagene Mx3000P PCR instrument (La Jolla, CA) using Brilliant II SYBR® Green Q-PCR Master Mix (Agilent Technologies). Each 25 µL reaction contained 2X Brilliant SYBR Green QPCR Master Mix, 75 nM ROX reference dye, forward and reverse primers at optimized concentrations (SI Table 1) and 5 µL diluted cDNA (1:20) in DEPC water. The thermocycle program included an enzyme activation step at 95°C (10 min) and 40 cycles of 95°C (30 sec) and 60°C (1 min). All reactions were performed in duplicate for five male samples per treatment group. No template controls (NTC) and no reverse transcriptase controls were conducted to monitor for contamination of reagents and RNA, respectively.

Cycle threshold (C_t) data were normalized to β-actin (control gene) as its expression was stable across all treatment groups. The fold change in target gene mRNA expression for treatment groups compared to the DMSO control was calculated using the $2^{-\Delta Ct}$ equation (Schmittgen and Livak 2008). Significant differences in mRNA expression ($p \leq 0.05$) were determined using a one-way ANOVA of $2^{-\Delta Ct}$ -transformed data followed by a Bonferroni correction for multiple comparisons (GraphPad Prism v. 5.02).

3.4. Results

3.4.1. Differentially expressed genes

Of the 43603 probes on the Agilent chicken microarray, 88 were differentially expressed (FDR $p \leq 0.1$, fold change≥1.5) in the liver of male chicken embryos exposed to 45 µg TDCPP/g egg (46 up-regulated, 42 down-regulated) and 13 probes were dysregulated at 7.6 µg TDCPP/g egg (3 up-regulated, 10 down-regulated). Grouping probes that spanned the same gene resulted in 47 DE genes at the HD and 5 at the LD (Fig. 3.1), with 100% overlap between the two doses. A detailed list of all probes that were differentially expressed following TDCPP exposure is included in SI Table 2. The ten genes with the greatest fold change in induction and suppression are shown in Table 3.1. Hierarchical clustering of the 88 DE probes (Fig. 3.2) resulted in two main branches separating the HD from the DMSO and LD samples, indicating a dose-dependent response that is also evident in Table 3.1. Real-time RT-PCR analysis of eight genes selected from the upstream regulator network (Fig. 3.3) showed an 87% (7/8) concordance in directional change in expression between the two technologies thereby validating the microarray results (SI Table 1).

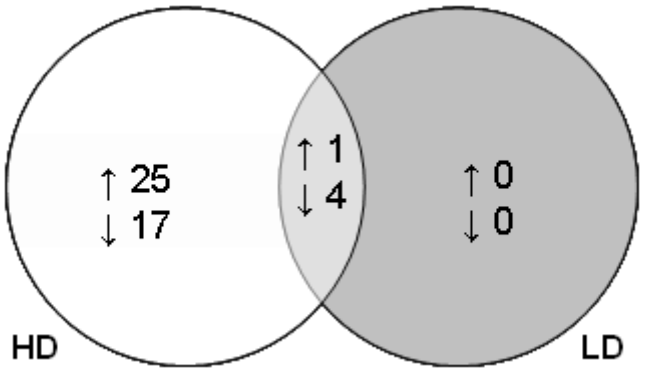

Figure 3.1. Venn diagram illustrating the number of unique genes up-(↑) or down-(↓) regulated (fold change ≥1.5, FDR $p \leq 0.1$) by 7.6 µg/g (LD) and 45 µg/g (HD) TDCPP.

Table 3.1. Top 10 up- or down-regulated genes in the livers of male chicken embryos exposed to 7.6 µg/g (LD) or 45 µg/g (HD) tris(1,3-dichloro-2-propyl)phosphate based on fold change.

Gene Symbol	Gene Name/Description	LD Fold Change	HD Fold Change
Most Up-regulated			
CYP2C45[a]	Rep: Cytochrome P-450 - Gallus gallus (Chicken), complete [TC348506]	1.52	6.55*
CYP3A7[a]	cytochrome P450 A 37	2.84	5.18*
ENPEP	PREDICTED: Gallus gallus similar to aminopeptidase A (LOC428771), mRNA	1.29	3.89*
BATF3	basic leucine zipper transcription factor, ATF-like 3	2.68*	3.70*
EGR1	early growth response 1	1.86	3.46*
WNT4[a]	wingless-type MMTV integration site family, member 4	1.70	3.45*
GAL8	gallinacin 8	1.46	2.93*
SPP1	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	1.08	2.92*
GSTA3[a]	glutathione S-transferase alpha 3	1.52	2.39*
GLUL[a]	glutamate-ammonia ligase (glutamine synthetase)	2.21	2.31*
Most Down-regulated			
CATHL2[a]	cathelicidin 2	-4.14*	-3.97*
ABI3BP	PREDICTED: Gallus gallus similar to NeshBP (LOC769237), mRNA	-1.25	-3.01*
GAL2[a]	gallinacin 2	-2.70	-2.93*
LECT2	leukocyte cell-derived chemotaxin 2	-2.03	-2.35*
TESC	tescalcin	-1.40	-2.27*
DECR1	2,4-dienoyl CoA reductase 1, mitochondrial	-1.44	-2.12*
CATHL3[a]	cathelicidin antimicrobial peptide	-2.53*	-2.58*
GAL7[a]	gallinacin 7	-2.07*	-2.03*
STEAP4	STEAP family member 4	-1.58	-1.87*
ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	-1.20	-1.83*

[a]The maximum fold change from multiple unique probes is presented

* FDR $p \leq 0.1$

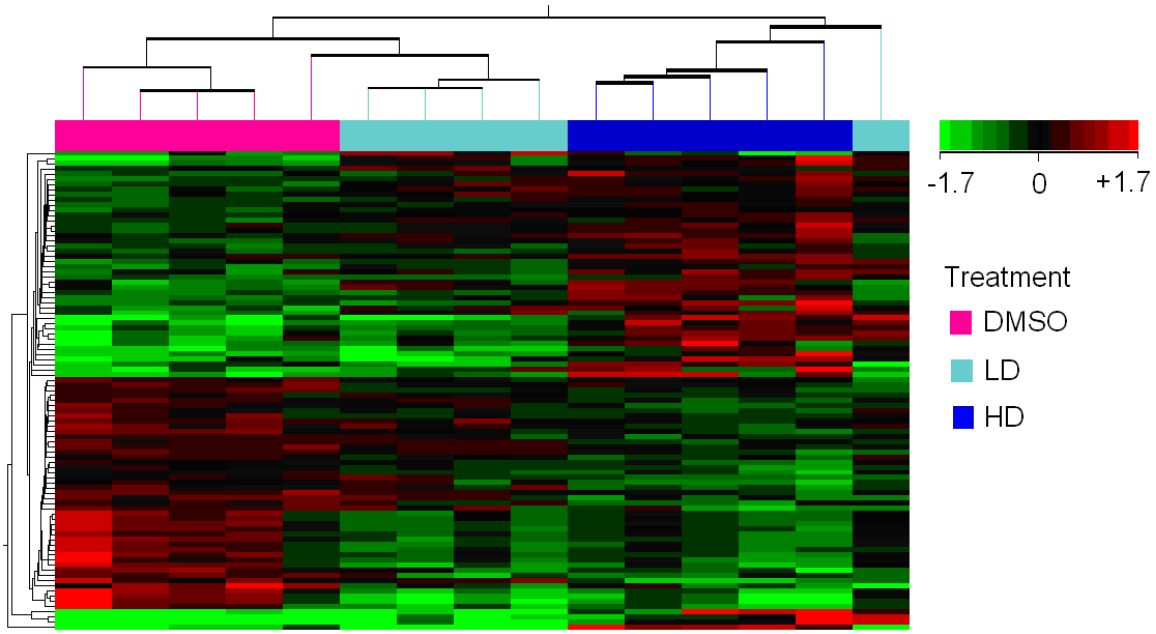

Figure 3.2. Hierarchical clustering of expression profiles of liver tissue from male chicken embryos exposed to the dimethyl sulfoxide (DMSO) solvent control, 7.6 µg TDCPP/g egg (LD) or 45 µg TDCPP/g egg (HD). Clustering was performed on 88 unique probes with a fold change ≥1.5 (FDR p ≤0.1).

3.4.2. Functional and Canonical Pathway Analyses

Eighty five percent (40 out of 47) of the significantly dysregulated genes were recognized by IPA and thus could be used for functional, pathway and regulatory analyses (SI Table 2). Functional enrichment analysis resulted in 76 significantly affected biological functions (p≤0.05; SI Table 3). A sub-set of enriched functions, including lipid metabolism and cancer, are summarized in Table 3.2 with their corresponding dysregulated genes. Some of the most highly dysregulated genes were essential for normal immune function, but were not recognized by IPA. Therefore immune function was included in Table 3.2 based on a literature search of each corresponding gene. The literature search also revealed an association between several dysregulated genes and cholestatic liver/biliary fibrosis.

Table 3.2. Most relevant biological functions affected by tris(1,3-dichloro-2-propyl)phosphate exposure (45 µg/g) as indicated by the dysregulation of genes in liver tissue of male chicken embryos.

Function	Annotation	Up-regulated	Down-regulated	Total DE genes
Immune Response	response to pathogens	GAL8	GAL2 GAL7 CATHL2 CATHL3	7
	dendritic cell differentiation and infection of liver	BATF3		
	early response to infection	SPP1		
Lipid metabolism	concentration of lipid	EGR1 SGK1 WNT4	CD36 DECR1 STEAP4	10
	oxidation of fatty acid	CYP2C9	CD36 DECR1	
	activation of fatty acid	CYP2C9		
	Transport, uptake, incorporation and secretion of fatty acids		CD36	
	metabolism of epoxyeicosatrienoic acids	CYP2C9		
Cholesterol metabolism	delay in initiation of absorption of cholesterol		CD36	
	concentration, efflux, uptake and storage of cholesterol	EGR1 LSS	CD36 STEAP4	
	homeostasis of cholesterol		CD36 MYLIP	
	oxidation of cholesterol		CD36	
Steroid hormone metabolism	concentration of hormone	EGR1 WNT4	CD36 STEAP4	
	metabolism and quantity of steroids (ex-esterdiols)	EGR1 SGK1 WNT4 CYP2C9 LSS	CD36 STEAP4	
Cancer	growth of secondary tumour	PTP4A3		20
	papilloma	EGR1 Gsta4		
	digestive organ tumour	CLDN3 CYP2C9 GLUL GPX3 PTP4A3 SGK1	CD36 LECT2 OIT3	

Note: The genes listed here are only a subset of the dysregulated genes associated with a particular function. Total DE genes reflects the actual number of dysregulated genes associated with a function, and the complete list of genes can be found in SI Table 3.

IPA was also used to identify signalling and metabolic pathways (canonical pathways) that were significantly affected following HD TDCPP exposure. The most significantly affected pathways were related to lipid homeostasis, oncogenesis, growth, and development. The top ranked canonical pathways are summarized in Table 3.3, and a full list of significantly affected canonical pathways is presented in SI Table 4.

Table 3.3: Top eight enriched canonical pathways for genes that were differentially expressed in the liver of male chicken embryos exposed to 45 µg/g tris(1,3-dichloro-2-propyl)phosphate. ↑=up-regulated and ↓= down-regulated.

Rank	Canonical Pathway	*p*-value	No. of molecules	Genes	Comments
1	Lanosterol Biosynthesis	3.63E-3	1 of 1	↑LSS	Catalyzes first step in synthesis of cholesterol and steroid hormones.
2	Glutamine Biosynthesis I	7.41E-3	1 of 2	↑GLUL	Involved in cell proliferation, inhibition of apoptosis and cell signalling.
3	L-serine Degradation	7.41E-3	1 of 3	↑SDSL	
4	Regulation of the Epithelial-Mesenchymal Transition (EMT) Pathway	1.78E-2	3 of 189	↑EGR1 ↑WNT4 ↑CLDN3	EMT is an essential process for embryo development and is also observed in the onset of migratory mesenchymal cancer.
5	GDP-mannose Biosynthesis	2.19E-2	1 of 6	↑PMM2	The key monosaccharide for glycosylation of proteins and lipids.
6	Calcium Transport I	2.57E-2	1 of 9	↓ATP2B2	Essential for energy homeostasis
7	Glycine Betaine Degradation	2.57E-2	1 of 10	↑SDSL	Mechanism for osmoregulation
8	LXR/RXR Activation	3.02E-2	3 of 189	↓MYLIP ↓CD36	Involved in regulation of lipid metabolism (e.g. cholesterol catabolism to bile acids)

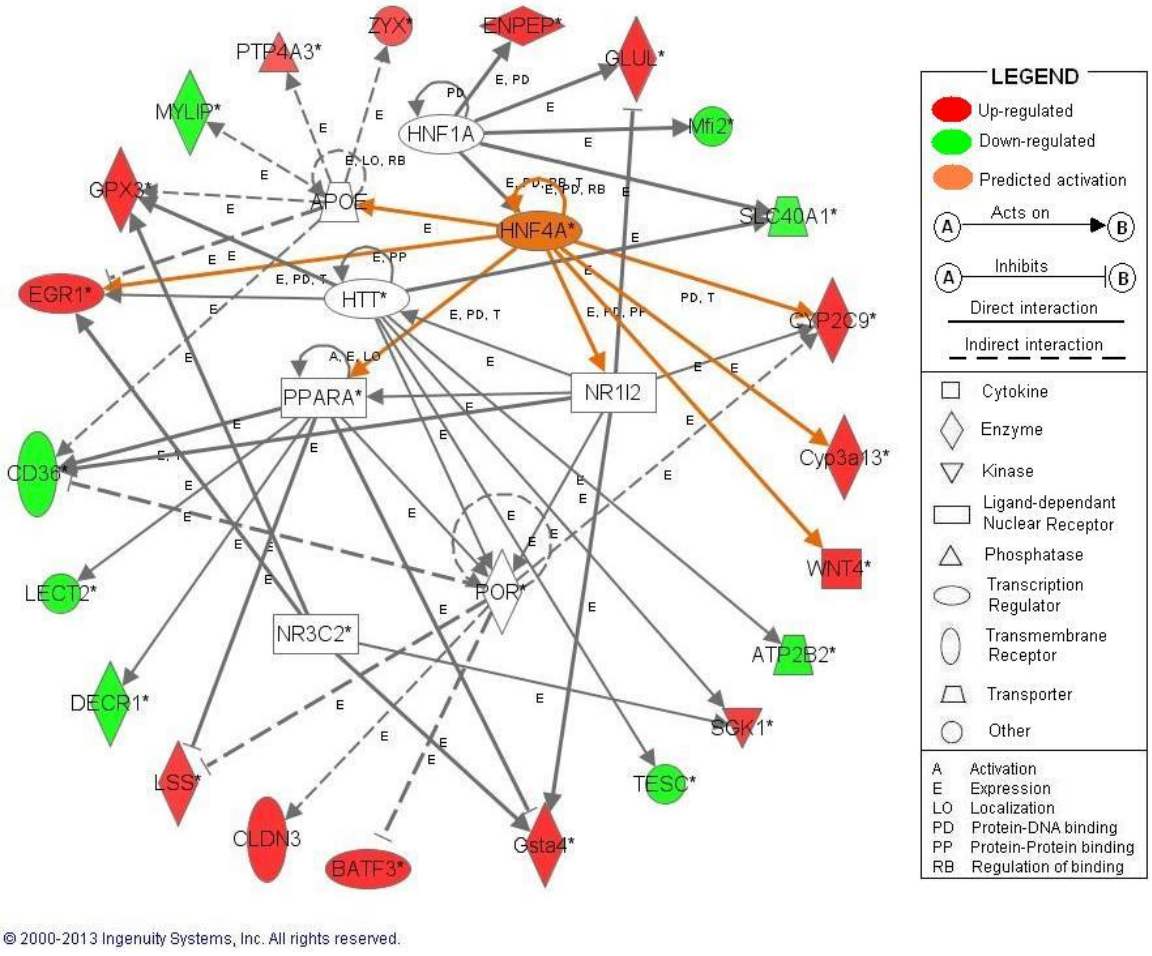

Figure 3.3. Interaction network of IPA-predicted upstream regulatory molecules (center) and the corresponding differentially expressed genes (circumference) from liver tissue of male chicken embryos exposed to 45 µg/g of tris(1,3-dichloro-2-propyl)phosphate. All regulators have four or more molecular interactions. HNF4A was the only regulator with a predicted activation state.

