Dioxin Resistance Alleles and Developmental Stage at Exposure as Determinants of Sensitivity to Dioxin-Induced Short-Term Toxicity and Male Reproductive Effects

Ulla Simanainen

National Public Health Institute
Department of Environmental Health
Laboratory of Toxicology
P.O.Box 95, FIN-70701 Kuopio, FINLAND

and

University of Kuopio
Institute of Applied Biotechnology
P.O.Box 1627, FIN-70211 Kuopio, FINLAND

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Publisher: National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki
FINLAND
Phone +358-9-47441
Fax +358-9-47448408

Author's address: National Public Health Institute
Department of Environmental Health
Laboratory of Toxicology
P.O.Box 95
FIN-70701 Kuopio
FINLAND
Phone +358-17-201177
Fax +358-17-201265
E-mail **ulla.simanainen@ktl.fi**

Supervisors: Docent Matti Viluksela
National Public Health Institute, Kuopio, Finland

Professor Richard E. Peterson
University of Wisconsin, Madison, WI, USA

Professor Sirpa Kärenlampi
University of Kuopio, Kuopio, Finland

Reviewers: Professor Risto Juvonen
University of Kuopio, Kuopio, Finland

Professor Sari Mäkelä
University of Turku, Turku, Finland

Opponent: Dr. Anne Marie Vinggaard
Danish Institute for Food and Veterinary Research, Department of Toxicology and Risk Assessment, Denmark

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Abstract

The term “dioxins” generally means a heterogenous mixture of chlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs). Dioxins are widespread and persistent environmental contaminants, and due to their lipophilicity and slow metabolism they accumulate in food chain. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic dioxin congener, and is referred to as ”the most toxic man-made compound”.

Humans are exposed to dioxins primarily via the food. In Finland, 80% of human exposure comes from fish, Baltic fish being the most crucial source. Therefore, the European Commission is planning actions to limit the consumption of Baltic fish, especially herring and salmon.

TCDD causes a wide variety of biochemical and toxic effects that are mostly mediated through the aryl hydrocarbon receptor (AHR). These effects have been reported in multiple species and at different stages of development. Defects in the developing male reproductive system and teeth are among the most sensitive endpoints of TCDD toxicity in laboratory animals.

This study outlines two important questions in dioxin risk assessment:

(1) What is the role of genetic factors in sensitivity to dioxins?
This issue was examined using two different animal models: a mouse model with *Ahr* null mutation and a rat model based on exceptional resistance to TCDD acute lethality. The TCDD resistance of these rats is due to a mutated *Ahr* allele (Ahr^{hw}) and an unknown allele B^{hw}.

Although the resistance alleles increase the resistance to hexachlorodibenzo-*p*-dioxin (HxCDD) - induced acute lethality remarkably less than to TCDD and results in exceptional relative sensitivity to HxCDD, the study showed that the rats with Ahr^{hw} and/or B^{hw} resistance alleles are not exceptionally sensitive to HxCDD–induced non-lethal short-term effects.

An efficacy (magnitude of effect) ratio of 0.5 between resistant and sensitive rats was found to categorize the dioxin short-term toxicity into different type I and type II endpoints. The resistance alleles do not modify type I endpoints (CYP1A1 induction, thymic atrophy and tooth defects), while especially the Ahr^{hw} reduce the efficacy of type II endpoints (decreased body weight and liver toxicity).

Although most if not all effects of TCDD on the developing prostate in mice were shown to be AHR mediated, the mutated Ahr^{hw} in rats was still able to normally mediate the TCDD-induced defects in male reproductive organ development after adult and perinatal TCDD exposure. However, the extent of the decrease in sperm numbers after perinatal TCDD exposure was reduced by the resistance alleles.

(2) How is dioxin sensitivity altered at different developmental stages?
Toxic effects of TCDD on male reproductive system were observed in fetuses exposed to at least 100-times lower doses compared to those that alter male reproductive system after adult exposure. The critical time window for TCDD defects in developing prostate was found to differ among different prostate lobes. The growth of ventral and anterior prostate was most affected during fetal period, while that of dorsolateral prostate required both fetal and postnatal exposure for maximal effect.

The development of resistance to acute lethality differed between rats having either one of the resistance alleles. Rats with Ahr^{hw} were resistant to TCDD when assessed a few days after birth, while rats with allele B^{hw} did not attain their resistance until four to six weeks after birth. This reflects basic differences in pathways mediating resistance linked to these alleles.

Simanainen, Ulla. Geneettiset herkkyystekijät ja kehitysvaihe dioksiinien lyhytaikaistoksisuutta ja lisääntymistoksisuutta määräävinä tekijöinä.
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Tiivistelmä (Abstract in Finnish)

Dioksiineilla tarkoitetaan yleensä epäyhtenäistä kemikaalien ryhmää, johon kuuluvat mm. klooratut dibentso-*p*-dioksiinit ja dibentsofuraanit (PCDD/F). Dioksiinit ovat laajalle levinneitä ja kestäviä ympäristösaasteita, jotka kertyvät ravintoketjussa. 2,3,7,8-Tetraklooridibentso-*p*-dioksiini (TCDD) on myrkyllisin dioksiinijohdos ja sitä pidetään yhtenä myrkyllisimmistä ihmisen syntetisoimista yhdisteistä.

Ihmiset altistuvat dioksiineille pääasiassa ravinnon kautta. Jopa 80% suomalaisten altistuksesta on peräisin kalasta, erityisesti Itämeren rasvaisista kaloista kuten silakasta ja lohesta. Euroopan komissio on rajoittanut Itämeren kalan käyttöä.

TCDD vaikuttaa AH-reseptorin kautta ja aiheuttaa monia biokemiallisia ja toksisia vaikutuksia, joita on havaittu eri eläinlajeilla ja eri kehitysvaiheissa. Vaikutukset hampaiden ja urosten lisääntymiselimistön kehitykseen ovat herkimpiä TCDD:n vaikutuksia koe-eläimillä.

Tässä väitöskirjassa selvitettiin kahta dioksiinien riskinarvioinnin kannalta keskeistä kysymystä:
(1) Kuinka geneettiset tekijät vaikuttavat dioksiiniherkkyyteen?
Työssä käytettiin eläinmalleja, jotka perustuivat toiminnallisen AH-reseptorin puuttumiseen (hiirimalli) sekä mutatoituneesta AH-reseptorialleelista (*Ahr*^{hw}) ja toistaiseksi tuntemattomasta alleelista *B*^{hw} johtuvaan poikkeuksellisen suureen TCDD:n sietokykyyn (rottamallit).

Vaikka rotat, joilla on *Ahr*^{hw} ja *B*^{hw} resistenssialleelit, ovat suhteellisesti herkempiä heksaklooridibentso-*p*-dioksiinin (HxCDD) aiheuttamalle kuolleisuudelle kuin TCDD:n aiheuttamalle kuolleisuudelle, eivät nämä rotat ole kuitenkaan poikkeuksellisen herkkiä HxCDD:n aiheuttamille biokemiallisille ja muille tyypillisille toksisuuden ilmenemismuodoille, kuten ruumiinpainon laskulle.

Kestävien ja herkkien rottien annos-vasteen analysoiminen osoitti, että lyhytaikaisen dioksiinialtistuksen aiheuttamat toksiset vasteet voidaan jakaa kahteen ryhmään, tyypin I ja tyypin II vasteisiin. Luotettavimmaksi luokittelukriteeriksi osoittautui vasteen teho (maksimivasteen suuruus). Tyyppi I:n vasteissa tehon suhdeluku kestävän ja herkän rottakannan välillä on ≥0.5 (eli kestävän kannan maksimivaste on yli puolet herkän kannan maksimivasteesta). Tällaisia vasteita ovat mm. CYP1A1 induktio, kateenkorvan pieneneminen ja hammasvauriot, ja resistenssialleelit eivät siis vaikuta niihin.Tyyppi II:n vasteissa dioksiinien teho pienenee erityisesti *Ahr*^{hw} alleelin vaikutuksesta ja tehon suhdeluku on <0.5 (eli kestävän kannan maksimivaste on alle puolet herkän kannan maksimivasteesta). Tyyppi II:n vasteita ovat mm. ruuminpainon lasku ja maksavaurio.

Vaikka suurin osa TCDD:n vaikutuksista kehittyvän hiiren eturauhaseen oli AH-reseptorivälitteisiä, ei mutatoitunut *Ahr*^{hw} muuttanut TCDD:n vaikutusta urosten lisääntymiselimiin rotilla. Sitävastoin TCDD-altistuksen aiheuttama siittiöiden määrän lasku oli suurin rotilla joilla ei ole kumpaakaan resistenssialleeleista.

(2) Miten dioksiiniherkkyys riippuu kehitysvaiheesta?
Kehittyvä urosrottien lisääntymiselimistö oli sikiöaikana ja imetyksen aikana jopa 100-kertaa herkempi kuin aikuisten rottien lisääntymiselimistö. Eturauhasen eri lohkot eivät ole saman aikaisesti herkkiä TCDD:n toksisille vaikutuksille. Ventraalinen ja anteriorinen lohko ovat herkkiä sikiöaikaiselle altistukselle. Sen sijaan dorsolateraalisen eturauhasen kehityshäiriöitä todettiin vain sekä sikiöaikaisen että syntymänjälkeinen TCDD altistuksen jälkeen.

Resistenssialleeleihin *Ahr*^{hw} ja *B*^{hw} liittyvä kestävyyden kehittymisaikataulu TCDD:n aiheuttaman akuutin kuolleisuuden suhteen oli selvästi erilainen. Rotat joilla oli *Ahr*^{hw} resistenssialleeli olivat kestäviä jo muutama päivä syntymän jälkeen, kun taas rotilla joilla oli *B*^{hw} alleeli kestävyys ilmeni vasta 4-6 viikon ikäisenä. Tämä viittaa eroihin alleeleihin liittyvissä kestävyyden mekanismeissa.

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I dedicate this work to my mother and father who have always believed in and supported me.

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Kuopio, May 2004

Ulla Simanainen

Contents

List of abbreviations

AH	Aryl hydrocarbon
AHR	Aryl hydrocarbon receptor, Ah receptor, dioxin receptor
Ahr	the gene encoding AHR
Ahr-/-	Aryl hydrocarbon knock-out (AHRKO); both alleles of *Ahr* are inactivated using gene targeting technology
Ahr^{b}	Allele of *Ahr* locus; high affinity; found in TCDD-responsive C57BL/6J mice
Ahr^{d}	Allele of *Ahr* locus; low affinity; found in TCDD-non-responsive DBA/2J mice
Ahr^{hw}	Allele of *Ahr* locus; found in exceptionally TCDD-resistant Han-Wistar rats.
Ahr^{wt}	Allele of *Ahr* locus; found in most rats strains, e.g. Long-Evans and Sprague Dawley rats.
AHRKO	Aryl hydrocarbon receptor knock-out; both alleles of *Ahr* are inactivated using gene targeting technology (*Ahr-/-*)
AHRR	Aryl hydrocarbon receptor repressor; inhibits AHR function by competing with AHR for dimerizing with ARNT and thereby inhibits binding to the DRE sequence
ARNT	Ah receptor nuclear translocator: dimerizes with AHR and this heterodimer binds to DNA
ASAT	Aspartate aminotransferase: enzyme released into the blood when certain organs or tissues, particularly the liver and heart, are injured
B	an unknown gene that affects TCDD-sensitivity; B^{hw} allele increases resistance indicated by some end points
bHLH/PAS	Basic helix-loop-helix/PAS: AHR belongs to this family of transcriptional regulators
C57BL/6J	Mouse strain; sensitive to the effects of aryl hydrocarbons
cDNA	Complementary DNA, reverse-transcribed from mRNA
c-src	A gene encoding a 60 kDa protein $p60^{c\text{-}src}$ possessing tyrosine kinase activity
CYP	Cytochrome P450: xenobiotic, oxidizing enzymes forming a CYP superfamily

DBA/2J	Mouse strain; resistant to the effects of aryl hydrocarbons
DHT	5α-Dihydrotestosterone; an androgen that is produced from testosterone by 5α-reductase activity. Stimulates the growth of prostate gland
3α-Diol	5α-Androstane-3α,17ß-diol, predominant androgen prior to puberty
DNA	Deoxyribonucleic acid: a double-stranded nucleic acid that contains the genetic information of organisms
DRE	Dioxin responsive element: a specific sequence of DNA that binds the AHR-ARNT complex
ED_{50}	Effective dose 50%: the dose that is estimated to give 50 % of the maximal response
ECEH-WHO	European Center of Environmental Health of the World Health Organization
EC SCF	European Commission Scientific Committee on Foods
EROD	Ethoxyresorufin-*O*-deethylase; EROD activity is almost exclusively due to the enzyme CYP1A
FFA	Free fatty acid; non-esterified fatty acids
GD	Gestation day; days from conception until birth
HIF-1α	Hypoxia inducible factor 1α; member of bHLH/PAS family of transcriptional regulators
HxCDD	1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin
HpCDD	1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin
H/W	Han/Wistar (*Kuopio*) rat strain; the most TCDD-resistant laboratory animal known
ICZ	Condensation product formed in stomach from dietary indole, indole-3-carbinol naturally occurring in i.e. cabbage. ICZ has high affinity to AHR and is a potent inducer of AHR-dependent gene expression *in vitro*.
KY-5	Commercial name for chlorophenol mixture used in Finland as a wood preservative
LD_{50}	Lethal Dose 50%: the dose that is estimated to kill 50 % of animals in acute toxicity test
L-E	Long-Evans (*Turku/AB*) rat strain: the most TCDD-sensitive rat strain known