3.4.3. Upstream Regulators

IPA identifies upstream regulator molecules that might explain the observed gene expression changes in the dataset. The analysis compares expected causal effects between upstream regulators and targets from the IPA Knowledge Base to the actual direction of change of target genes, and issues a predicted activation state (activated or inhibited) for each regulator molecule. If the expression of target genes results in conflicting states of activation for a

single regulator, an activation state is not predicted. Significant ($p \leq 0.05$) predicted regulators with 4 or more interactions with DE genes for liver tissue of male chicken embryos exposed to HD TDCPP are indicated in Figure 3.3. HNF4A was the only upstream regulator to be assigned an activation state and was predicted to be activated, but it was not differentially expressed on the chicken microarray.

3.5. Discussion

We evaluated the effects of *in ovo* TDCPP exposure on global gene expression in liver tissue of male chicken embryos at the pipping stage of development. The microarray analysis revealed 47 DE genes at the HD, five of which were also dysregulated at the LD. The changes in gene expression indicated a dose-dependent effect that was supported by hierarchical cluster analysis. However, the effect of TDCPP on gene expression was rather modest given the observed reduction in embryo growth, gallbladder size, and circulating T_4 levels (Farhat *et al.* 2013). This moderate transcriptional effect is likely the result of low residual TDCPP concentrations in the livers at the time of tissue collection, due to its extensive metabolism (Farhat *et al.* 2013). A greater disruption of TH-responsive genes in particular was expected, due to the importance of THs in avian embryonic growth and development (McNabb 2007). Rather, the expression profile indicated effects on immune function, and lipid and steroid metabolism, which likely represent downstream effects of TH disruption, as THs are known to play a significant role in their regulation (McNabb 2007; Zhu

and Cheng 2010). The TDCPP-induced expression profile also indicated a state of cholestatic liver/biliary fibrosis and the dysregulation of cancer related genes, which is consistent with the classification of TDCPP as a carcinogen.

3.5.1. Immune Response

A number of transcripts that play critical roles in immune response were among the most dysregulated genes in TDCPP-treated chicken embryos. Gallinacin (GAL) 8 was up-regulated while GAL2, GAL7, cathelicidin (CATHL) 2 and CATHL3 were suppressed. GALs and CATHLs are antimicrobial peptides associated with an immune response against pathogenic assault (Meade *et al.* 2009). Newly-hatched chicks showed elevated levels of GALs that declined during the first week of life, which can be considered a preparatory mechanism for the bacterial colonization of the gut at the onset of foraging (Bar-Shira and Friedman 2006). The suppression of antimicrobial peptides at pipping in TDCPP-exposed embryos could leave them susceptible to microbial attack.

Secreted phosphoprotein 1 (SPP1), a pro-inflammatory cytokine involved in early response to infection was induced. Interestingly, mice with sclerosing cholangitis (bile duct inflammation and subsequent obstruction) and biliary fibrosis exhibit pronounced induction of SPP1 in bile duct epithelial cells and hepatocytes (Fickert *et al.* 2007). Wingless-type MMTV integration site family member 4 (WNT4) and early growth response 1 (EGR1), which regulate cellular differentiation and proliferation (Heikkila *et al.* 2002; McMullen *et al.* 2005) and are associated with cholestasis-induced liver fibrosis (Cheng *et al.* 2008; Kim *et*

al. 2006), were also up-regulated in this study. This provides further support that the TDCPP-exposed chicken embryos were in a state of cholestatic liver fibrosis. A cholestatic state is consistent with our previous observation that HD-treated embryos had significantly smaller gallbladders (Farhat *et al.* 2013), as obstruction of the bile ducts would lead to reduced bile flow into the gallbladder.

Depleted T_4 levels in TDCPP-treated chicken embryos (Farhat *et al.* 2013) is consistent with the observed immune response because THs play an important role in immune function (Barreiro Arcos *et al.* 2010). Hypothyroidic chickens had a reduced total white blood cell count and number of lymphocytes (Scott and Glick 1987), similar to TDCPP-exposed mice which showed lymphoid depletion, a depressed lymphoproliferative response to mitogens and an increased frequency of tumours following tumour cell challenge (Luster *et al.* 1981). In concordance with this, a number of genes associated with lymphoma and leukemia, diseases that compromise the immune system, were dysregulated in this study.

It is important to note that of the seven dysregulated transcripts involved in immune response, only BATF3 was recognized as a mammalian orthologue and mapped into IPA. Therefore, the downstream functional and canonical pathway analyses may underestimate the effects of TDCPP on the chicken immune system. Furthermore, these immuno-responsive genes composed four of the five DE genes in the LD group. The fifth DE gene in the LD was myosin regulatory light chain interacting protein, which is involved in cholesterol homeostasis.

Therefore, immune function and cholesterol homeostasis appear to be the functions most sensitive to TDCPP exposure.

3.5.2. Metabolism

The two genes that were most induced following TDCPP exposure were cytochrome P450 (CYP) 2C45 and CYP3A37, which catalyze the oxidative metabolism of a wide range of endogenous and exogenous compounds including steroids, fatty acids, and xenobiotics (Nebert and Gonzalez 1987; Zhou *et al.* 2009). CYP3A37 induction following TDCPP exposure is consistent with results from Farhat *et al.* (2013) that showed a 7.9-fold induction of CYP3A37 by real-time RT-PCR. Glutathione S-transferase (GST) alpha 3, a phase II metabolizing enzyme that mainly binds xenobiotics and products of oxidative stress (Hayes and Pulford 1995), was also induced. The induction of genes specialized in detoxification of xenobiotics is in concordance with the rapid and extensive metabolism of TDCPP *in vitro* and *in* vivo (Chen *et al.* 2012; Lynn *et al.* 1981; Nomeir *et al.* 1981). Furthermore, we previously reported that the liver of chicken embryos exposed to 45 µg TDCPP/g egg at the onset of development contained only 0.002 µg TDCPP/g liver tissue at pipping (same HD samples used for this study) (Farhat *et al.* 2013) indicating that extensive metabolism had occurred. Although the concentrations of metabolites in liver tissue were not measured (method under development), metabolites are not likely the cause of the observed effects on gene expression. TDCPP was rapidly excreted in urine primarily as bis(1,3-dichloro-2-propyl) phosphate in rats, and tissue

concentrations of TDCPP and metabolites were negligible after 120 hrs (Lynn *et al.* 1981). Thus, many of the genes exhibiting altered expression at pipping are likely downstream consequences of TDCPP exposure during development rather than direct effects.

Lipid and steroid metabolism are biological functions that were significantly affected in this study. For example, lanosterol biosynthesis, the first step in cholesterol biosynthesis, was the most significantly affected canonical pathway in TDCPP-treated chicken embryos. Furthermore, WNT4, six-transmembrane epithelial antigen of the prostate member 4 (STEAP4), CD36 and EGR1 are DE genes involved in multiple metabolic processes including hormone homeostasis and regulation of glucose and lipids (Goudriaan *et al.* 2003; Heikkila *et al.* 2002; Lee *et al.* 1996; Wellen and Hotamisligil 2005). WNT4 induction elevated aldosterone levels and reduced corticosterone and testosterone levels in mice (Heikkila *et al.* 2002; Jordan *et al.* 2003), which is consistent with the altered CYP11B2 expression (involved in aldosterone synthesis) and testosterone levels observed in TDCPP-exposed zebrafish (Liu *et al.* 2012). CD36 and STEAP4 were down-regulated in chicken liver tissue following TDCPP exposure and have been associated with reduced body weight and elevated plasma cholesterol levels in knockout mice (Goudriaan *et al.* 2003; Wellen *et al.* 2007); this is consistent with reduced body weight of TDCPP-treated chicken embryos (Farhat *et al.* 2013) and elevated plasma cholesterol levels in TDCPP-exposed rats (Luster *et al.* 1981). THs stimulate lipid synthesis, mobilization and degradation (Muller and Seitz 1984) and are therefore implicated in the disruption of

cholesterol homeostasis in TDCPP-treated chickens, as circulating T_4 levels were depleted (Farhat *et al.* 2013). Hypothyroidism leads to hypercholesterolemia due to diminished clearance of cholesterol and reduction in its conversion to bile acids (Erem *et al.* 1999). Young thyroidectomised chickens exhibited reduced body weight and elevated plasma phospholipids, triglycerides and cholesterol (Shibata *et al.* 2003), which is consistent with the phenotypic alterations caused by TDCPP in chickens and rats (Farhat *et al.* 2013; Luster *et al.* 1981).

Interactome analysis revealed potential upstream regulators that may be involved in transcriptional changes following TDCPP exposure, and a number of these are involved in reverse cholesterol transport (a net flux of cholesterol from extra-hepatic tissues to the liver and subsequent disposal through the bile). For example HNF4A, which induces bile synthesis from cholesterol (Crestani *et al.* 2004), was predicted to be in an activated state as all downstream DE genes were induced. HNF4A also regulates APOE, a transporter that is critical for the clearance of chyolomicron remnants enriched with cholesterol (Mahley and Ji 1999). These results suggest that bile synthesis is increased in TDCPP-treated chicken embryos; however, mRNA expression levels of bile acid synthases 7α-hydroxylase, sterol 27-hydroxylase and oxysterol 7α-hydroxylase were unaltered. The lack of an effect on bile acid synthases may be due to the counter-active effect of PPARA, which also promotes lipoprotein assembly for reverse cholesterol transport and stimulates fatty acid and cholesterol catabolism (Michalik *et al.* 2006). PPARA appears to be in an inhibited state based on the directional change in expression of downstream genes. The activation states of

HNF4A and PPARA might seem contradictory but may be explained by the phenotypic observations in TDCPP-treated chicken embryos. As previously described, the reduced gallbladder size may be due to cholestasis, which could lead to conflicting signalling cascades: the intestine requires more bile acids for adequate lipid uptake because chicken embryos are heavily reliant on yolk fatty acids for maintenance and growth (De Oliveira *et al.* 2008), but the liver is saturated with bile acids that are hepatotoxic at high concentrations (De Fabiani *et al.* 2001). Indeed, PPARA plays a significant role in the prevention of cholestasis in mice (Hays *et al.* 2005) and may be playing a similar role in TDCPP-treated chicken embryos.

3.5.3. Cancer

Twenty of the 47 genes that were dysregulated by TDCPP in chicken embryo liver were associated with cancer-related pathways. The suppression of oncoprotein induced transcript 3 in human liver and the induction of CYP2C9 in mice have been associated with hepatocellular carcinoma (Chang *et al.* 2009; Griffitts *et al.* 2009); these genes were also dysregulated in this study. Moreover, the expression of leukocyte cell-derived chemotaxin 2, which was suppressed in this study, has been suggested as a biomarker for hepatocellular carcinoma as its induction inhibits the invasion of cancer cells (Wu *et al.* 2010). Although hepatic tumours were not observed in the TDCPP-treated chicken embryos, the hepatic expression profile supports the literature that describes liver tumour

formation following chronic TDCPP exposure in rats (Freudenthal and Henrich 2000; OEHHA 2011b).

3.5.4. Conclusions

Our results add insight into the possible mechanisms of TDCPP action that may have led to the adverse phenotypic effects observed in our companion study (Farhat *et al.* 2013). TDCPP treatment resulted in an expression profile that indicated disruption of immune response and reflected a state of cholestatic liver/biliary fibrosis. This state was accompanied by the dysregulation of genes involved in inflammatory pathways and steroid and lipid metabolism. Although few TH-responsive genes were differentially expressed at pipping, the importance of THs in immune response and metabolism led to the prediction that TH-disruption, early in development, was responsible for the observed genomic profile. Measurement of mRNA levels of TH-responsive genes throughout incubation is required to confirm the role of TH disruption in the toxic action of TDCPP. Such studies should be conducted in concert with liver histological analysis and measurement of circulating TH, steroid and lipid levels to determine whether the molecular alterations translate to phenotypic changes.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

4.1. Conclusions

This thesis contains novel findings regarding the toxicological effects of TCPP and TDCPP in an avian embryo. Its results support the current literature that describes TCPP and TDCPP as having developmental and endocrine-disrupting effects. Both flame retardants delayed pipping (≥9.24 µg TCPP/g; 45 µg TDCPP/g) and reduced growth parameters (51.6 µg TCPP/g; 45 µg TDCPP/g) in treated chicken embryos, indicating developmental toxicity. TCPP induced TH-responsive genes and TDCPP decreased circulating thyroxine (T_4) levels, which indicate a disruption of the endocrine system. Furthermore, a microarray mRNA expression analysis of liver from TDCPP-treated embryos revealed specific biochemical pathways affected by TDCPP, the most significant of which was lanosterol biosynthesis (the first step in cholesterol biosynthesis). The global expression profile of the TDCPP-treated chicken embryos indicated a state of hepatic inflammation and the onset of cholestatic liver/biliary fibrosis. It also identified the disruption of lipid and steroid metabolism as being a main target of TDCPP toxicity. Although the specific mechanism(s) of action of TDCPP remains unclear, this work has narrowed the vast pool of possibilities to the general area of energy homeostasis, with a focus on lipid metabolism. The disruption of thyroid hormone (TH) action is suspected as the root cause of this

energy imbalance, but further studies are required to confirm this hypothesis. The specific conclusions to the hypotheses of this thesis are as follows:

A. Neither TCPP nor TDCPP were embryo-lethal up to 51.6 µg TCPP/g egg and 45 µg TDCPP/g egg under the specified conditions. Since these concentrations are well above environmental exposure levels, TCPP and TDCPP are not expected to affect the viability of wild avian species.
B. As predicted, TDCPP exposure reduced circulating T_4 levels and reduced embryo growth (reduced head plus bill length and embryo weight) but only dysregulated the expression of xenobiotic metabolizing enzymes; the five TH-responsive genes measured in this study were unaffected by TDCPP exposure. This finding confirms that TDCPP has endocrine disrupting potential in chicken embryos, which is in concordance with the mammalian and aquatic literature.
C. As expected, >92% of the injected TCPP or TDCPP was absorbed into the contents of the treated egg shortly after administration, and levels were reduced to <1% by day 19 of incubation. This indicates that the flame retardants were metabolized rather than being concentrated in tissues that were not specifically tested.
D. Only a small portion of TH-responsive genes out of ~44,000 on the microarray were dysregulated by TDCPP. The lack of a significant thyroid response in the global expression profile of TDCPP-treated embryos is likely due to the low residual concentrations at pipping.