mRNA	Messenger RNA: formed from a DNA template by transcription
NF-κB	Nuclear factor κB: universal transcription factor
OCDD	Octachlorinated dibenzo-*p*-dioxin
$p27^{Kip1}$	27 kDa protein that belongs to the family of cell cycle regulators called cyclin-dependent kinase inhibitors, which cause cell cycle arrest in the G1 phase.
$p60^{c\text{-}src}$	60 kDa protein encoded by *c-src* gene (see *c-src*)
PCBs	Polychlorinated biphenyls
PCDDs PCDFs	Polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans; belong to the family of "dioxins"
PeCDD	1,2,3,7,8-pentachlorodibenzo-*p*-dioxin
PEPCK	Phosphoenolpyruvate carboxykinase; a rate-limiting enzyme in gluconeogenesis
PND	Postnatal day; days from birth
REP	Relative potency value; the potency ratio PCDD:TCDD value when the value has been generated by a single laboratory examining a single endpoint
RNA	Ribonucleic acid: a single-stranded nucleic acid
S-D	Sprague-Dawley rat strain
SNP	Single nucleotide polymorphism
TEF	Toxic equivalency factor (see WHO-TEF)
TEQ	Toxic equivalency quantity (see WHO-TEQ)
T_3	3,3`,5-triiodothyronine; thyroid hormone
T_4	3,3`,5,5`-tetraiodo-L-thyronine, thyroxine; thyroid hormone
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin: the most toxic congener of dioxins
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase; an enzyme involved in glucuronidation of e.g. thyroid hormones in order to increase their solubility to water and aid in their excretion.

UGS	Urogenital sinus; the small bodily cavity where the fetal urinary and genital organ development begins
USEPA	US Environmental Protection Agency
WHO	World Health Organization
WHO-TEF	WHO-Toxic equivalency factor: the factor that compares toxicity of other dioxin-like compounds to TCDD, which has a TEF value of 1
WHO-TEQ	Toxic equivalency quantity: a sum of dioxin congener concentrations multiplied by their TEF values from the WHO-TEF standards (see WHO-TEF)
XRE	Xenobiotic responsive element (compare to DRE)

List of original publications

I **Simanainen U.,** Tuomisto J.T., Tuomisto J., and Viluksela M. (2002). Structure-activity relationships and dose responses of polychlorinated dibenzo-*p*-dioxins for short-term effects in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant and –sensitive rat strains. *Toxicology and Applied Pharmacology* 181, 38-47.

II **Simanainen U.,** Tuomisto J.T., Tuomisto J., and Viluksela M. (2003). Dose-response analysis on short-term effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in three differentially susceptible rat lines. *Toxicology and Applied Pharmacology* 187, 128-136.

III **Simanainen U.**, Haavisto T., Tuomisto J.T., Paranko J., Toppari J., Tuomisto J., Peterson R.E., and Viluksela M. (2004). Pattern of male reproductive system effects after *in utero* and lactational 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in three differentially TCDD-sensitive rat lines. *Toxicological Sciences, In Press.*

IV **Simanainen U.**, Adamson A., Tuomisto J.T., Miettinen H.M., Toppari J., Tuomisto J. and Viluksela M. (2004). Adult 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure and effects on male reproductive organs in three differentially TCDD-susceptible rat lines. *Submitted.*

V Lin T-M., Ko K., Moore R.W., **Simanainen U.**, Oberley T.D., and Peterson R.E. (2002). Effects of aryl hydrocarbon receptor null mutation and *in utero* and lactational 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure on prostate and seminal vesicle development in C57BL/6 mice. *Toxicological Sciences* 68, 479-487.

VI Lin T-M., **Simanainen U.**, Moore R.W., and Peterson R.E. (2002). Critical windows of vulnerability for effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on prostate and seminal vesicle development in C57BL/6 mice. *Toxicological Sciences* 69, 202-209.

VII **Simanainen U.**, Tuomisto J.T., Pohjanvirta R., Syrjälä P., Tuomisto J., and Viluksela M. (2004). Postnatal development of resistance to short-term high-dose toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in TCDD-resistant and –semiresistant rat strains. *Toxicology and Applied Pharmacology* 196, 11-19.

1 General introduction and literature review

1.1 Introduction

The effects of dioxins[1], especially the most toxic congener 2,3,7,8-tertrachlorodibenzo-*p*-dioxin (TCDD; Fig. 1) on animals/humans have been extensively studied over the past 40 years. Such interest is maintained by the intriguing features of TCDD, including the many toxic endpoints it influences (i.e. enzyme induction, teratogenicity, carcinogenicity, immunosuppression, male reproductive defects), large inter-species and intra-species variability in responses to TCDD, and its aryl hydrocarbon receptor (AHR) -mediated toxicity.

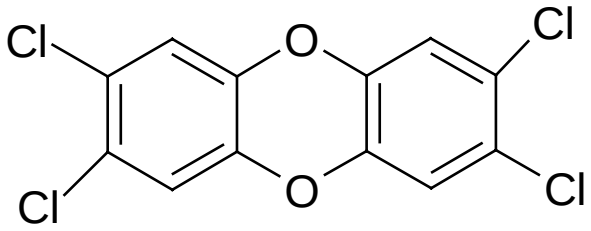


Figure 1: Chemical structure of dioxin model compound TCDD.

Food is the main source of human exposure to dioxins, accounting for 90-98 % of the total exposure (Fries 1995; Llobet *et al.* 2003; Parzefall 2002; Sweetman *et al.* 2000). As highly lipophilic and biopersistent compounds, dioxins are deposited in animal fat and accumulate in the food chain. In Finland the major source is Baltic herring, which is a commercially important fatty fish. In recent years the efforts to control pollution have resulted in a measurable reduction of human dietary exposures (Hays and Aylward 2003; Kiviranta *et al.* 2001; Llobet *et al.* 2003; Tsutsumi *et al.* 2001). The estimated human daily intake of dioxins is about 1-3 pg/kg/day for adults, and is highly dependent on the diet (USEPA, 2000). However, food may become accidentally contaminated with dioxins and there are some highly contaminated areas e.g. Baltic Sea. The most recent food contamination incident took place in 1999, when the control authorities in Belgium identified elevated dioxin levels in beef and chicken (Huwe 2002). The source of contamination was found to be PCB-containing oil mixed feloniously with recycled animal fats destined for animal feed.

[1] Polychlorinated dibenzo-*p*-dioxins and dibenzofurans and dioxin-like polychlorinated biphenyls; collectively called “dioxins”

Despite the enormous amount of data produced in the field of dioxin research, there is no general agreement on the most sensitive and relevant endpoints for human risk assessment. Neither is there an agreement on "safe" daily intake of dioxins from the diet. The U.S. Environmental Protection Agency (USEPA) has based its assessment on low-dose linear extrapolations of potential cancer risks using human data from highly exposed industrial cohorts, and proposes that the acceptable daily dose of dioxins is equivalent to 0.001-0.01 pg TCDD/kg/day (USEPA 2000). USEPA has consistently derived much more stringent tolerable daily doses of dioxins than have other U.S. and international agencies. Recently, the World Health Organization (WHO) established the revisited tolerable daily intake of dioxins as 1-4 pg/kg/day (van Leeuwen *et al.* 2000). Their assessment was based on developmental defects, shown at low doses in animal studies. Additionally, in an opinion by the European Commission Scientific Committee on Food (ECSCF), they identified developmental effects on the male rat reproductive system as the most sensitive endpoints and derived a tolerable weekly dose of 14 pg/kg/week for dioxins (ECSCF 2001).

Studies on animal models have indicated that genetic variability is a significant factor affecting on effects of dioxin. In our laboratory, the highly TCDD-resistant Han/Wistar (*Kuopio*, H/W) rat strain and TCDD- sensitive Long-Evans (*Turku/AB*, L-E) rats have been used to study a wide variety of mechanistic aspects of dioxin toxicity (reviewed by Pohjanvirta and Tuomisto 1994). The exceptional resistance of H/W rats to acute lethality caused by TCDD is related to a mutation in *Ahr* and to an unknown allele "*B*" (Pohjanvirta 1990; Pohjanvirta *et al.* 1998; Tuomisto *et al.* 1999). H/W rats are over 1000-fold resistant to TCDD compared to the L-E rats. These resistance alleles were recently segregated into new rat lines: line A rats have the H/W-type mutated *Ahr*, line B rats the "*B* allele", and line C rats no resistance alleles (Tuomisto *et al.* 1999). The availability of these new rat lines permits studies on the influence of genetic factors on dioxin toxicity by analyzing the influence of the deviant *Ahr* and *B* alleles on the sensitivity of the rats to various endpoints of dioxin toxicity.

Variability in the sensitivity of animal strains to different dioxin congeners could influence the utility of the concept of toxic equivalency factor (TEF), which has been used as the basis of human risk assessment. In addition, the wide inter- and intra-species variation in the potency and spectrum of dioxin toxicity leads to many assumptions in the extrapolation of animal data to humans, thereby complicating the assessment of dioxin risk to humans. The differences in sensitivity are mainly observed in acute, high-dose exposures, but there is only a limited amount of information about their significance in low-dose and developmental exposures.

Furthermore, increasing data on the sensitivity of developmental processes as well as the recognition that nursing infants may have considerably high intakes of dioxins (estimated on body weight basis), have questioned the use of cancer as the critical endpoint in dioxin

risk assessment. Therefore, the developmental stage during exposure appears to be a highly important factor in dioxin risk assessment.

This thesis provides further information on two important questions in dioxin toxicity: (1) what is the influence of genetic factors (resistance alleles) on toxicity of dioxins and, particularly on one of the most sensitive endpoints of dioxins, male reproductive toxicity and (2) how is dioxin sensitivity altered at different developmental stages?

1.2 Dioxins and TCDD

The term “dioxin” refers in common language to the group of polychlorinated dibenzo-*p*-dioxins (PCDD), -dibenzofurans (PCDF) and –biphenyls (PCB) (Figure 2).

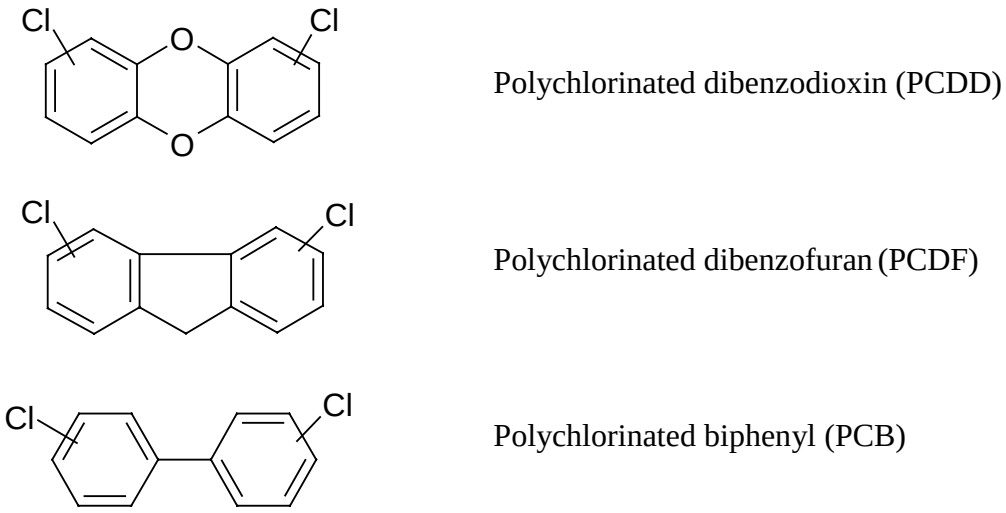


Figure 2: Chemical structures of PCDDs, PCDFs and PCBs.

The number of congeners and isomers is high; altogether there are 75 PCDDs, 135 PCDFs, and 209 PCBs. The term isomer refers to compounds with the same empirical formulae, but different localization of substituents in the molecule. The term congener refers to compounds with same basic structure but different number of substituents. Incorporation of chlorine to the lateral 2, 3, 7, and 8 positions of PCDD and PCDF congeners results in the characteristic “dioxin-like” toxic and chemical effects. Additionally, only about one twentieth of the PCBs have dioxin-like structure and toxicity. Those PCBs that are unsubstituted (non-ortho) or monosubstituted (mono-ortho) in the ortho positions are referred to as coplanar PCBs. (Safe 1986)

All 2,3,7,8-substituted PCDD and PCDF congeners induce similar biological effects through the cytosolic aryl hydrocarbon receptor (AHR), except at vastly different doses (Safe 1990; Van den Berg *et al.* 1998). Toxic equivalency factor (TEF) is used to normalize for differences in pharmacokinetics and ligand binding, and to compare the potency of each congener to that of TCDD, which is the most potent dioxin and generally used as a model compound for this class of compounds. The TEF approach assumes that the compounds must show a structural relationship to the PCDDs and PCDFs, bind the AHR, elicit AHR-mediated biochemical and toxic responses, and accumulate in the food chain.