E. Cholesterol metabolism was confirmed to be a major target of TDCPP toxicity, but no effects on the expression of bile acid synthases was observed. The reduced gallbladder size in TDCPP-treated embryos is more likely the result of physical obstruction of the bile ducts due to excessive inflammation and/or biliary fibrosis, rather than decreased bile acid synthesis.

F. TDCPP dysregulated numerous genes involved in the metabolism and homeostasis of steroid hormones. As steroid hormones are synthesized from cholesterol, this is likely a downstream effect of the dysregulation of cholesterol biosynthesis.

4.2. Future Directions

TCPP and TDCPP continue to be produced in high volumes and demand for their production continues to increase. The stability and persistence of these compounds in the environment makes it essential to understand the toxicological implications of exposure. Although much data exist on the overt toxicological effects of TCPP and TDCPP, very little is known about their specific mechanisms of toxicity. The results of this study illuminated potential targets of interference but further research is necessary to better understand the root cause of the observed developmental and biochemical effects. The following recommendations are made for future research on TCPP and TDCPP:

1. Perform a histological examination of the liver, gallbladder and their ducts in TDCPP-treated chicken embryos to determine whether the expression

profile that implies liver/biliary fibrosis translates to visible lesions. An examination of the bile ducts will also reveal whether the small gallbladders observed in TDCPP-treated embryos were due to a physical obstruction of bile flow.

2. Perform clinical chemistry assays on the plasma and liver of TDCPP-treated embryos to determine the levels of cholesterol, fatty acids, glucose, and steroid hormones since the most highly affected genes reflect disruption in lipid and steroid metabolism.
3. Determine circulating TH levels and mRNA expression levels of TH-responsive genes throughout embryonic development of TCPP/TDCPP-treated embryos beginning at day 7-8 of incubation. The organs are fully functional at this stage and development should be advanced enough to collect sufficient samples. The disruption of TH action within the second half of incubation has the greatest effect on embryo growth (King and May 1984); therefore, if TH-disruption is the root cause of the adverse phenotypic effects observed, a significant disruption of these endpoints should be observed.
4. Conduct protein binding studies to determine the interaction of TCPP and TDCPP with the TH-receptor, transthyretin, peroxisome proliferator-activated receptor alpha and the thrombospondin receptor, as well as other implicated receptors/transporters, to determine their involvement in the action of these flame retardants.

5. Measure the activity of deiodinase enzymes and the most induced xenobiotic metabolizing enzymes in response to TCPP and TDCPP treatment. Changes in deiodinase activity would indicate an effect on TH metabolism and xenobiotic activation would support the involvement of those enzymes in flame retardant metabolism.
6. Understanding the relevance of the toxic effects of TCPP and TDCPP in an environmental context is hindered by the fact that they are metabolized so quickly. Although the chicken embryos were exposed to about 50 µg/g of TCPP or TDCPP, only 1 and 5 ng/g were detected in the egg contents at pipping, respectively. The effects observed at pipping likely stem from a major disruption that occurred when xenobiotic concentrations were high in the embryo; therefore, the final tissue concentrations are not reflective of the exposure burden of the embryo. This prevents the direct comparison of our dosing levels to TCPP and TDCPP concentrations detected in wild avian species. It is paradoxical that these compounds are extensively and rapidly metabolized, yet are detected in wild species. This may indicate that the daily exposure burden in wild birds is higher than expected, and effort needs to be directed at determining the actual exposure levels in wild birds based on rate of intake of contaminated food/water. This information would help clarify the actual risk of adverse effects in wild species, and aid in designing studies that test more relevant concentrations. It would also be of great use to determine the maternal

transfer of TCPP and TDCPP from female birds to their eggs to better understand the exposure of wild avian embryos.

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Appendix I: Supplementary Information

(Pertaining to Chapter 3)

SI Table 1: Validation of differentially expressed genes in livers of male chicken embryos exposed to 7.6 (LD) or 45 (HD) µg/g TDCPP by real-time RT-PCR. PCR primer/probe sequences are indicated with reaction concentrations in brackets. MA=microarray fold change, PCR= Real-time RT-PCR fold change, SE=standard error of means, F=forward, R=reverse, *statistically significant (FDR p-value ≤0.01for MA; p-value ≤0.05 for PCR from one-way ANOVA followed by Bonferroni correction). Bolded values indicate consistent directionality and significance between MA and PCR.

Gene Symbol	Gene Name		PCR Primer/ Probe Sequence (5'→3')	Accession number	LD			HD		
					MA	PCR	SE	MA	PCR	SE
CYP3A37	Cytochrome P450 3A37	F	AGCCTGCGGTTGTTGTCATG (900nM)	NM_001001751	2.84	4.55	1.63	**5.18***	**7.86***	2.58
		R	CTTCAGCTAATGAGACAGCGTTTC (900nM)							
LSS	lanosterol synthase	F	GTCAACCTGCACAGCAAAGG (150nM)	NM_001006514	1.11	0.98	0.16	1.78*	0.95	0.12
		R	TCATGGGGAGGTAGACCTGG (100nM)							
BATF3	basic leucine zipper transcription factor, ATF-like 3	F	CAAACGCAGAAAGCGGACAA (150nM)	XM_419428	2.68*	2.81	0.44	3.70*	2.91	0.96
		R	GCATCAGGCCTTGGTATGGT (100nM)							
GLUL	glutamine synthetase	F	TGGGATGGAGCAGGAGTACA (100nM)	NM_205493	2.01	1.69	0.38	2.31*	1.55	0.15
		R	ACTTCTGCGTTGGTTCCTCC (100nM)							
CD36	thrombospondin receptor	F	GCGCTTCTTCTCCTCTGACA (100nM)	NM_001030731	0.72	0.72	0.14	**0.55***	**0.33***	0.07
		R	TCTAGGACTCCAGCCAGTGT (150nM)							
GFRA1	GDNF family receptor alpha 1	F	CGCTCCTCTACTTGGCTCTG (100nM)	NM_205102	0.81	0.61	0.16	**0.57***	**0.49***	0.09
		R	CTCTCTTTGCCGGCTACACA (100nM)							
LECT2	leukocyte cell-derived chemotaxin 2	F	CAGGGGATCAGGCTTCTGTG (100nM)	NM_205478	0.49	0.39	0.14	**0.43***	**0.10***	0.03
		R	AGCGATCGCAGTTCTCAACA (100nM)							
TESC	tescalcin	F	GAGGACTTCGACACCATCCC (150nM)	NM_001080884	0.72	1.19	0.35	0.44*	0.48	0.16
		R	CCTCGTCCATGTCCATCTCG (150nM)							

SI Table 2. Significantly dysregulated probes (FDR p ≤0.1, fold change ≥ ±1.5; Shaded) in the liver of male chicken embryos exposed to 7.6 (LD) or 45 (HD) µg/g tris(1,3-dichloro-2-propyl)phosphate. Probes that were mapped into Ingenuity Pathway Analysis (IPA) are indicated by M. Genes with duplicate probes are indicated with a *. Bolded mammalian gene symbols differ from chicken gene symbols.

Probe ID	Gene Bank ID	Gene Symbol	Gene Name/Description	LD		HD		IPA
				FDR *p*	Fold Change	FDR *p*	Fold Change	
A_87_P080486			Rep: Cytochrome P-450	0.9999	1.52	0.0000	6.55	M*
A_87_P115288	BX932650		finished cDNA, clone ChEST495k1	0.3106	-2.18	0.0000	5.43	
A_87_P037490	NM_001001751	CYP3A7	cytochrome P450 A 37	0.7297	2.84	0.0000	5.18	M*
A_87_P132438	AJ002600		peripheral Retina, embryonal stage E6-cDNA	0.9999	1.44	0.0000	4.68	
A_87_P210273	NM_001001751	CYP3A7	cytochrome P450 A 37	0.9788	2.39	0.0765	4.66	M*
A_87_P095826	XM_426327	ENPEP	PREDICTED: similar to aminopeptidase A	0.9999	1.29	0.0673	3.89	M*
A_87_P023505	XM_419428	BATF3	basic leucine zipper transcription factor, ATF-like 3	0.0519	2.68	0.0000	3.70	M*
A_87_P073406	CR389000	EGR1	early growth response 1	0.9999	1.86	0.0765	3.46	M*
A_87_P009788	NM_204783	WNT4	wingless-type MMTV integration site family, member 4	0.9999	1.70	0.0000	3.45	M*
A_87_P024307	BX930422		finished cDNA, clone ChEST629g14	0.9999	1.05	0.0507	3.19	
A_87_P313142	XM_424435	LOC426826	PREDICTED: hypothetical LOC426826	0.9999	1.58	0.0507	3.06	
A_87_P313147	XM_424433	LOC426826	PREDICTED: hypothetical LOC426826	0.9999	1.58	0.0507	2.95	
A_87_P100601	NM_001001781	GAL8	Gal 8	0.9999	1.46	0.0884	2.93	
A_87_P140263	NM_204535	SPP1	secreted phosphoprotein 1	0.9999	1.08	0.0000	2.92	
A_87_P013422	CR391487		finished cDNA, clone ChEST760m2	0.9999	1.11	0.0000	2.86	
A_87_P097806	BX932427		basic leucine zipper transcription factor, ATF-like 3	0.3345	1.98	0.0000	2.71	
A_87_P065921	NM_204783	WNT4	wingless-type MMTV integration site family, member 4	0.9999	1.44	0.0000	2.59	M*
A_87_P123761	CR389246		finished cDNA, clone ChEST249n8	0.4433	-1.65	0.0000	2.46	
A_87_P037870	NM_204818	GSTA3	glutathione S-transferase alpha 3	0.9999	1.49	0.0507	2.39	M*
A_87_P009202	NM_205493	GLUL	glutamate-ammonia ligase	0.4199	2.01	0.0765	2.31	M*
A_87_P149778	NM_204818	GSTA3	glutathione S-transferase alpha 3	0.9874	1.52	0.0000	2.31	M*
A_87_P056936	NM_205493	GLUL	glutamate-ammonia ligase	0.1514	2.21	0.0673	2.25	M*
A_87_P037678	NM_204202	CLDN3	claudin 3	0.9999	-1.01	0.0000	2.21	M
A_87_P024284	BX930460		finished cDNA, clone ChEST599e12.	0.9999	1.25	0.0612	2.19	
A_87_P023894	NM_001163232	GPX3	glutathione peroxidase 3	0.9999	1.19	0.0765	2.16	M*
A_87_P101046	NM_001166674	CRYGN	crystallin, gamma N	0.9999	1.30	0.0000	2.06	M
A_87_P059236	NM_001030612	LOC416235	ovoinhibitor	0.9999	1.31	0.0765	2.05	M*
A_87_P036306	NM_001031468	PMM2	phosphomannomutase 2	0.8488	1.46	0.0398	1.93	M*
A_87_P070016	NM_001198643	SDSL	serine dehydratase-like	0.9999	1.25	0.0884	1.86	M*
A_87_P080495	NM_001001752	CYP2C45	cytochrome P-450 2C45	0.9999	1.05	0.0673	1.84	M*
A_87_P111913	NM_001005346	TSKU	tsukushin	0.9999	1.11	0.0000	1.82	M*

Probe ID	Gene Bank ID	Gene Symbol	Gene Name/Description	LD		HD		IPA
				FDR *p*	Fold Change	FDR *p*	Fold Change	
A_87_P117148	NM_204476	SGK1	serum/glucocorticoid regulated kinase 1	0.2373	1.64	0.0255	1.78	M*
A_87_P036122	NM_001006514	LSS	lanosterol synthase	0.9999	1.11	0.0825	1.78	M*
A_87_P018410	CR352717		finished cDNA, clone ChEST39p3.	0.9999	1.26	0.0398	1.76	
A_87_P030353	XM_419241	LAD1	PREDICTED: similar to linear IgA disease antigen homolog	0.9999	1.02	0.0000	1.71	
A_87_P145243	BU232665	LSS	lanosterol synthase	0.9999	1.11	0.0255	1.71	M*
A_87_P119193	NM_001114972	SEPN1	selenoprotein N, 1	0.9999	1.21	0.0673	1.66	M*
A_87_P119958	CR353684		finished cDNA, clone ChEST931i7.	0.1063	1.67	0.0733	1.65	M*
A_87_P013884	CR390863		finished cDNA, clone ChEST966o16.	0.2249	1.65	0.0950	1.62	
A_87_P023046	BX933401		finished cDNA, clone ChEST62k22.	0.1321	1.62	0.0950	1.62	
A_87_P032667	XM_001237034	ARL4C	PREDICTED: ADP-ribosylation factor-like 4C	0.5383	1.37	0.0398	1.59	M*
A_87_P011966	CR522928		finished cDNA, clone ChEST543g21.	0.2050	1.54	0.0255	1.58	M
A_87_P122993	CR388456		finished cDNA, clone ChEST583b15.	0.6405	1.27	0.0000	1.57	
A_87_P017389	NM_001037271	GDPD5	glycerophosphodiester phosphodiesterase domain containing 5	0.9999	-1.02	0.0255	1.53	M*
A_87_P095776	XM_420635	SEC24D	PREDICTED: Gallus gallus SEC24 related gene family, member D	0.9606	1.27	0.0507	1.51	M*
A_87_P105348	XM_001231578	PTP4A3	protein tyrosine phosphatase type IVA, member 3	0.4526	1.33	0.0000	1.51	M*
A_87_P016464	CR386456		finished cDNA, clone ChEST31m24.	0.0831	1.58	0.9754	1.10	
A_87_P015764	XM_001232644		finished cDNA, clone ChEST507o18.	0.0000	2.98	0.9951	1.09	
A_87_P018393	CR352749		finished cDNA, clone ChEST102f23.	0.9999	-1.11	0.0612	-1.50	
A_87_P050806	NM_001012913	SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	0.9999	-1.14	0.0673	-1.52	M*
A_87_P120788	CR385330		finished cDNA, clone ChEST411l5.	0.9999	-1.25	0.0673	-1.56	M*
A_87_P200363	XM_422605	PID1	PREDICTED: similar to putative protein product of HMFN2073	0.9999	-1.13	0.0398	-1.57	
A_87_P254148	XM_415601	FN3K	fructosamine 3 kinase	0.9999	-1.23	0.0398	-1.57	M*
A_87_P037180	NM_001031565	LOC429115	methylcrotonoyl-Coenzyme A carboxylase 2-like	0.9999	-1.32	0.0950	-1.60	
A_87_P013010	CR406089		finished cDNA, clone ChEST972m23.	0.9999	-1.19	0.0398	-1.62	
A_87_P080266	CR354362	OIT3	oncoprotein induced transcript 3	0.9999	-1.16	0.0825	-1.66	M*
A_87_P125708	CR391453		finished cDNA, clone ChEST902p11.	0.9999	-1.20	0.0673	-1.66	M*
A_87_P258958	XM_001235504	LOC772356	PREDICTED: hypothetical protein LOC772356	0.9999	-1.29	0.0398	-1.66	
A_87_P012557	CR406755		finished cDNA, clone ChEST979f12.	0.9999	-1.14	0.0000	-1.68	
A_87_P129033	NM_001001193	GAL6	Gal 6	0.4433	-1.54	0.0950	-1.68	
A_87_P077406	NM_205207	MFI2	antigen p97 (melanoma associated)	0.9999	-1.14	0.0993	-1.70	M*
A_87_P056411	NM_001012561	MYLIP	myosin regulatory light chain interacting protein	0.0519	-1.73	0.0255	-1.70	M*
A_87_P081236	NM_205102	GFRA1	GDNF family receptor alpha 1	0.9999	-1.24	0.0673	-1.74	M*
A_87_P017371	CR354362	OIT3	oncoprotein induced transcript 3	0.9999	-1.16	0.0612	-1.77	M*
A_87_P008791	NM_205207	MFI2	antigen p97 (melanoma associated)	0.9999	-1.10	0.0673	-1.78	M*
A_87_P192938	NM_001030731	CD36	thrombospondin receptor	0.9699	-1.38	0.0398	-1.81	M*