Typically, the rank order of potency is TCDD > pentachlorodibenzo-*p*-dioxin (PeCDD) > hexachlorodibenzo-*p*-dioxin (HxCDD) > heptachlorodibenzo-*p*-dioxin (HpCDD) >

octachlorodibenzo-*p*-dioxin. The current WHO-TEFs (Table 1) are based on scientific assessment of all available data (Van den Berg *et al.* 1998). Virtually all biological systems studied so far follow this rank order. The relative potency (REP) value denotes the relative potency of PCDDs compared to TCDD when a single laboratory examining a single endpoint has generated the value.

Table 1: WHO-TEF values for mammals, fish and birds.

CONGENER	TOXIC EQUIVALENCY FACTOR (TEF)		
	Mammals	Fish [a]	Bird [a]
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	1	1	1 [c]
1,2,3,4,7,8-HxCDD	0.1 [a]	0.5	0.05 [c]
1,2,3,6,7,8-HxCDD	0.1 [a]	0.01	0.01 [c]
1,2,3,7,8,9-HxCDD	0.1 [a]	0.01 [b]	0.1 [c]
1,2,3,4,6,7,8-HpCDD	0.01	0.001	<0.001 [c]
OCDD	0.0001 [a]	-	-

"-" Indicates no TEF value because of the lack of data

a) Limited data set

b) Cytochrome P450 (CYP) 1A induction *in vitro*

c) CYP1A induction *in vivo* after *in ovo* exposure

For all mammals, as well as for avian and fish species studied so far, the TEFs seem to be applicable for lethality (van den Berg *et al.* 2000). A conspicuous exception to the universal TEF concept is the acute lethality (follow-up time 42 days) of PCDDs in TCDD-resistant H/W rats (Pohjanvirta *et al.* 1993, 1995). The H/W rats are more sensitive to the higher chlorinated congeners, and their rank order for acute lethality is HxCDD > HpCDD > PeCDD > TCDD. The unusual potency pattern of PCDDs in H/W rats in terms of acute lethality implies that TEFs may be response specific.

Using the TEF concept, toxic equivalents (TEQs) can be calculated for any sample by summing the normalized TEF consentrations of each dioxin-like compound. The TEQ concentrations are thus calculated using the following equation:

$$TEQ = \Sigma n_1[PCDD_i \times TEF_i] + \Sigma n_2 [PCDF_i \times TEF_i] + \Sigma n_3 [PCB_i \times TEF_i]. \quad [1]$$

The limitations associated with the TEF concept are the possible additive and antagonistic interactions between dioxin-like and non-dioxin-like congeners, the differences in species responsiveness, and differences in the shape of the dose-response curves for individual AHR agonists (van den Berg *et al.* 2000). In addition, pharmacokinetic differences between animal species can influence the TEF value. In spite of all assumptions and limitations of the TEF concept, the prediction of TEQs for humans still seems to be the best approach for risk management of dioxin-like compounds (van den Berg *et al.* 2000).

Dioxins have no known industrial uses, but are formed as trace contaminants during waste combustion and in the synthesis of products such as the chlorophenoxy acids and chlorophenols that are used as herbicides, fungicides and mold inhibitors. In Finland the worst known source is the chlorophenol containing wood preservative KY-5 that was manufactured from 1948 until 1984 in a factory located along the Kymijoki river. High concentrations of KY-5-derived dioxins accumulated in the Kymijoki river bottom sediments (Korhonen *et al.* 2001). Chemical accidents may also result in the formation of dioxins and lead to environmental or occupational exposure to these compounds. The most severe accident took place in Seveso, Italy, in 1976, where an explosion of a chemical plant producing trichlorophenol resulted in an escape of about 2 kg of TCDD to the environment (Bertazzi *et al.* 1998).

Dioxins are highly lipophilic molecules and are generally recalcitrant to chemical and biological degradation. The lipophilicity and stability of dioxins increase with increasing ring chlorination. Because of their high lipophilicity and water insolubility, dioxins concentrate in sediments and accumulate in tissue fat of fish, birds, and mammals (Isosaari *et al.* 2002a, b; Kiviranta *et al.* 2002; Loonen *et al.* 1996). Dioxins are also universally detected in human tissues, including mother's milk and serum (Eskenazi *et al.* 2003, 2004; Kiviranta *et al.* 1999; Wang *et al.* 2004). In breast fed infants the daily intake of dioxins is even 1-2 orders of magnitude higher than in adults on a per body weight basis due to their small body size (Päpke 1998). In addition, developing offspring can be exposed to dioxins via the placenta (Chen *et al.* 2001; Hurst *et al.* 2000a,b; Kreuzer *et al*. 1997; Li *et al.* 1995). The estimation for human half-life of TCDD in adults is 7 years, while in newborns the estimation is only 0.4 years (Kreuzer *et al.* 1997; LaKind and Filser 1999; Pirkle *et al.* 1989; Poiger and Schlatter 1986).

In the average human population, the main exposure to dioxins occurs via food. In Europe the main source is meat and dairy products. However, in Finland, the main source is Baltic fish. The European Commission laid down the maximum limits for dioxin levels in fish and other foodstuffs (ECSCF, 2001). For fish and fishery products the limit was set to 4 pg TEQ/g wet weight, thereby practically banning the use of Baltic herring and salmon for human consumption, as the major part of herring consumed by Finns seems to exceed the limit value (Kiviranta *et al.* 2003). Finland and Sweden were, however, allowed a transition period until 2006, at which time the limit value will be re-evaluated. This will be a challenge for dioxin risk assessment, and calls for a careful consideration of the risks associated with low-dose exposure to dioxins in the light of the beneficial effects of eating fish.

1.3 AH receptor, a key mediator of TCDD toxicity

AHR is known to mediate most of the biochemical and toxic effects of dioxins (Poland and Glover 1980; Okey *et al.* 1994; Fernandez-Salguero *et al.* 1996; Lahvis and Bradfield 1998; Tuomisto *et al.* 1999). The AHR is a soluble intracellular protein present in a wide variety of animal species and tissues, although the amount is variable (Pohjanvirta and Tuomisto 1994; Hahn 2002). The AHR is bound in the cytoplasm to the molecular chaperone hsp90, the co-chaperone p23 and to a 38kD tetratricopeptide repeat protein of the immunophilin family (ARA9, AIP, Xap2) (Kazlauskas *et al.* 1999, 2000; LaPres *et al.* 2000; Ma and Whitlock 1997; Petrulis *et al.* 2000).

The AHR binds the ligand in the cytosol, and the receptor-ligand complex is subsequently translocated into the nucleus (Figure 3), where it dimerizes with the AHR nuclear translocator (ARNT) and releases hsp90. The AHR and ARNT proteins are structurally related, prototypic members of basic helix-loop-helix/PAS (bHLH/PAS) transcription factors (Hahn 2002; Gu *et al.* 2000). The AHR-ARNT heterodimer binds to the dioxin-responsive elements (DREs) upstream of the promoters that regulate the transcription of a battery of xenobiotic metabolizing enzymes (Mimura and Fujii-Kuriyama 2003). These enzymes include, but are not limited to cytocrome CYP1A1, CYP1B1 and CYP1A2, and the phase II enzymes UDP-glucuronosyltransferase (UGT*01) and glutathione S-transferase Ya (Nebert *et al.* 1993; Okey *et al.* 1994).

1.3.1 Regulation of AHR function

The AHR complex activates the gene encoding the AHR repressor (AHRR). The AHRR inhibits AHR function by competing with AHR for dimerization with ARNT and for binding to the DRE sequence (Mimura *et al.* 1999). The binding of AHRR-ARNT complex to DRE does not activate the AHR regulated gene. The regulation of AHR function by AHRR seems to be evolutionarily conserved across mammals and fish (Karchner *et al.* 2002). Additionally, after a single dose of TCDD in rats, the AHR is up-regulated after initial depletion (Franc *et al.* 2001). However, the impact of AHR up-regulation on TCDD responsiveness in animals is not known.

1.3.2 Other AHR ligands

Besides dioxins, polyaromatic hydrocarbons (PAHs) such as 3-methylcholanthrene, benzo(a)pyrene, benzoflavones, and related compounds bind to AHR. The PAHs bind to AHR with relatively low affinity compared to TCDD. These compounds are formed during combustion processes and may have been involved in the evolution of the AHR from a developmental regulatory gene to a regulator of biotransformation enzymes (Hahn 2002).

Although AHR binds PCDDs and other xenobiotics selectively and with high affinity, it is also likely to have endogenous ligands. On the other hand, AHR may play a biological role

that is not yet understood. Possible endogenous ligands have been isolated from such sources as human urine and porcine lung tissue (Adachi *et al.* 2001; Song *et al.* 2002). Candidate endogenous ligands include lipoxin A(4) (Schaldach *et al.* 1999), bilirubin-related compounds (Phelan *et al.* 1998; Sinal and Bend 1997), and tryptophan-related compounds (Heath-Pagliuso *et al.* 1998). Tryptophan derivatives are suggested to function as hormones that, mediate the neuro-endocrine signaling of light (Rannug *et al.* 2003).

Several dietary constituents can also act as ligands for AHR. For example, indolo[3,2-b]carbazole (ICZ), a metabolite of indole carbinoles found in the gastrointestinal tract has been shown to function as an AHR agonist. The AHR-binding affinity of ICZ is high, being within the range of that for TCDD (Bjeldanes *et al.* 1991; Gillner *et al.* 1993). However, the potency of TCDD to induce CYP1A1, as measured by ethoxyresorufin *O*-deethylase (EROD) activity in Hepa-1 mouse hepatoma cells is 10^3-10^4 times higher than that of ICZ (Chen *et al.* 1995). Additionally, ICZ is not able to elicit AHR mediated toxicity in rats (Pohjanvirta *et al.* 2002). This lack of potency of ICZ *in vivo* is probably caused by factors such as low bioavailability and rapid elimination.

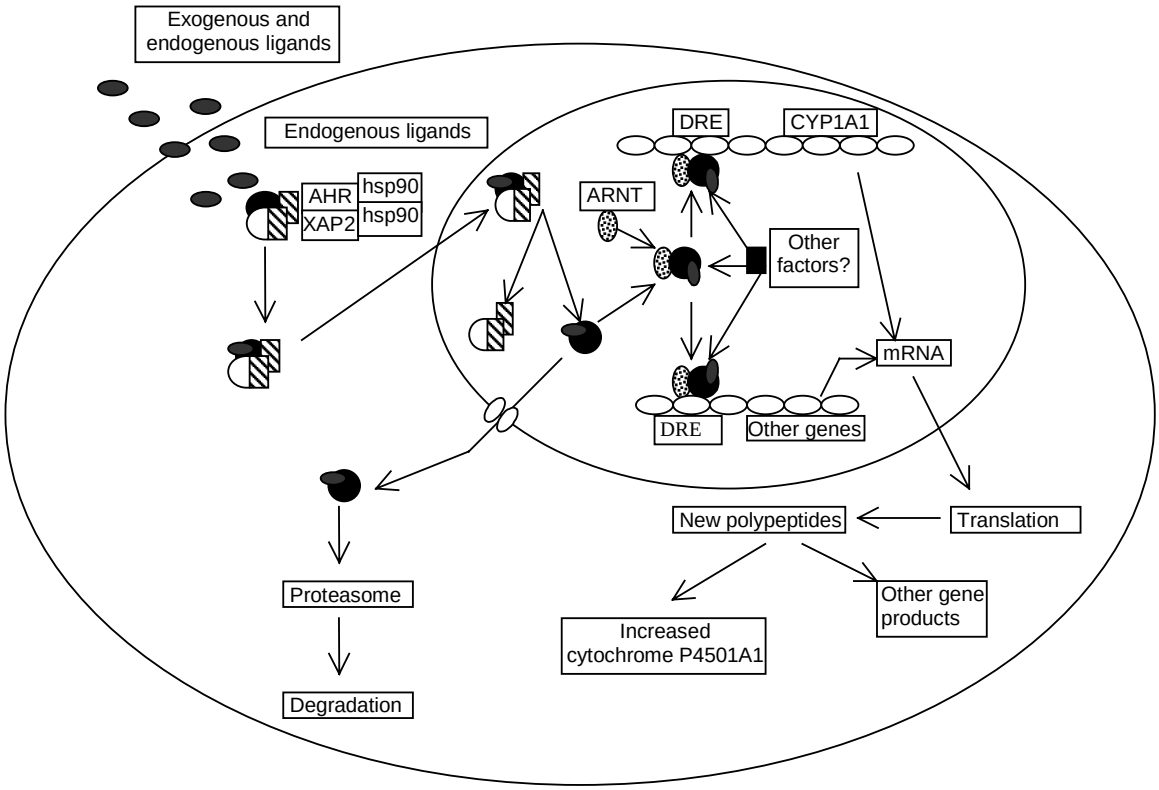


Figure 3: Model for AHR mediated gene activation (modified from Denison and Nagy 2003). See text for explanations. Appreviations: AHR, Aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; hsp90, 90 kDa heat shock protein; DRE, dioxin responsive element; XAP2, 38kD tetratricopeptide repeat protein of the immunophilin family.