Probe ID	Gene Bank ID	Gene Symbol	Gene Name/Description	LD		HD		IPA
				FDR *p*	Fold Change	FDR *p*	Fold Change	
A_87_P036885	NM_001030731	CD36	thrombospondin receptor	0.9999	-1.36	0.0000	-1.82	M*
A_87_P073711	XM_001231641	ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	0.9999	-1.20	0.0884	-1.83	M*
A_87_P101486		STEAP4	STEAP family member 4	0.4702	-1.58	0.0255	-1.87	M
A_87_P011702	CR523309		finished cDNA, clone ChEST857k6.	0.9999	-1.17	0.0000	-1.94	M*
A_87_P100606	NM_001001194	GAL7	Gal 7	0.2050	-1.88	0.0733	-1.95	
A_87_P129133	NM_001001194	GAL7	Gal 7	0.0519	-2.07	0.0398	-2.00	
A_87_P128803	DQ092353	CATHL3	cathelicidin antimicrobial peptide	0.1896	-1.81	0.0507	-2.01	
A_87_P035105	NM_001001194	GAL7	Gal 7	0.0519	-2.02	0.0398	-2.03	
A_87_P128798	DQ092351	CATHL3	cathelicidin antimicrobial peptide	0.0000	-2.25	0.0673	-2.07	
A_87_P010585	XM_418328	DECR1	PREDICTED: 2,4-dienoyl CoA reductase 1, mitochondrial	0.9999	-1.44	0.0398	-2.12	M*
A_87_P014960	CR389220		finished cDNA, clone ChEST501i4.	0.1321	-2.29	0.0612	-2.13	
A_87_P268253	NM_001080884	TESC	tescalcin	0.9999	-1.40	0.0733	-2.27	M*
A_87_P100611			Gallinacin-7 Precursor	0.0000	-2.23	0.0000	-2.27	
A_87_P009368	NM_205478	LECT2	leukocyte cell-derived chemotaxin 2	0.4028	-2.03	0.0507	-2.35	M*
A_87_P016402	CR386546		finished cDNA, clone ChEST771i1.	0.2180	-2.13	0.0612	-2.41	
A_87_P018185	CR353073		finished cDNA, clone ChEST157l21.	0.0831	-3.15	0.0733	-2.47	
A_87_P129690	NM_001001605	CATHL3	cathelicidin antimicrobial peptide	0.1063	-2.53	0.0255	-2.58	
A_87_P059356	NM_001201399	GAL2	gallinacin 2	0.2717	-2.25	0.0398	-2.67	
A_87_P129023	NM_001201399	GAL2	gallinacin 2	0.2565	-2.70	0.0765	-2.93	
A_87_P158318	XM_001232471	ABI3BP	PREDICTED: ABI gene family, member 3 (NESH) binding protein	0.9999	-1.25	0.0000	-3.01	
A_87_P009700	DQ092352	CATHL2	Cathelicidin-2 Precursor	0.0000	-4.14	0.0000	-4.11	
A_87_P100946	FJ938358	CATHL2	Cathelicidin-2 Precursor	0.0000	-3.47	0.0000	-3.53	
A_87_P059231	NM_001024830	CATHL2	cathelicidin 2	0.0000	-3.60	0.0000	-3.97	
A_87_P100951	NM_001024830	CATHL2	cathelicidin 2	0.0000	-2.42	0.0000	-2.46	

SI Table 3. Detailed list of functions and genes within each functional enrichment category in liver tissue of male chicken embryos exposed to 45 µg/g of TDCPP.

Rank	Category	Function Annotation	p-value	Genes	# Genes
1	Infectious Disease	severe acute respiratory syndrome	1.18E-04	ARL4C, EGR1, GLUL, ZYX	7
	Infectious Disease	infection by Pseudomonas aeruginosa	3.67E-03	Gsta4	
	Infectious Disease	infection by Staphylococcus aureus	1.10E-02	CD36	
	Infectious Disease	infection of spleen and liver	2.54E-02 - 2.90E-02	BATF3	
	Infectious Disease	Parasitic Infection	4.64E-02	BATF3, CD36	
2	Respiratory Disease	severe acute respiratory syndrome	1.18E-04	ARL4C, EGR1, GLUL, ZYX	4
	Respiratory Disease	remodeling of pulmonary alveolus	1.82E-02	EGR1	
	Respiratory Disease	pulmonary interstitial fibrosis	3.26E-02	EGR1	
3	Cellular Function and Maintenance	homeostasis of metal ion (ex. Na+)	5.83E-04 - 1.82E-02	ATP2B2, SGK1, SLC40A1, TESC	12
	Cellular Function and Maintenance	cellular homeostasis	1.32E-02	ATP2B2, CD36, EGR1, Gsta4, SGK1, SLC40A1, SPINK5, TESC, WNT4	
	Cellular Function and Maintenance	Lymphocyte homeostasis	4.73E-02	EGR1, SLC40A1, SPINK5, WNT4	
	Cellular Function and Maintenance	function of microglia	1.52E-03	CD36, EGR1	
	Cellular Function and Maintenance	function of vestibular hair cells	1.10E-02	ATP2B2	
	Cellular Function and Maintenance	function of antigen presenting cells and phagocytes	2.02E-02 - 3.60E-02	BATF3, CD36, EGR1	
	Cellular Function and Maintenance	function of leukocytes	4.28E-02	BATF3, CD36, EGR1, LECT2	
	Cellular Function and Maintenance	metabolism of thymocytes	3.67E-03	EGR1	
	Cellular Function and Maintenance	uptake of eosinophils	3.67E-03	CD36	
	Cellular Function and Maintenance	phagocytosis by microglia and fibroblasts	1.10E-02	CD36	
	Cellular Function and Maintenance	respiration of fibroblast cell lines	2.54E-02	Gsta4	
	Cellular Function and Maintenance	formation of adherens junctions	4.67E-02	ZYX	
4	Small Molecule Biochemistry	homeostasis of cholesterol	1.44E-02	CD36, MYLIP	17

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Small Molecule Biochemistry	abnormal quantity of progesterone	3.67E-03	EGR1	
	Small Molecule Biochemistry	abnormal quantity of lipid	8.88E-03	CD36, EGR1, STEAP4	
	Small Molecule Biochemistry	abnormal quantity of hormone	9.82E-03	CD36, EGR1	
	Small Molecule Biochemistry	abnormal quantity of triacylglycerol	2.18E-02	CD36	
	Small Molecule Biochemistry	hydroxylation of lauric acid	7.33E-03	CYP2C9	
	Small Molecule Biochemistry	incorporation of fatty acids	7.33E-03 - 3.97E-02	CD36	
	Small Molecule Biochemistry	metabolism of epoxyeicosatrienoic acids	7.33E-03 - 1.10E-02	CYP2C9	
	Small Molecule Biochemistry	metabolism of 17-alpha-ethinylestradiol	1.10E-02	CYP2C9	
	Small Molecule Biochemistry	metabolism of linoleic acid	1.46E-02	CYP2C9	
	Small Molecule Biochemistry	metabolism of 5-hydroxytryptamine	2.18E-02	ATP2B2	
	Small Molecule Biochemistry	steroid metabolism	2.87E-02	CYP2C9, LSS, WNT4	
	Small Molecule Biochemistry	metabolism of arachidonic acid	3.26E-02	CYP2C9	
	Small Molecule Biochemistry	metabolism of cyclic GMP	3.61E-02	ATP2B2	
	Small Molecule Biochemistry	oxidation of fatty acid	8.47E-03	CD36, CYP2C9, DECR1	
	Small Molecule Biochemistry	oxidation of cholesterol	1.10E-02	CD36	
	Small Molecule Biochemistry	oxidation of 2-hydroxyestradiol	1.46E-02	CYP2C9	
	Small Molecule Biochemistry	oxidation of beta-estradiol	2.18E-02	CYP2C9	
	Small Molecule Biochemistry	quantity of lactose	3.67E-03	ATP2B2	
	Small Molecule Biochemistry	quantity of steroid	9.37E-03	CD36, EGR1, SGK1, STEAP4, WNT4	
	Small Molecule Biochemistry	quantity of uric acid in blood	1.46E-02	OIT3	
	Small Molecule Biochemistry	quantity of phosphatidylethanolamine	1.82E-02	CD36	
	Small Molecule Biochemistry	quantity of lysophosphatidylcholine	3.26E-02	CD36	
	Small Molecule Biochemistry	reduction of monohydroperoxy-linoleic acid	3.67E-03	Gsta4	
	Small Molecule Biochemistry	release of amino acids	3.67E-03 - 7.33E-03	ENPEP	
	Small Molecule Biochemistry	release of L-aspartic acid	1.10E-02	ENPEP	
	Small Molecule Biochemistry	uptake of fatty acids	3.67E-03 - 3.97E-002	CD36	
	Small Molecule Biochemistry	uptake of D-glucose	7.31E-03	CD36, EGR1, STEAP4	
	Small Molecule Biochemistry	delay in initiation of absorption of cholesterol	7.33E-03	CD36	
	Small Molecule Biochemistry	accumulation of fatty acids	1.10E-02 - 1.82E-02	CD36	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Small Molecule Biochemistry	efflux of cholesterol	1.13E-02	CD36, LSS	
	Small Molecule Biochemistry	conversion of retinaldehyde and vitamin A	1.46E-02	CYP2C9	
	Small Molecule Biochemistry	synthesis of GABA	1.82E-02	EGR1	
	Small Molecule Biochemistry	synthesis of 5-hydroxytryptamine	2.18E-02	CYP2C9	
	Small Molecule Biochemistry	synthesis of androgen and hydrocortisone	2.18E-02 - 3.26E-02	WNT4	
	Small Molecule Biochemistry	activation of fatty acid	2.18E-02	CYP2C9	
	Small Molecule Biochemistry	concentration of lipid	2.92E-02	CD36, DECR1, EGR1, SGK1, STEAP4, WNT4	
	Small Molecule Biochemistry	concentration of hormone	3.03E-02	CD36, EGR1, STEAP4, WNT4	
	Small Molecule Biochemistry	storage of cholesterol	4.32E-02	CD36	
	Small Molecule Biochemistry	transport of palmitic acid	4.32E-02	CD36	
	Small Molecule Biochemistry	catabolism of hydrogen peroxide	4.67E-02	GPX3	
	Small Molecule Biochemistry	secretion of triacylglycerol	4.67E-02	CD36	
5	Cancer	serous ovarian carcinoma process	2.87E-04	ARL4C, CLDN3, ENPEP, GPX3	20
	Cancer	clear-cell ovarian carcinoma	1.52E-03	CLDN3, GPX3	
	Cancer	epithelial ovarian cancer	2.64E-03	ARL4C, CLDN3, ENPEP, GPX3, SEC14L1	
	Cancer	binding of acute myeloblastic leukemia cells	3.67E-03	GFRA1	
	Cancer	tumorigenesis of leukemia cell lines	3.67E-03	EGR1	
	Cancer	growth of secondary tumor	2.54E-02	PTP4A3	
	Cancer	malignant mixed Mullerian tumor	2.54E-02	CLDN3	
	Cancer	endometrial ovarian cancer	3.16E-02	CLDN3, GPX3, SEC14L1	
	Cancer	ovarian endometrioid carcinoma	3.26E-02	CLDN3	
	Cancer	development of T-cell non-Hodgkin lymphoma	3.61E-02	EGR1	
	Cancer	papilloma	3.87E-02	EGR1, Gsta4	
	Cancer	invasion of metanephric bud	4.32E-02	GFRA1	
	Cancer	lymphohematopoietic cancer	4.41E-02	ATP2B2, CD36, EGR1, GPX3, RPLP1, SGK1	
	Cancer	mammary tumor	4.63E-02	ATP2B2, CLDN3, DECR1, EGR1, GFRA1, SLC40A1, ZYX	
	Cancer	hereditary gingival fibromatosis	4.67E-02	EGR1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Cancer	digestive organ tumor	4.93E-02	CD36, CLDN3, CYP2C9, GLUL, GPX3, LECT2, OIT3, PTP4A3, SGK1	
	Cancer	non-Hodgkin's disease	4.94E-02	CD36, EGR1, SGK1	
	Endocrine System Disorders	serous ovarian carcinoma process	2.87E-04	ARL4C, CLDN3, ENPEP, GPX3	
	Endocrine System Disorders	clear-cell ovarian carcinoma	1.52E-03	CLDN3, GPX3	
6	Endocrine System Disorders	epithelial ovarian cancer	2.64E-03	ARL4C, CLDN3, ENPEP, GPX3, SEC14L1	5
	Endocrine System Disorders	endometrial ovarian cancer	3.16E-02	CLDN3, GPX3, SEC14L1	
	Endocrine System Disorders	ovarian endometrioid carcinoma	3.26E-02	CLDN3	
	Reproductive System Disease	serous ovarian carcinoma process	2.87E-04	ARL4C, CLDN3, ENPEP, GPX3	
	Reproductive System Disease	clear-cell ovarian carcinoma	1.52E-03	CLDN3, GPX3	
	Reproductive System Disease	epithelial ovarian cancer	2.64E-03	ARL4C, CLDN3, ENPEP, GPX3, SEC14L1	
	Reproductive System Disease	mullerian aplasia	3.67E-03	WNT4	
7	Reproductive System Disease	endometriosis	3.94E-03	ATP2B2, CADM3, EGR1, GPX3, SGK1	10
	Reproductive System Disease	malignant mixed Mullerian tumor	2.54E-02	CLDN3	
	Reproductive System Disease	secondary sex reversal	2.90E-02	WNT4	
	Reproductive System Disease	endometrial ovarian cancer	3.16E-02	CLDN3, GPX3, SEC14L1	
	Reproductive System Disease	ovarian endometrioid carcinoma	3.26E-02	CLDN3	
	Nervous System Development and Function	quantity of motor neurons	6.60E-04	GDPD5, GFRA1, WNT4	
	Nervous System Development and Function	quantity of sensory neurons	1.61E-02	ATP2B2, GFRA1	
	Nervous System Development and Function	quantity of thyrotrophs	2.18E-02	WNT4	
8	Nervous System Development and Function	quantity of GABAergic neurons	2.54E-02	GFRA1	9
	Nervous System Development and Function	quantity of somatotrophs and gonadotropes	2.90E-0.2 - 3.97E-02	WNT4	
	Nervous System Development and Function	abnormal morphology of auditory cortex	3.67E-03	ATP2B2	
	Nervous System Development and Function	abnormal morphology of neurons	4.22E-02	ATP2B2, GFRA1, WNT4	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Nervous System Development and Function	abnormal morphology of petrosal ganglion	4.32E-02	GFRA1	
	Nervous System Development and Function	long-term potentiation of dentate gyrus	7.33E-03	EGR1	
	Nervous System Development and Function	morphology of somatotrophs	7.33E-03	EGR1	
	Nervous System Development and Function	morphology of brain cells	4.38E-02	ATP2B2, EGR1	
	Nervous System Development and Function	morphology of nerves	4.73E-02	ATP2B2, GFRA1	
	Nervous System Development and Function	long-term memory	7.63E-03	EGR1, SGK1	
	Nervous System Development and Function	differentiation of GABAergic neurons	1.10E-02	GFRA1	
	Nervous System Development and Function	differentiation of neurons	2.19E-02	ATP2B2, GDPD5, GFRA1, SPINK5	
	Nervous System Development and Function	function of vestibular hair cells	1.10E-02	ATP2B2	
	Nervous System Development and Function	loss of enteric neurons	1.10E-02	GFRA1	
	Nervous System Development and Function	loss of outer hair cells	1.10E-02	ATP2B2	
	Nervous System Development and Function	loss of trigeminal motor neurons	1.10E-02	GFRA1	
	Nervous System Development and Function	loss of nodose ganglion neurons	2.18E-02	GFRA1	
	Nervous System Development and Function	recruitment of microglia	1.10E-02	CD36	
	Nervous System Development and Function	retraction of axons	1.46E-02	WNT4	
	Nervous System Development and Function	cell division of neurons	1.82E-02	GFRA1	
	Nervous System Development and Function	lack of hair cells	1.82E-02	ATP2B2	
	Nervous System Development and Function	lack of enteric neurons	2.54E-02	GFRA1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Nervous System Development and Function	development of adenohypophysis	2.54E-02	EGR1	
	Nervous System Development and Function	development of enteric nervous system	3.61E-02	GFRA1	
	Nervous System Development and Function	chemotaxis of nervous tissue cell lines	2.90E-02	CD36	
	Nervous System Development and Function	plasticity of synapse	3.95E-02	ATP2B2, EGR1	
	Nervous System Development and Function	abnormal auditory evoked potential	3.97E-02	ATP2B2	
	Nervous System Development and Function	pain behavior	4.67E-02	GFRA1	
9	Tissue Morphology	quantity of neurons	8.41E-03	ATP2B2, GDPD5, GFRA1, WNT4	13
	Tissue Morphology	quantity of myeloma cell lines	1.10E-02	SGK1	
	Tissue Morphology	quantity of cells	1.14E-02	ATP2B2, BATF3, CD36, EGR1, GDPD5, GFRA1, LECT2, SEPN1, SGK1, STEAP4, WNT4	
	Tissue Morphology	quantity of leukocytes	4.62E-02	BATF3, CD36, EGR1, LECT2, STEAP4, WNT4	
	Tissue Morphology	quantity of Peyer's patches	4.67E-02	GFRA1	
	Tissue Morphology	abnormal morphology of lateral motor column	3.67E-03	GFRA1	
	Tissue Morphology	abnormal morphology of adipose tissue	2.09E-02	CD36, STEAP4	
	Tissue Morphology	abnormal morphology of white adipose tissue	2.54E-02	STEAP4	
	Tissue Morphology	abnormal morphology of Wolffian duct	3.97E-02	GFRA1	
	Tissue Morphology	abnormal morphology of neurons	4.22E-02	ATP2B2, GFRA1, WNT4	
	Tissue Morphology	abnormal morphology of petrosal ganglion	4.32E-02	GFRA1	
	Tissue Morphology	erosion of tarsal bone	3.67E-03	LECT2	
	Tissue Morphology	induction of renal vesicle	3.67E-03	WNT4	
	Tissue Morphology	lack of tunnel of Corti	1.10E-02	ATP2B2	
	Tissue Morphology	lack of mullerian duct	1.46E-02	WNT4	
	Tissue Morphology	lack of hair cells	1.82E-02	ATP2B2	
	Tissue Morphology	relaxation of aortic ring tissue	3.97E-02	CYP2C9	
	Tissue Morphology	morphology of nerves	4.73E-02	ATP2B2, GFRA1	
	Tissue Morphology	size of infarct	4.91E-02	EGR1, GPX3	