1.3.3 Physiological role of AHR

By generating AHR knock-out (AHRKO) mice it has been shown that the AHR plays an important role in normal mammalian development. AHRKO mice have a smaller liver (Fernandez-Salguero *et al.* 1995, 1997; Schmidt *et al.* 1996) and defects in the heart (Lund *et al.* 2003; Thackaberry *et al.* 2002, 2003; Vasquez *et al.* 2003) and certain reproductive organs (Benedict *et al.* 2000, 2003; Lin *et al.* 2001), as well as in the vascular (Lahvis *et al.* 2000) and immune systems (Fernandez-Salguero *et al.* 1995, 1997; Gonzalez *et al.* 1995). AHR may also be involved in the hepatic accumulation of retinoids (Andreola *et al.* 1997, 2004). Many other phenotypic changes in AHRKO mice are manifested only as the animal ages, implying more rapid aging in AHRKO mice compared to wild-type mice (Fernandez-Salguero *et al.* 1997; Gonzalez and Fernandez-Salguero 1998).

1.3.4 Other possible mediators of TCDD toxicity

In addition to the basic AHR-mediated mechanism described above, other mechanisms by which the biological effects of dioxins are mediated have been suggested. Signal transduction through cellular factors such as tyrosine kinase $p60^{src}$, retinoblastoma protein, steroid receptors, nuclear factor -κB (NF-κB), and hypoxia-inducible factor 1α (HIF1α) has been shown to be modulated by TCDD in a manner independent of the transcriptional activation of AHR (Chan *et al.* 1999; Enan and Matsumura 1996; Enan *et al.* 1998; Ge and Elferink 1998; Gradin *et al.* 1996; Klinge *et al.* 2000; Ohtake *et al.* 2003; Puga *et al.* 2000).

1.4 Factors affecting toxicity of dioxins

A characteristic feature of TCDD is the dramatic difference in sensitivity to its acute toxicity among different animal species. In laboratory mammals, the lethal dose 50% (LD_{50}) values range from about 2 µg/kg in guinea pigs to more than 3000 µg/kg in hamsters (Olson *et al.* 1980). Table 2 presents the LD50 values for some species and some strains of the same species. No acute mortality has been reported among humans occupationally or accidentally exposed to dioxin. The highest human TCDD exposure has been estimated to be 15-20 µg/kg (Geusau *et al.* 2001).

Variation in the sensitivity of different species to TCDD is not due to appreciable differences in the half-life of TCDD elimination from the body. For example, the half-life of TCDD elimination from the whole body is estimated as 24-31 days in the rat (Rose *et al.* 1976), 30 days in the guinea pig (Gasiewicz and Neal 1979), and 11-12 days in the hamster (Olson *et al.* 1980). Perhaps the most significant variable affecting TCDD sensitivity/resistance is the genotype, and most probably the structure of AHR. Recent studies have indicated that, in the TCDD-resistant hamster and TCDD–sensitive guinea pig, the C-terminal ends of AHR differ, leading to differences in the transactivation properties of AHR (Korkalainen *et al.* 2000, 2001). Although these wide differences in sensitivity

have primarily been reported for acute, high-dose effects, they may exert major difficulties in interspecies extrapolations. Therefore, a crucial question for dioxin risk assessment is whether these sensitivity differences also exist in low-dose outcomes such as in developmental defects.

Table 2: LD50 values of TCDD for different animal species and strains (males only).

Species/Strain	LD50 (µg/kg)	Reference
Lake trout sack fry	0.074	(Walker *et al.* 1996)
Guinea pig	2	(McConnell *et al.* 1978)
Rhesus monkey	70	(McConnell *et al.* 1978)
Hamster	3000	(Olson *et al.* 1980)
Mouse, C57BI/6	180	(Chapman and Schiller 1985)
DBA/2	2600	(Chapman and Schiller 1985)
Rat, Long-Evans	18	(Pohjanvirta *et al.* 1993)
Han/Wistar	> 9600	(Unkila *et al*., 1994)
Line A	> 10000	(Tuomisto *et al.* 1999)
Line B	830	(Tuomisto *et al.* 1999)
Line C	40	(Tuomisto *et al.* 1999)

1.4.1 Genetic factors

There is a great difference in sensitivity to acute lethality among the different strains of the same species. The molecular basis for these differences appears to be associated with the polymorphism of the AHR. The differences in sensitivity to TCDD may be described by differences in TCDD potency and/or efficacy to induce toxic effects. Potency is a measure of the concentration or dose of TCDD that produces a toxic response (i.e. ED50), while efficacy characterizes the magnitude of effect induced by TCDD.

Animal models

C57BL/6J and DBA/2J mice. Poland and Glover (1975) found a 10- to 14-fold difference in TCDD susceptibility between the Ah-responsive C57BL/6J mice and the less responsive DBA/2J mice. The C57BL/6J mice have a wild-type Ahr^b allele, resulting in the presence of a high-affinity AHR. In DBA/2J mice the non-responsive Ahr^d allele results in a structurally altered receptor with lower affinity for the ligand (Ema *et al.* 1994; Okey *et al.* 1989).

The 10-14 –fold difference in TCDD potency to induce acute lethality between C57BL/6 and DBA/2 mouse strains is also shown in non-lethal endpoints, including CYP1A1 induction (Chapman and Schiller 1985; Ema *et al.* 1994; Okey *et al.* 1989; Poland and Glover 1980). However, TCDD efficacy was only slightly affected. Similarly, in C57BL/6J mice congenic at *Ahr* locus, the potency difference in acute lethality segregated with the *Ahr* allele and the same difference was also seen in body weight and organ weight changes

as well as clinical pathology (Birnbaum *et al.* 1990). But again, the efficacy was only slightly affected.

AHRKO mice. Three independently generated *Ahr* knockout mouse strains have been generated (Fernandez-Salguero *et al.* 1995; Mimura *et al.* 1997; Schmidt *et al.* 1996). AHRKO mice are resistant to toxic effects of TCDD including acute lethality and developmental defects (Fernandez-Salguero *et al.* 1995, 1996; Lin *et al.* 2001; Mimura *et al.* 1997). However, at high doses of TCDD, AHRKO mice display retinoid accumulation in liver and abnormal vascular structures in liver and kidney (Lahvis *et al.* 2000). Furthermore, placental exposure to TCDD causes an increase in prenatal mortality in AHRKO fetuses (Mimura *et al.* 1997; Peters *et al.* 1999). These findings suggest the presence of a novel, alternative pathway for TCDD toxicity.