Rank	Category	Function Annotation	p-value	Genes	# Genes
10	Hematological System Development and Function	function of microglia	1.52E-03	CD36, EGR1	10
	Hematological System Development and Function	erythropoiesis of splenocytes	3.67E-03	EGR1	
	Hematological System Development and Function	metabolism of thymocytes	3.67E-03	EGR1	
	Hematological System Development and Function	uptake of eosinophils	3.67E-03	CD36	
	Hematological System Development and Function	abnormal morphology of foam cells	7.33E-03	CD36	
	Hematological System Development and Function	abnormal morphology of neutrophils	1.82E-02	EGR1	
	Hematological System Development and Function	cell rolling of red blood cells	7.33E-03	CD36	
	Hematological System Development and Function	maturation of monocytes	7.33E-03	GFRA1	
	Hematological System Development and Function	anticoagulation	1.10E-02	CYP2C9	
	Hematological System Development and Function	phagocytosis by microglia	1.10E-02	CD36	
	Hematological System Development and Function	recruitment of microglia	1.10E-02	CD36	
	Hematological System Development and Function	differentiation of blood cells	1.55E-02	BATF3, CD36, EGR1, SPINK5, TESC, WNT4	
	Hematological System Development and Function	differentiation of B-1 lymphocytes	2.18E-02	EGR1	
	Hematological System Development and Function	differentiation of leukocytes	2.72E-02	BATF3, CD36, EGR1, SPINK5, WNT4	
	Hematological System Development and Function	differentiation of myeloid dendritic cells	3.26E-02	BATF3	
	Hematological System Development and Function	differentiation of lymphocytes	3.79E-02	CD36, EGR1, SPINK5, WNT4	
	Hematological System Development and Function	differentiation of antigen presenting cells	4.91E-02	BATF3, EGR1	
	Hematological System Development and Function	quantity of myeloid dendritic cells	3.61E-02	BATF3	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Hematological System Development and Function	quantity of leukocytes	4.62E-02	BATF3, CD36, EGR1, LECT2, STEAP4, WNT4	
	Hematological System Development and Function	adhesion of red blood cells	3.97E-02	CD36	
	Hematological System Development and Function	accumulation of eosinophils	4.32E-02	EGR1	
	Hematological System Development and Function	formation of macrophages	4.67E-02	EGR1	
	Hematological System Development and Function	morphology of myeloid cells	4.82E-02	CD36, EGR1	
11	Amino Acid Metabolism	release of amino acids	3.67E-03 - 7.33E-03	ENPEP	2
	Amino Acid Metabolism	synthesis of GABA	1.82E-02	EGR1	
12	Auditory and Vestibular System Development and Function	mineralization of otoliths	3.67E-03	ATP2B2	1
	Auditory and Vestibular System Development and Function	lack of pillar cells	1.10E-02	ATP2B2	
	Auditory and Vestibular System Development and Function	lack of supporting cell layer of inner ear	1.10E-02	ATP2B2	
	Auditory and Vestibular System Development and Function	lack of hair cells	1.82E-02	ATP2B2	
	Auditory and Vestibular System Development and Function	lack of otoliths	2.54E-02	ATP2B2	
	Auditory and Vestibular System Development and Function	auditory brainstem response	2.54E-02	ATP2B2	
13	Carbohydrate Metabolism	glycogenesis of soleus muscle	3.67E-03	CD36	6
	Carbohydrate Metabolism	metabolism of fructosamine	3.67E-03	FN3K	
	Carbohydrate Metabolism	metabolism of etoposide	7.33E-03	CYP2C9	
	Carbohydrate Metabolism	quantity of lactose	3.67E-03	ATP2B2	
	Carbohydrate Metabolism	quantity of phosphatidylethanolamine	1.82E-02	CD36	
	Carbohydrate Metabolism	quantity of lysophosphatidylcholine	3.26E-02	CD36	
	Carbohydrate Metabolism	uptake of D-glucose	7.31E-03	CD36, EGR1, STEAP4	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Carbohydrate Metabolism	absorption of D-glucose	7.33E-03	CD36	
	Carbohydrate Metabolism	gluconeogenesis	1.38E-02	CD36, STEAP4	
	Carbohydrate Metabolism	depletion of glycogen	2.18E-02	CD36	
	Cell Cycle	metaphase/anaphase transition of liver cells	3.67E-03	EGR1	
14	Cell Cycle	entry into cell cycle progression of kidney cell lines	7.33E-03	WNT4	3
	Cell Cycle	cell division of neurons	1.82E-02	GFRA1	
	Cell Death and Survival	cytotoxicity of hepatoma cell lines	3.67E-03	SGK1	
	Cell Death and Survival	cell death of bone marrow stromal cells	7.33E-03	EGR1	
	Cell Death and Survival	cell death of enteric neurons	7.33E-03	GFRA1	
	Cell Death and Survival	cell death of epithelial cells	3.24E-02	ATP2B2, DECR1, EGR1, SGK1	
	Cell Death and Survival	apoptosis of breast cell lines	1.03E-02	DECR1, SGK1	
	Cell Death and Survival	apoptosis of epithelial cell lines	2.33E-02	ATP2B2, DECR1, SGK1	
15	Cell Death and Survival	apoptosis of motor neurons	3.97E-02	GFRA1	6
	Cell Death and Survival	loss of outer hair cells	1.10E-02	ATP2B2	
	Cell Death and Survival	loss of trigeminal motor neurons	1.10E-02	GFRA1	
	Cell Death and Survival	loss of nodose ganglion neurons	2.18E-02	GFRA1	
	Cell Death and Survival	cytolysis of fibroblast cell lines	1.82E-02	CLDN3	
	Cell Death and Survival	survival of superior cervical ganglion neurons	2.18E-02	GFRA1	
	Cell Death and Survival	survival of dopaminergic neurons	3.97E-02	GFRA1	
	Cell Morphology	morphology of germ cell tumor cell lines	3.67E-03	EGR1	
	Cell Morphology	morphology of somatotrophs	7.33E-03	EGR1	
	Cell Morphology	morphology of phagocytes	3.87E-02	CD36, EGR1	
	Cell Morphology	morphology of brain cells	4.38E-02	ATP2B2, EGR1	
	Cell Morphology	morphology of myeloid cells	4.82E-02	CD36, EGR1	
	Cell Morphology	abnormal morphology of foam cells	7.33E-03	CD36	
	Cell Morphology	abnormal morphology of neutrophils	1.82E-02	EGR1	
16	Cell Morphology	abnormal morphology of enteric neurons	3.26E-02	GFRA1	7
	Cell Morphology	abnormal morphology of neurons	4.22E-02	ATP2B2, GFRA1, WNT4	
	Cell Morphology	abnormal morphology of Leydig cells	4.67E-02	EGR1	
	Cell Morphology	integrity of adherens junctions	7.33E-03	ZYX	
	Cell Morphology	keratinization of skin	1.82E-02	SPINK5	
	Cell Morphology	lack of hair cells	1.82E-02	ATP2B2	
	Cell Morphology	plasticity of synapse	3.95E-02	ATP2B2, EGR1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
17	Cell-To-Cell Signaling and Interaction	binding of acute myeloblastic leukemia cells	3.67E-03	GFRA1	11
	Cell-To-Cell Signaling and Interaction	activation of kidney cancer cell lines	7.33E-03	ENPEP	
	Cell-To-Cell Signaling and Interaction	cell-cell adhesion of squamous cell carcinoma cell lines	7.33E-03	ZYX	
	Cell-To-Cell Signaling and Interaction	cell-cell adhesion	1.23E-02	CADM3, CLDN3, ZYX	
	Cell-To-Cell Signaling and Interaction	chemoattraction of GABAergic neurons	7.33E-03	GFRA1	
	Cell-To-Cell Signaling and Interaction	long-term potentiation of dentate gyrus	7.33E-03	EGR1	
	Cell-To-Cell Signaling and Interaction	phagocytosis by microglia and fibroblasts	1.10E-02	CD36	
	Cell-To-Cell Signaling and Interaction	recruitment of microglia	1.10E-02	CD36	
	Cell-To-Cell Signaling and Interaction	attachment of brain cancer cell lines	1.46E-02	EGR1	
	Cell-To-Cell Signaling and Interaction	metabolism of 5-hydroxytryptamine	2.18E-02	ATP2B2	
	Cell-To-Cell Signaling and Interaction	synthesis of 5-hydroxytryptamine	2.18E-02	CYP2C9	
	Cell-To-Cell Signaling and Interaction	response of endothelial cells	2.54E-02	CD36	
	Cell-To-Cell Signaling and Interaction	adhesion of tumor cell lines	3.07E-02	EGR1, PTP4A3, ZYX	
	Cell-To-Cell Signaling and Interaction	adhesion of red blood cells	3.97E-02	CD36	
	Cell-To-Cell Signaling and Interaction	plasticity of synapse	3.95E-02	ATP2B2, EGR1	
	Cell-To-Cell Signaling and Interaction	abnormal auditory evoked potential	3.97E-02	ATP2B2	
	Cell-To-Cell Signaling and Interaction	immune response of antigen presenting cells	4.20E-02	BATF3, CD36	
	Cell-To-Cell Signaling and Interaction	formation of adherens junctions	4.67E-02	ZYX	
18	Cell-mediated Immune	metabolism of thymocytes	3.67E-03	EGR1	1

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Response				
19	Cellular Development	erythropoiesis of splenocytes	3.67E-03	EGR1	12
	Cellular Development	maturation of acute myeloblastic leukemia cells	3.67E-03	GFRA1	
	Cellular Development	maturation of monocytes	7.33E-03	GFRA1	
	Cellular Development	maturation of plasmacytoid dendritic cells	1.46E-02	EGR1	
	Cellular Development	maturation of phagocytes	1.66E-02	EGR1, GFRA1	
	Cellular Development	metabolism of thymocytes	3.67E-03	EGR1	
	Cellular Development	differentiation of GABAergic neurons	1.10E-02	GFRA1	
	Cellular Development	differentiation of blood cells	1.55E-02	BATF3, CD36, EGR1, SPINK5, TESC, WNT4	
	Cellular Development	differentiation of B-1 lymphocytes	2.18E-02	EGR1	
	Cellular Development	differentiation of neurons	2.19E-02	ATP2B2, GDPD5, GFRA1, SPINK5	
	Cellular Development	differentiation of leukocytes	2.72E-02	BATF3, CD36, EGR1, SPINK5, WNT4	
	Cellular Development	differentiation of myeloid dendritic cells	3.26E-02	BATF3	
	Cellular Development	differentiation of lymphocytes	3.79E-02	CD36, EGR1, SPINK5, WNT4	
	Cellular Development	differentiation of Schwann cells	3.97E-02	GLUL	
	Cellular Development	differentiation of cells	4.26E-02	ATP2B2, BATF3, CD36, EGR1, GDPD5, GFRA1, GLUL, SPINK5, TESC, WNT4	
	Cellular Development	differentiation of granule cells	4.32E-02	ATP2B2	
	Cellular Development	differentiation of antigen presenting cells	4.91E-02	BATF3, EGR1	
	Cellular Development	arrest in growth of melanoma cells	1.10E-02	EGR1	
	Cellular Development	proliferation of acute myeloblastic leukemia cells	1.46E-02	GFRA1	
	Cellular Development	proliferation of epithelial cell lines	2.02E-02	DECR1, EGR1, PTP4A3	
	Cellular Development	proliferation of fibrosarcoma cell lines	3.97E-02	EGR1	
	Cellular Development	development of oocytes	3.26E-02	WNT4	
20	Developmental Disorder	SERKAL syndrome, sex reversal, dysgenesis of kidneys, adrenals and lungs	3.67E-03	WNT4	6
	Developmental Disorder	congenital disorder of glycosylation type 1a	3.67E-03	PMM2	
	Developmental Disorder	autosomal dominant hemochromatosis	3.67E-03	SLC40A1	
	Developmental Disorder	hemochromatosis Type IV	3.67E-03	SLC40A1	
	Developmental Disorder	mullerian aplasia	3.67E-03	WNT4	
	Developmental Disorder	rigid spine syndrome	3.67E-03	SEPN1	
	Developmental Disorder	congenital myopathy with fiber type disproportion	7.33E-03	SEPN1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Developmental Disorder	Niemann-Pick disease type C	1.82E-02	CD36	
	Developmental Disorder	secondary sex reversal	2.90E-02	WNT4	
	Developmental Disorder	Hirschsprung disease	3.97E-02	GFRA1	
	Developmental Disorder	developmental delay of kidney	3.97E-02	WNT4	
	Developmental Disorder	agenesis of kidney	4.67E-02	GFRA1	
21	Digestive System Development and Function	abnormal size of liver	3.67E-03	EGR1	3
	Digestive System Development and Function	contraction of intestine	1.10E-02	GFRA1	
	Digestive System Development and Function	function of liver	1.49E-02	CD36, EGR1	
	Digestive System Development and Function	abnormal morphology of intestine	2.50E-02	CD36, GFRA1	
	Digestive System Development and Function	food preference	2.54E-02	CD36	
	Digestive System Development and Function	lack of enteric neurons	2.54E-02	GFRA1	
22	Embryonic Development	abnormal morphology of enlarged anterior chamber of eye	3.67E-03	EGR1	6
	Embryonic Development	erythropoiesis of splenocytes	3.67E-03	EGR1	
	Embryonic Development	formation of renal vesicle	7.33E-03	WNT4	
	Embryonic Development	development of spleen	8.48E-03	EGR1, SLC40A1	
	Embryonic Development	development of mullerian duct	1.82E-02	WNT4	
	Embryonic Development	development of mesonephros	2.18E-02	WNT4	
	Embryonic Development	development of adenohypophysis	2.54E-02	EGR1	
	Embryonic Development	development of oocytes	3.26E-02	WNT4	
	Embryonic Development	development of hair	4.67E-02	SPINK5	
	Embryonic Development	abnormal size of eye	1.10E-02	EGR1	
	Embryonic Development	lack of mullerian duct	1.46E-02	WNT4	
	Embryonic Development	keratinization of skin	1.82E-02	SPINK5	
	Embryonic Development	secondary sex determination	1.82E-02	WNT4	
	Embryonic Development	morphogenesis of nephrogenic mesenchyme	2.18E-02	WNT4	
	Embryonic Development	invasion of embryonic cell lines	3.26E-02	PTP4A3	
	Embryonic Development	invasion of metanephric bud	4.32E-02	GFRA1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
23	Endocrine System Development and Function	abnormal quantity of progesterone	3.67E-03	EGR1	5
	Endocrine System Development and Function	abnormal quantity of hormone	9.82E-03	CD36, EGR1	
	Endocrine System Development and Function	morphology of pituitary gland	6.07E-03	EGR1, WNT4	
	Endocrine System Development and Function	morphology of somatotrophs	7.33E-03	EGR1	
	Endocrine System Development and Function	metabolism of 17-alpha-ethinylestradiol	1.10E-02	CYP2C9	
	Endocrine System Development and Function	oxidation of beta-estradiol	2.18E-02	CYP2C9	
	Endocrine System Development and Function	quantity of thyrotrophs	2.18E-02	WNT4	
	Endocrine System Development and Function	quantity of somatotrophs	2.90E-02	WNT4	
	Endocrine System Development and Function	quantity of gonadotropes	3.97E-02	WNT4	
	Endocrine System Development and Function	synthesis of androgen	2.18E-02	WNT4	
	Endocrine System Development and Function	synthesis of hydrocortisone	3.26E-02	WNT4	
	Endocrine System Development and Function	development of adenohypophysis	2.54E-02	EGR1	
	Endocrine System Development and Function	concentration of hormone	3.03E-02	CD36, EGR1, STEAP4, WNT4	
24	Energy Production	oxidation of fatty acid	8.47E-03	CD36, CYP2C9, DECR1	3
	Energy Production	oxidation of cholesterol	1.10E-02	CD36	
	Energy Production	oxidation of 2-hydroxyestradiol	1.46E-02	CYP2C9	
	Energy Production	oxidation of beta-estradiol	2.18E-02	CYP2C9	
25	Free Radical Scavenging	reduction of monohydroperoxy-linoleic acid	3.67E-03	Gsta4	4
	Free Radical Scavenging	secretion of reactive oxygen species	7.33E-03	CD36	
	Free Radical Scavenging	metabolism of reactive oxygen species	1.98E-02	CD36, DECR1, GPX3, Gsta4	
	Free Radical Scavenging	production of superoxide	2.78E-02	CD36, Gsta4	
	Free Radical Scavenging	production of reactive oxygen species	3.69E-02	CD36, DECR1, Gsta4	
	Free Radical Scavenging	catabolism of hydrogen peroxide	4.67E-02	GPX3	

Rank	Category	Function Annotation	p-value	Genes	# Genes
26	Hematological Disease	CD36 deficiency	3.67E-03	CD36	4
	Hematological Disease	autosomal dominant hemochromatosis	3.67E-03	SLC40A1	
	Hematological Disease	hemochromatosis Type IV	3.67E-03	SLC40A1	
	Hematological Disease	tumorigenesis of leukemia cell lines	3.67E-03	EGR1	
	Hematological Disease	non-Hodgkin's disease	4.94E-02	CD36, EGR1, SGK1	
27	Hematopoiesis	erythropoiesis of splenocytes	3.67E-03	EGR1	6
	Hematopoiesis	metabolism of thymocytes	3.67E-03	EGR1	
	Hematopoiesis	maturation of monocytes	7.33E-03	GFRA1	
	Hematopoiesis	maturation of plasmacytoid dendritic cells	1.46E-02	EGR1	
	Hematopoiesis	maturation of phagocytes	1.66E-02	EGR1, GFRA1	
	Hematopoiesis	differentiation of B-1 lymphocytes	2.18E-02	EGR1	
	Hematopoiesis	differentiation of leukocytes	2.72E-02	BATF3, CD36, EGR1, SPINK5, WNT4	
	Hematopoiesis	differentiation of myeloid dendritic cells	3.26E-02	BATF3	
	Hematopoiesis	differentiation of lymphocytes	3.79E-02	CD36, EGR1, SPINK5, WNT4	
	Hematopoiesis	differentiation of antigen presenting cells	4.91E-02	BATF3, EGR1	
	Hematopoiesis	adhesion of red blood cells	3.97E-02	CD36	
28	Hepatic System Development and Function	abnormal size of liver	3.67E-03	EGR1	2
	Hepatic System Development and Function	metaphase/anaphase transition of liver cells	3.67E-03	EGR1	
	Hepatic System Development and Function	function of liver	1.49E-02	CD36, EGR1	
29	Hereditary Disorder	CD36 deficiency	3.67E-03	CD36	9
	Hereditary Disorder	SERKAL syndrome, sex reversal, dysgenesis of kidneys, adrenals and lungs	3.67E-03	WNT4	
	Hereditary Disorder	congenital disorder of glycosylation type 1a	3.67E-03	PMM2	
	Hereditary Disorder	glutamine synthase deficiency	3.67E-03	GLUL	
	Hereditary Disorder	autosomal dominant hemochromatosis	3.67E-03	SLC40A1	
	Hereditary Disorder	hemochromatosis Type IV	3.67E-03	SLC40A1	
	Hereditary Disorder	rigid spine syndrome	3.67E-03	SEPN1	
	Hereditary Disorder	congenital myopathy with fiber type disproportion	7.33E-03	SEPN1	
	Hereditary Disorder	nonsyndromic recessive deafness DFNB12	7.33E-03	ATP2B2	
	Hereditary Disorder	Netherton syndrome	1.10E-02	SPINK5	
	Hereditary Disorder	Niemann-Pick disease type C	1.82E-02	CD36	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Hereditary Disorder	hereditary gingival fibromatosis	4.67E-02	EGR1	
30	Hypersensitivity Response	uptake of eosinophils	3.67E-03	CD36	2
	Hypersensitivity Response	accumulation of eosinophils	4.32E-02	EGR1	
31	Lipid Metabolism	abnormal quantity of progesterone	3.67E-03	EGR1	10
	Lipid Metabolism	abnormal quantity of lipid	8.88E-03	CD36, EGR1, STEAP4	
	Lipid Metabolism	abnormal quantity of triacylglycerol	2.18E-02	CD36	
	Lipid Metabolism	incorporation of 15-(4-iodophenyl)-3-methylpentadecanoic acid	3.67E-03	CD36	
	Lipid Metabolism	incorporation of diacyl- and triacylglycerol	7.33E-03 - 1.82E-02	CD36	
	Lipid Metabolism	incorporation of palmitic acid	2.18E-02	CD36	
	Lipid Metabolism	incorporation of oleic acid	3.97E-02	CD36	
	Lipid Metabolism	oxidation of fatty acid	8.47E-03	CD36, CYP2C9, DECR1	
	Lipid Metabolism	oxidation of cholesterol	1.10E-02	CD36	
	Lipid Metabolism	oxidation of 2-hydroxyestradiol	1.46E-02	CYP2C9	
	Lipid Metabolism	oxidation of beta-estradiol	2.18E-02	CYP2C9	
	Lipid Metabolism	reduction of monohydroperoxy-linoleic acid	3.67E-03	Gsta4	
	Lipid Metabolism	uptake of omega-(4-iodophenyl)pentadecanoic acid	3.67E-03	CD36	
	Lipid Metabolism	uptake of 15-(4-iodophenyl)-3-methylpentadecanoic acid	7.33E-03	CD36	
	Lipid Metabolism	uptake of myristic acid	7.33E-03	CD36	
	Lipid Metabolism	uptake of oleic acid	1.82E-02	CD36	
	Lipid Metabolism	uptake of cholesterol ester	3.61E-02	CD36	
	Lipid Metabolism	uptake of palmitic acid	3.97E-02	CD36	
	Lipid Metabolism	delay in initiation of absorption of cholesterol	7.33E-03	CD36	
	Lipid Metabolism	hydroxylation of lauric acid	7.33E-03	CYP2C9	
	Lipid Metabolism	metabolism of epoxyeicosatrienoic acids	7.33E-03 - 1.10E-02	CYP2C9	
	Lipid Metabolism	metabolism of 17-alpha-ethinylestradiol	1.10E-02	CYP2C9	
	Lipid Metabolism	metabolism of linoleic acid	1.46E-02	CYP2C9	
	Lipid Metabolism	steroid metabolism	2.87E-02	CYP2C9, LSS, WNT4	
	Lipid Metabolism	metabolism of arachidonic acid	3.26E-02	CYP2C9	
	Lipid Metabolism	quantity of steroid	9.37E-03	CD36, EGR1, SGK1, STEAP4, WNT4	
	Lipid Metabolism	quantity of phosphatidylethanolamine	1.82E-02	CD36	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Lipid Metabolism	quantity of lysophosphatidylcholine	3.26E-02	CD36	
	Lipid Metabolism	accumulation of linoleic acid	1.10E-02	CD36	
	Lipid Metabolism	accumulation of oleic acid	1.10E-02	CD36	
	Lipid Metabolism	accumulation of stearic acid	1.10E-02	CD36	
	Lipid Metabolism	accumulation of palmitic acid	1.82E-02	CD36	
	Lipid Metabolism	efflux of cholesterol	1.13E-02	CD36, LSS	
	Lipid Metabolism	homeostasis of cholesterol	1.44E-02	CD36, MYLIP	
	Lipid Metabolism	conversion of retinaldehyde	1.46E-02	CYP2C9	
	Lipid Metabolism	conversion of vitamin A	1.46E-02	CYP2C9	
	Lipid Metabolism	synthesis of GABA	1.82E-02	EGR1	
	Lipid Metabolism	synthesis of androgen	2.18E-02	WNT4	
	Lipid Metabolism	synthesis of hydrocortisone	3.26E-02	WNT4	
	Lipid Metabolism	activation of fatty acid	2.18E-02	CYP2C9	
	Lipid Metabolism	concentration of lipid	2.92E-02	CD36, DECR1, EGR1, SGK1, STEAP4, WNT4	
	Lipid Metabolism	concentration of cholesterol	3.93E-02	CD36, EGR1, STEAP4	
	Lipid Metabolism	concentration of triacylglycerol	4.47E-02	CD36, EGR1, STEAP4	
	Lipid Metabolism	storage of cholesterol	4.32E-02	CD36	
	Lipid Metabolism	transport of palmitic acid	4.32E-02	CD36	
	Lipid Metabolism	secretion of triacylglycerol	4.67E-02	CD36	
32	Lymphoid Tissue Structure and Development	erythropoiesis of splenocytes	3.67E-03	EGR1	3
	Lymphoid Tissue Structure and Development	metabolism of thymocytes	3.67E-03	EGR1	
	Lymphoid Tissue Structure and Development	development of spleen	8.48E-03	EGR1, SLC40A1	
	Lymphoid Tissue Structure and Development	quantity of Peyer's patches	4.67E-02	GFRA1	
33	Metabolic Disease	congenital disorder of glycosylation type 1a	3.67E-03	PMM2	6
	Metabolic Disease	glutamine synthase deficiency	3.67E-03	GLUL	
	Metabolic Disease	autosomal dominant hemochromatosis	3.67E-03	SLC40A1	
	Metabolic Disease	hemochromatosis Type IV	3.67E-03	SLC40A1	
	Metabolic Disease	coumarin resistance	7.33E-03	CYP2C9	
	Metabolic Disease	xanthomatosis	1.10E-02	CD36	
	Metabolic Disease	hyperkalemia	1.46E-02	SGK1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Metabolic Disease	Niemann-Pick disease type C	1.82E-02	CD36	
	Metabolic Disease	cerebral amyloid angiopathy	3.61E-02	CD36	
	Molecular Transport	abnormal quantity of progesterone	3.67E-03	EGR1	
	Molecular Transport	abnormal quantity of lipid	8.88E-03	CD36, EGR1, STEAP4	
	Molecular Transport	abnormal quantity of hormone	9.82E-03	CD36, EGR1	
	Molecular Transport	abnormal quantity of triacylglycerol	2.18E-02	CD36	
	Molecular Transport	concentration of protein	3.67E-03	ATP2B2	
	Molecular Transport	concentration of lipid	2.92E-02	CD36, DECR1, EGR1, SGK1, STEAP4, WNT4	
	Molecular Transport	concentration of hormone	3.03E-02	CD36, EGR1, STEAP4, WNT4	
	Molecular Transport	concentration of cholesterol	3.93E-02	CD36, EGR1, STEAP4	
	Molecular Transport	concentration of triacylglycerol	4.47E-02	CD36, EGR1, STEAP4	
	Molecular Transport	quantity of lactose	3.67E-03	ATP2B2	
	Molecular Transport	quantity of steroid	9.37E-03	CD36, EGR1, SGK1, STEAP4, WNT4	
	Molecular Transport	quantity of phosphatidylethanolamine	1.82E-02	CD36	
	Molecular Transport	quantity of lysophosphatidylcholine	3.26E-02	CD36	
	Molecular Transport	uptake of D-glucose	7.31E-03	CD36, EGR1, STEAP4	
34	Molecular Transport	uptake of 15-(4-iodophenyl)-3-methylpentadecanoic acid	7.33E-03	CD36	9
	Molecular Transport	uptake of fatty acids	3.67E-03 - 3.97E-002	CD36	
	Molecular Transport	delay in initiation of absorption of cholesterol	7.33E-03	CD36	
	Molecular Transport	secretion of lipoprotein	7.33E-03	CD36	
	Molecular Transport	secretion of reactive oxygen species	7.33E-03	CD36	
	Molecular Transport	secretion of triacylglycerol	4.67E-02	CD36	
	Molecular Transport	accumulation of fatty acids	1.10E-02 - 1.82E-02	CD36	
	Molecular Transport	efflux of cholesterol	1.13E-02	CD36, LSS	
	Molecular Transport	efflux of Na+	1.82E-02	SGK1	
	Molecular Transport	depletion of glycogen	2.18E-02	CD36	
	Molecular Transport	release of heavy metal	2.18E-02	SLC40A1	
	Molecular Transport	reabsorption of Na+	2.90E-02	SGK1	
	Molecular Transport	transport of iron ion	3.61E-02	SLC40A1	
	Molecular Transport	transport of palmitic acid	4.32E-02	CD36	

Rank	Category	Function Annotation	p-value	Genes	# Genes
35	Neurological Disease	delay in hyperalgesia	3.67E-03	SGK1	5
	Neurological Disease	congenital myopathy with fiber type disproportion	7.33E-03	SEPN1	
	Neurological Disease	nonsyndromic recessive deafness DFNB12	7.33E-03	ATP2B2	
	Neurological Disease	noise-induced hearing loss	3.26E-02	ATP2B2	
	Neurological Disease	age-related hearing loss	3.61E-02	ATP2B2	
	Neurological Disease	cerebral amyloid angiopathy	3.61E-02	CD36	
	Neurological Disease	Hirschsprung disease	3.97E-02	GFRA1	
36	Nucleic Acid Metabolism	formation of 5-fluorouracil	3.67E-03	CYP2C9	2
	Nucleic Acid Metabolism	oxidation of voriconazole	3.67E-03	CYP2C9	
	Nucleic Acid Metabolism	demethylation of sildenafil	7.33E-03	CYP2C9	
	Nucleic Acid Metabolism	metabolism of theophylline	1.10E-02	CYP2C9	
	Nucleic Acid Metabolism	metabolism of cyclic GMP	3.61E-02	ATP2B2	
37	Nutritional Disease	autosomal dominant hemochromatosis	3.67E-03	SLC40A1	1
	Nutritional Disease	hemochromatosis Type IV	3.67E-03	SLC40A1	
38	Organ Development	abnormal morphology of enlarged anterior chamber of eye	3.67E-03	EGR1	6
	Organ Development	erythropoiesis of splenocytes	3.67E-03	EGR1	
	Organ Development	mineralization of otoliths	3.67E-03	ATP2B2	
	Organ Development	development of spleen	8.48E-03	EGR1, SLC40A1	
	Organ Development	development of adenohypophysis	2.54E-02	EGR1	
	Organ Development	development of oocytes	3.26E-02	WNT4	
	Organ Development	development of hair	4.67E-02	SPINK5	
	Organ Development	abnormal size of eye	1.10E-02	EGR1	
	Organ Development	function of liver	1.49E-02	CD36, EGR1	
	Organ Development	function of adrenal gland	2.90E-02	WNT4	
	Organ Development	keratinization of skin	1.82E-02	SPINK5	
39	Organ Morphology	abnormal morphology of auditory cortex	3.67E-03	ATP2B2	7
	Organ Morphology	abnormal morphology of enlarged anterior chamber of eye	3.67E-03	EGR1	
	Organ Morphology	abnormal morphology of cornified envelope	1.82E-02	SPINK5	
	Organ Morphology	abnormal morphology of ovary	2.36E-02	EGR1, WNT4	
	Organ Morphology	abnormal morphology of intestine	2.50E-02	CD36, GFRA1	
	Organ Morphology	abnormal morphology of skin	2.57E-02	GFRA1, SPINK5	
	Organ Morphology	abnormal morphology of hair shaft	4.32E-02	SPINK5	
	Organ Morphology	abnormal morphology of Leydig cells	4.67E-02	EGR1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Organ Morphology	abnormal size of liver	3.67E-03	EGR1	
	Organ Morphology	abnormal size of eye	1.10E-02	EGR1	
	Organ Morphology	lack of kidney	5.70E-03	GFRA1, WNT4	
	Organ Morphology	lack of pillar cells	1.10E-02	ATP2B2	
	Organ Morphology	lack of supporting cell layer of inner ear	1.10E-02	ATP2B2	
	Organ Morphology	lack of hair cells	1.82E-02	ATP2B2	
	Organ Morphology	lack of otoliths	2.54E-02	ATP2B2	
	Organ Morphology	morphology of pituitary gland	6.07E-03	EGR1, WNT4	
	Organ Morphology	morphology of somatotrophs	7.33E-03	EGR1	
	Organ Morphology	morphology of skin	3.88E-02	EGR1, GFRA1, SPINK5	
	Organ Morphology	morphology of kidney	4.37E-02	CD36, GFRA1, WNT4	
	Organ Morphology	morphology of brain cells	4.38E-02	ATP2B2, EGR1	
	Organ Morphology	contraction of intestine	1.10E-02	GFRA1	
	Organ Morphology	mass of muscle	1.82E-02	SEPN1	
	Organ Morphology	mass of gastrocnemius	4.32E-02	SEPN1	
	Organ Morphology	remodeling of pulmonary alveolus	1.82E-02	EGR1	
40	Organismal Development	abnormal morphology of enlarged anterior chamber of eye	3.67E-03	EGR1	4
	Organismal Development	erythropoiesis of splenocytes	3.67E-03	EGR1	
	Organismal Development	formation of renal vesicle	7.33E-03	WNT4	
	Organismal Development	development of spleen	8.48E-03	EGR1, SLC40A1	
	Organismal Development	development of mullerian duct	1.82E-02	WNT4	
	Organismal Development	development of mesonephros	2.18E-02	WNT4	
	Organismal Development	development of adenohypophysis	2.54E-02	EGR1	
	Organismal Development	development of oocytes	3.26E-02	WNT4	
	Organismal Development	development of hair	4.67E-02	SPINK5	
	Organismal Development	abnormal size of eye	1.10E-02	EGR1	
	Organismal Development	keratinization of skin	1.82E-02	SPINK5	
	Organismal Development	secondary sex determination	1.82E-02	WNT4	
	Organismal Development	morphogenesis of nephrogenic mesenchyme	2.18E-02	WNT4	
41	Organismal Injury and Abnormalities	erosion of tarsal bone	3.67E-03	LECT2	5
	Organismal Injury and Abnormalities	delay in hyperalgesia	3.67E-03	SGK1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Organismal Injury and Abnormalities	atrophy of Leydig cells	7.33E-03	EGR1	
	Organismal Injury and Abnormalities	injury of hepatocytes	1.46E-02	EGR1	
	Organismal Injury and Abnormalities	remodeling of pulmonary alveolus	1.82E-02	EGR1	
	Organismal Injury and Abnormalities	failure of heart	2.54E-02	CD36	
	Organismal Injury and Abnormalities	pulmonary interstitial fibrosis	3.26E-02	EGR1	
	Organismal Injury and Abnormalities	size of infarct	4.91E-02	EGR1, GPX3	
42	Skeletal and Muscular Disorders	erosion of tarsal bone	3.67E-03	LECT2	3
	Skeletal and Muscular Disorders	rigid spine syndrome	3.67E-03	SEPN1	
	Skeletal and Muscular Disorders	congenital myopathy with fiber type disproportion	7.33E-03	SEPN1	
	Skeletal and Muscular Disorders	hereditary gingival fibromatosis	4.67E-02	EGR1	
43	Skeletal and Muscular System Development and Function	glycogenesis of soleus muscle	3.67E-03	CD36	3
	Skeletal and Muscular System Development and Function	mass of extensor digitorum longus muscle	1.82E-02	SEPN1	
	Skeletal and Muscular System Development and Function	mass of soleus muscle	1.82E-02	SEPN1	
	Skeletal and Muscular System Development and Function	mass of anterior tibial muscle	2.54E-02	SEPN1	
	Skeletal and Muscular System Development and Function	mass of gastrocnemius	4.32E-02	SEPN1	
	Skeletal and Muscular System Development and Function	formation of osteoclast-like cells	2.54E-02	EGR1	
	Skeletal and Muscular System Development and	quantity of skeletal muscle satellite cells	3.26E-02	SEPN1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Function				
44	Tissue Development	abnormal morphology of enlarged anterior chamber of eye	3.67E-03	EGR1	11
	Tissue Development	erythropoiesis of splenocytes	3.67E-03	EGR1	
	Tissue Development	cell-cell adhesion of squamous cell carcinoma cell lines	7.33E-03	ZYX	
	Tissue Development	cell-cell adhesion	1.23E-02	CADM3, CLDN3, ZYX	
	Tissue Development	formation of epithelial tubule	7.33E-03	WNT4	
	Tissue Development	formation of renal vesicle	7.33E-03	WNT4	
	Tissue Development	formation of adherens junctions	4.67E-02	ZYX	
	Tissue Development	development of spleen	8.48E-03	EGR1, SLC40A1	
	Tissue Development	development of mullerian duct	1.82E-02	WNT4	
	Tissue Development	development of mesonephros	2.18E-02	WNT4	
	Tissue Development	development of adenohypophysis	2.54E-02	EGR1	
	Tissue Development	development of oocytes	3.26E-02	WNT4	
	Tissue Development	development of hair	4.67E-02	SPINK5	
	Tissue Development	abnormal size of eye	1.10E-02	EGR1	
	Tissue Development	attachment of brain cancer cell lines	1.46E-02	EGR1	
	Tissue Development	keratinization of skin	1.82E-02	SPINK5	
	Tissue Development	morphogenesis of nephrogenic mesenchyme	2.18E-02	WNT4	
	Tissue Development	morphogenesis of endothelial tube	2.54E-02	WNT4	
	Tissue Development	organization of extracellular matrix	2.43E-02	ABI3BP, SPINK5	
	Tissue Development	adhesion of tumor cell lines	3.07E-02	EGR1, PTP4A3, ZYX	
	Tissue Development	adhesion of red blood cells	3.97E-02	CD36	
	Tissue Development	plasticity of synapse	3.95E-02	ATP2B2, EGR1	
	Tissue Development	accumulation of eosinophils	4.32E-02	EGR1	
45	Tumor Morphology	maturation of acute myeloblastic leukemia cells	3.67E-03	GFRA1	2
	Tumor Morphology	arrest in growth of melanoma cells	1.10E-02	EGR1	
	Tumor Morphology	proliferation of acute myeloblastic leukemia cells	1.46E-02	GFRA1	
46	Visual System Development and Function	abnormal morphology of enlarged anterior chamber of eye	3.67E-03	EGR1	1
	Visual System Development and Function	abnormal size of eye	1.10E-02	EGR1	
47	Organismal Functions	homeostasis of rodents	4.34E-03	CD36, EGR1	4
	Organismal Functions	metabolism of mammalia	7.23E-03	Gsta4, STEAP4	

Rank	Category	Function Annotation	p-value	Genes	# Genes
48	Renal and Urological System Development and Function	lack of kidney	5.70E-03	GFRA1, WNT4	3
	Renal and Urological System Development and Function	lack of ureter	3.97E-02	GFRA1	
	Renal and Urological System Development and Function	entry into cell cycle progression of kidney cell lines	7.33E-03	WNT4	
	Renal and Urological System Development and Function	development of oocytes	3.26E-02	WNT4	
	Renal and Urological System Development and Function	morphology of kidney	4.37E-02	CD36, GFRA1, WNT4	
49	Cardiovascular Disease	Infarction	6.93E-03	CD36, CYP2C9, EGR1, GPX3	4
	Cardiovascular Disease	failure of heart	2.54E-02	CD36	
	Cardiovascular Disease	cerebral amyloid angiopathy	3.61E-02	CD36	
	Cardiovascular Disease	size of infarct	4.91E-02	EGR1, GPX3	
50	Auditory Disease	nonsyndromic recessive deafness DFNB12	7.33E-03	ATP2B2	1
	Auditory Disease	noise-induced hearing loss	3.26E-02	ATP2B2	
	Auditory Disease	age-related hearing loss	3.61E-02	ATP2B2	
51	Cellular Assembly and Organization	integrity of adherens junctions	7.33E-03	ZYX	2
	Cellular Assembly and Organization	remodeling of actin stress fibers	7.33E-03	ZYX	
	Cellular Assembly and Organization	retraction of axons	1.46E-02	WNT4	
	Cellular Assembly and Organization	formation of adherens junctions	4.67E-02	ZYX	
52	Cellular Compromise	atrophy of Leydig cells	7.33E-03	EGR1	2
	Cellular Compromise	injury of hepatocytes	1.46E-02	EGR1	
	Cellular Compromise	retraction of axons	1.46E-02	WNT4	
	Cellular Compromise	degeneration of oocytes	2.18E-02	WNT4	
53	Cellular Movement	cell rolling of red blood cells	7.33E-03	CD36	3
	Cellular Movement	chemoattraction of GABAergic neurons	7.33E-03	GFRA1	
	Cellular Movement	migration of melanoma cell lines	1.84E-02	CD36, PTP4A3	
	Cellular Movement	chemotaxis of skin cell lines	2.54E-02	CD36	
	Cellular Movement	chemotaxis of nervous tissue cell lines	2.90E-02	CD36	
	Cellular Movement	invasion of embryonic cell lines	3.26E-02	PTP4A3	
54	Inflammatory Response	abnormal morphology of foam cells	7.33E-03	CD36	3

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Inflammatory Response	abnormal morphology of neutrophils	1.82E-02	EGR1	
	Inflammatory Response	phagocytosis by microglia	1.10E-02	CD36	
	Inflammatory Response	phagocytosis of fibroblasts	1.10E-02	CD36	
	Inflammatory Response	pulmonary interstitial fibrosis	3.26E-02	EGR1	
	Inflammatory Response	quantity of myeloid dendritic cells	3.61E-02	BATF3	
	Inflammatory Response	morphology of phagocytes	3.87E-02	CD36, EGR1	
	Inflammatory Response	immune response of antigen presenting cells	4.20E-02	BATF3, CD36	
	Inflammatory Response	accumulation of eosinophils	4.32E-02	EGR1	
	Inflammatory Response	formation of macrophages	4.67E-02	EGR1	
55	Protein Trafficking	secretion of lipoprotein	7.33E-03	CD36	1
56	Behavior	long-term memory	7.63E-03	EGR1, SGK1	5
	Behavior	food preference	2.54E-02	CD36	
	Behavior	invasive behavior	4.67E-02	SEC24D	
	Behavior	pain behavior	4.67E-02	GFRA1	
57	Post-Translational Modification	homotetramerization of protein	8.48E-03	DECR1, GPX3	3
	Post-Translational Modification	palmitoylation of protein	2.90E-02	WNT4	
58	Protein Synthesis	homotetramerization of protein	8.48E-03	DECR1, GPX3	8
	Protein Synthesis	quantity of vldl triglyceride in blood	3.61E-02	CD36	
	Protein Synthesis	quantity of leptin in blood	3.62E-02	CD36, STEAP4	
	Protein Synthesis	degradation of protein	4.21E-02	ENPEP, MYLIP, SPINK5, TESC	
59	Cellular Growth and Proliferation	arrest in growth of melanoma cells	1.10E-02	EGR1	4
	Cellular Growth and Proliferation	proliferation of acute myeloblastic leukemia cells	1.46E-02	GFRA1	
	Cellular Growth and Proliferation	proliferation of epithelial cell lines	2.02E-02	DECR1, EGR1, PTP4A3	
	Cellular Growth and Proliferation	proliferation of fibrosarcoma cell lines	3.97E-02	EGR1	
	Cellular Growth and Proliferation	formation of osteoclast-like cells	2.54E-02	EGR1	
	Cellular Growth and Proliferation	formation of macrophages	4.67E-02	EGR1	
60	Connective Tissue Development and Function	phagocytosis of fibroblasts	1.10E-02	CD36	3

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Connective Tissue Development and Function	quantity of labyrinth supporting cells	2.18E-02	ATP2B2	
	Connective Tissue Development and Function	formation of osteoclast-like cells	2.54E-02	EGR1	
61	Dermatological Diseases and Conditions	Netherton syndrome	1.10E-02	SPINK5	5
	Dermatological Diseases and Conditions	desquamation of epidermis	1.10E-02	SPINK5	
	Dermatological Diseases and Conditions	xanthomatosis	1.10E-02	CD36	
	Dermatological Diseases and Conditions	regression of hair follicle	3.26E-02	GFRA1	
	Dermatological Diseases and Conditions	papilloma	3.87E-02	EGR1, Gsta4	
62	Immune Cell Trafficking	recruitment of microglia	1.10E-02	CD36	2
	Immune Cell Trafficking	accumulation of eosinophils	4.32E-02	EGR1	
63	Vitamin and Mineral Metabolism	metabolism of 17-alpha-ethinylestradiol	1.10E-02	CYP2C9	6
	Vitamin and Mineral Metabolism	steroid metabolism	2.87E-02	CYP2C9, LSS, WNT4	
	Vitamin and Mineral Metabolism	homeostasis of cholesterol	1.44E-02	CD36, MYLIP	
	Vitamin and Mineral Metabolism	conversion of retinaldehyde	1.46E-02	CYP2C9	
	Vitamin and Mineral Metabolism	conversion of vitamin A	1.46E-02	CYP2C9	
	Vitamin and Mineral Metabolism	loading of Ca2+	1.46E-02	ATP2B2	
	Vitamin and Mineral Metabolism	synthesis of hydrocortisone	3.26E-02	WNT4	
64	Cell Signaling	loading of Ca2+	1.46E-02	ATP2B2	2
	Cell Signaling	cGMP-mediated signaling	4.32E-02	CD36	
65	Gastrointestinal Disease	injury of hepatocytes	1.46E-02	EGR1	12
	Gastrointestinal Disease	infection of liver	2.90E-02	BATF3	
	Gastrointestinal Disease	hypoperistalsis	3.61E-02	GFRA1	
	Gastrointestinal Disease	Hirschsprung disease	3.97E-02	GFRA1	
	Gastrointestinal Disease	hereditary gingival fibromatosis	4.67E-02	EGR1	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Gastrointestinal Disease	digestive organ tumor	4.93E-02	CD36, CLDN3, CYP2C9, GLUL, GPX3, LECT2, OIT3, PTP4A3, SGK1	
66	Hepatic System Disease	injury of hepatocytes	1.46E-02	EGR1	2
	Hepatic System Disease	infection of liver	2.90E-02	BATF3	
67	Hair and Skin Development and Function	abnormal morphology of cornified envelope	1.82E-02	SPINK5	5
	Hair and Skin Development and Function	abnormal morphology of hair shaft	4.32E-02	SPINK5	
	Hair and Skin Development and Function	keratinization of skin	1.82E-02	SPINK5	
	Hair and Skin Development and Function	proliferation of epithelial cell lines	2.02E-02	DECR1, EGR1, PTP4A3	
	Hair and Skin Development and Function	chemotaxis of skin cell lines	2.54E-02	CD36	
	Hair and Skin Development and Function	development of hair	4.67E-02	SPINK5	
68	Organismal Survival	thermosensitivity	1.82E-02	GPX3	1
69	Humoral Immune Response	differentiation of B-1 lymphocytes	2.18E-02	EGR1	1
70	Reproductive System Development and Function	quantity of breast cell lines	2.18E-02	SGK1	3
	Reproductive System Development and Function	abnormal morphology of ovary	2.36E-02	EGR1, WNT4	
	Reproductive System Development and Function	abnormal morphology of Leydig cells	4.67E-02	EGR1	
	Reproductive System Development and Function	development of adenohypophysis	2.54E-02	EGR1	
	Reproductive System Development and Function	development of oocytes	3.26E-02	WNT4	
71	Cardiovascular System Development and Function	morphogenesis of endothelial tube	2.54E-02	WNT4	4
	Cardiovascular System Development and Function	cardiac output of heart	3.26E-02	CD36	
	Cardiovascular System Development and Function	chemotaxis of vascular endothelial cells	3.26E-02	CD36	
	Cardiovascular System Development and Function	chemotaxis of endothelial cell lines	3.61E-02	CD36	

Rank	Category	Function Annotation	p-value	Genes	# Genes
	Cardiovascular System Development and Function	function of blood vessel	3.97E-02	GPX3	
	Cardiovascular System Development and Function	relaxation of aortic ring tissue	3.97E-02	CYP2C9	
72	Immunological Disease	infection of spleen	2.54E-02	BATF3	2
	Immunological Disease	development of T-cell non-Hodgkin lymphoma	3.61E-02	EGR1	
73	Connective Tissue Disorders	pulmonary interstitial fibrosis	3.26E-02	EGR1	1
	Connective Tissue Disorders	hereditary gingival fibromatosis	4.67E-02	EGR1	
74	Inflammatory Disease	pulmonary interstitial fibrosis	3.26E-02	EGR1	2
	Inflammatory Disease	systemic inflammatory response syndrome	3.61E-02	CD36	
75	Renal and Urological Disease	developmental delay of kidney	3.97E-02	WNT4	3
	Renal and Urological Disease	diabetic nephropathy	4.20E-02	ENPEP, WNT4	
	Renal and Urological Disease	agenesis of kidney	4.67E-02	GFRA1	
76	Protein Degradation	degradation of protein	4.21E-02	ENPEP, MYLIP, SPINK5, TESC	4

SI Table 4. Ingenuity Canonical Pathways disrupted by the dysregulation of genes in liver tissue following male chicken embryo exposure to 45 µg/g of TDCPP. Ratio is the number of dysregulated genes divided by the total number of molecules in a pathway.

Ingenuity Canonical Pathway	p-value	Ratio	Genes
Lanosterol Biosynthesis	3.63E-03	1.00E+00	LSS
Glutamine Biosynthesis I	7.41E-03	5.00E-01	GLUL
L-serine Degradation	7.41E-03	3.33E-01	SDSL
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.78E-02	1.59E-02	EGR1,WNT4,CLDN3
GDP-mannose Biosynthesis	2.19E-02	1.67E-01	PMM2
Calcium Transport I	2.57E-02	1.11E-01	ATP2B2
Glycine Betaine Degradation	2.57E-02	1.00E-01	SDSL
LXR/RXR Activation	3.02E-02	1.59E-02	MYLIP,CD36
Bupropion Degradation	3.98E-02	3.85E-02	CYP2C9
Cholesterol Biosynthesis I	3.98E-02	7.69E-02	LSS
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	3.98E-02	7.69E-02	LSS
Cholesterol Biosynthesis III (via Desmosterol)	3.98E-02	7.69E-02	LSS
Acetone Degradation I (to Methylglyoxal)	3.98E-02	3.70E-02	CYP2C9
Glutathione-mediated Detoxification	4.37E-02	3.57E-02	Gsta4
Colanic Acid Building Blocks Biosynthesis	4.68E-02	7.14E-02	PMM2
Glutathione Redox Reactions I	5.01E-02	5.88E-02	GPX3
Nicotine Degradation III	5.37E-02	2.00E-02	CYP2C9
Melatonin Degradation I	6.03E-02	1.89E-02	CYP2C9
Estrogen Biosynthesis	6.46E-02	2.63E-02	CYP2C9
Nicotine Degradation II	6.46E-02	1.69E-02	CYP2C9
Superpathway of Melatonin Degradation	7.08E-02	1.72E-02	CYP2C9
Superpathway of Cholesterol Biosynthesis	8.13E-02	3.45E-02	LSS
PPARα/RXRα Activation	9.12E-02	1.09E-02	CD36,CYP2C9
Inhibition of Angiogenesis by TSP1	1.01E-01	2.94E-02	CD36
PXR/RXR Activation	1.34E-01	1.47E-02	CYP2C9
Glutamate Receptor Signaling	1.53E-01	1.61E-02	GLUL
Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza	1.68E-01	1.23E-02	WNT4
Pyridoxal 5'-phosphate Salvage Pathway	1.68E-01	1.59E-02	SGK1
Remodeling of Epithelial Adherens Junctions	1.71E-01	1.49E-02	ZYX
ERK5 Signaling	1.75E-01	1.56E-02	SGK1
Basal Cell Carcinoma Signaling	1.95E-01	1.39E-02	WNT4
Atherosclerosis Signaling	1.95E-01	7.58E-03	CD36
GDNF Family Ligand-Receptor Interactions	1.99E-01	1.43E-02	GFRA1
CDK5 Signaling	2.16E-01	1.12E-02	EGR1
Salvage Pathways of Pyrimidine Ribonucleotides	2.22E-01	1.19E-02	SGK1
Amyotrophic Lateral Sclerosis Signaling	2.53E-01	9.80E-03	GLUL
Granulocyte Adhesion and Diapedesis	2.64E-01	5.81E-03	CLDN3
Agranulocyte Adhesion and Diapedesis	2.75E-01	5.46E-03	CLDN3

Ingenuity Canonical Pathway	p-value	Ratio	Genes
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	2.86E-01	8.77E-03	WNT4
Role of Tissue Factor in Cancer	2.86E-01	8.93E-03	EGR1
Type II Diabetes Mellitus Signaling	3.01E-01	7.52E-03	CD36
Insulin Receptor Signaling	3.06E-01	7.41E-03	SGK1
GNRH Signaling	3.14E-01	7.14E-03	EGR1
Human Embryonic Stem Cell Pluripotency	3.24E-01	6.62E-03	WNT4
Tight Junction Signaling	3.32E-01	6.33E-03	CLDN3
Ovarian Cancer Signaling	3.34E-01	7.19E-03	WNT4
Epithelial Adherens Junction Signaling	3.37E-01	6.90E-03	ZYX
B Cell Receptor Signaling	3.66E-01	6.10E-03	EGR1
Glioblastoma Multiforme Signaling	3.68E-01	6.33E-03	WNT4
CXCR4 Signaling	3.72E-01	6.25E-03	EGR1
Aldosterone Signaling in Epithelial Cells	3.72E-01	6.37E-03	SGK1
Calcium Signaling	3.75E-01	4.98E-03	ATP2B2
Germ Cell-Sertoli Cell Junction Signaling	3.78E-01	6.33E-03	ZYX
Sertoli Cell-Sertoli Cell Junction Signaling	3.82E-01	5.38E-03	CLDN3
Wnt/β-catenin Signaling	3.89E-01	5.78E-03	WNT4
LPS/IL-1 Mediated Inhibition of RXR Function	3.95E-01	4.33E-03	CYP2C9
EIF2 Signaling	3.98E-01	5.21E-03	RPLP1
Leukocyte Extravasation Signaling	4.27E-01	5.03E-03	CLDN3
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	4.44E-01	4.39E-03	WNT4
Integrin Signaling	4.44E-01	4.83E-03	ZYX
Huntington's Disease Signaling	4.60E-01	4.27E-03	SGK1
Glucocorticoid Receptor Signaling	4.97E-01	3.57E-03	SGK1
Colorectal Cancer Metastasis Signaling	4.97E-01	4.00E-03	WNT4
Xenobiotic Metabolism Signaling	4.99E-01	3.41E-03	CYP2C9
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	5.46E-01	3.08E-03	WNT4