Han/Wistar and Long-Evans rats. Another useful model, discovered in our laboratory, is the large intraspecies variation between the sensitive Long-Evans (*Turku/AB*; L-E) and the resistant Han/Wistar (*Kuopio*; H/W) rat strains to the lethality of TCDD. The LD50 value for H/W rats (>9600 µg/kg) may be even 1000-fold larger than that of L-E rats (18 µg/kg) (Unkila *et al*. 1994; Pohjanvirta *et al*. 1993). The apparent molecular weight of AHR (~106 kDa) is similar in L-E rats and Sprague-Dawley (S-D) rats, but is smaller (~98 kDa) in H/W rats (Pohjanvirta *et al.* 1999). The reason for the smaller size proved to be a deletion/insertion-type change in the 3´ end of exon 10 in the cDNA (Pohjanvirta *et al.* 1998). The deletion has resulted in loss of 343 amino acids in respective protein. Two diverse insertions, a shorter inversion (29 bases), and a longer insertion (134 bases) variant contained a misplaced translation termination codon (UAA) in their sequence. These gene sequences translate into the same protein with a total loss of 38 amino acids. However, the binding affinity of TCDD to AHR did not differ between these strains (Pohjanvirta *et al.* 1999).

Line A, B and C rats. Tuomisto and coworkers used conventional cross-breeding to segregate the resistance alleles, Ahr^{hw} and the unknown gene B, of H/W rats into different rat lines designated A, B and C (Tuomisto *et al.* 1999). Line A has the mutated Ahr^{hw} allele and the wild-type *B* allele (genotype $Ahr^{hw/hw}$, $B^{wt/wt}$). Line B lacks the resistant Ahr^{hw} allele, but is homozygous for B^{hw} ($Ahr^{wt/wt}$, $B^{hw/hw}$). Line C possesses neither of the resistance alleles ($Ahr^{wt/wt}$, $B^{wt/wt}$). Lines A, B and C exhibit highly different LD50 values for TCDD (Table 2). Line A rats are as resistant as H/W rats, line C rats almost as sensitive as L-E rats, and line B rats have intermediate resistance (Fig. 4).

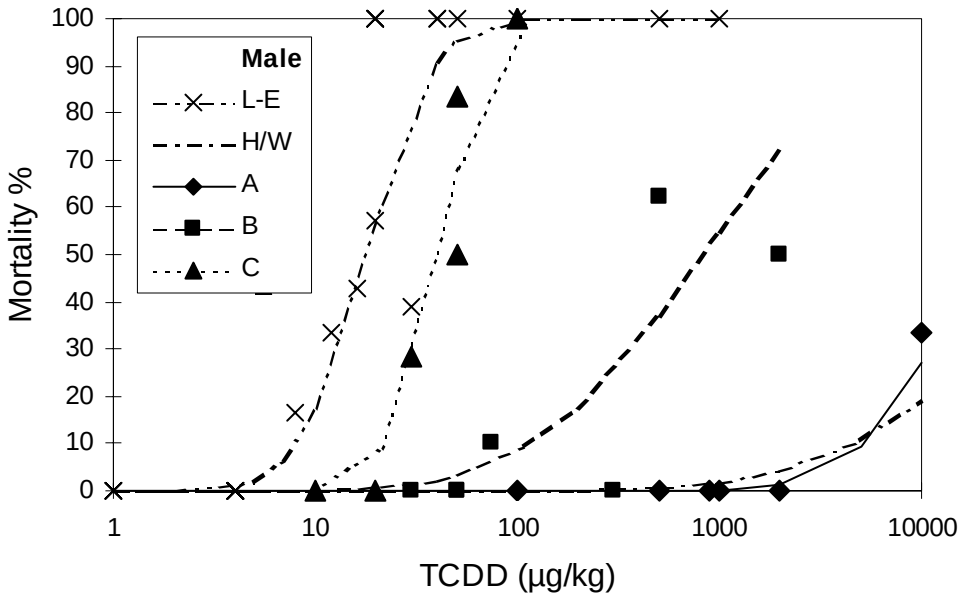


Figure 4: Mortality dose-responses for TCDD in male H/W, L-E and line A, B, and C rats (modified from Tuomisto *et al*., 1999).

Endpoint-dependent resistance

As is different from the mouse model, in H/W and L-E rats as well as in line A, B and C rats the vast sensitivity difference seen in acute lethality, was not observed in all short-term effects of TCDD. CYP1A1 expression and thymic atrophy are similarly induced between the TCDD-sensitive and -resistant rats (Pohjanvirta *et al.* 1988; Tuomisto *et al.* 1999; Tuomisto 1999). However, the resistance alleles greatly reduced the sensitivity the TCDD-induced increase in serum bilirubin.

Genetic variation in humans

As indicated previously, genetic variation in the AHR leads to differences in sensitivity to biochemical and toxic effects of dioxins in laboratory animals. In human *Ahr*, six single nucleotide polymorphisms (SNPs) have been identified, four of which result in changes in the amino acid sequence of the AHR protein. The majority of these SNPs locate in exon 10, which covers the major portion of the C-terminal transactivation domain (Cauchi *et al.* 2001; Harper *et al.* 2002; Kawajiri *et al.* 1995; Smart and Daly 2000; Wong *et al.* 2001). The impact of AHR polymorphism on human health is not clear, but most studies have been unable to connect specific AHR alleles either to CYP1A1 expression/induction, lung cancer incidence or chloracne (Anttila *et al.* 2000; Cauchi *et al.* 2001; Kawajiri *et al.* 1995; Wanner *et al.* 1999). Interestingly, a recent study shows that a combination of two or three of these SNPs prevents the TCDD-dependent CYP1A1 induction *in vitro* (Wong *et al.* 2001). The potential clinical significance of this finding, to cancer risk, or other toxic effects remains to be determined.

1.4.2 Developmental stage

Acute toxicity

Acute toxicity data that address the question of age at the time of exposure as a factor affecting toxicity are insufficient. In most acute toxicity studies sexually mature, adult animals have been used. However, it is generally believed that young animals are more susceptible to biological and toxic effects of TCDD than adults are. Therefore, it is important to elucidate the sensitivity at various ages, and the possible sensitivity differences of young animals of different animal species/strains to dioxins. If a sensitivity difference between young and adult animals exists, development of resistance during maturing could further explain the resistance.

Developmental toxicity

More data are available to compare the sensitivity of TCDD exposure during the prenatal and neonatal periods of life. Strain differences in adulthood are poor predictors for strain differences in embryo/fetal mortality, and the potency of TCDD in prenatal mortality tends to be more similar across species (Peterson *et al.* 1993). The LD50 of TCDD in adult hamsters and guinea pigs is >3000 and 2 µg/kg TCDD, respectively, the difference being over 1500-fold. However, there is only 12-fold difference in the maternal dose of TCDD causing increased prenatal mortality in hamster and guinea pig embryo/fetus (18 and 1.5 µg/kg TCDD, respectively) (Olson and McGarrigle 1992). Similarly, the 1000-fold difference in acute lethality to TCDD between L-E and H/W rats is clearly narrowed when TCDD is administered during gestation (Huuskonen *et al.* 1994).

Critical window of sensitivity during the prenatal period

The time period during which the embryo/fetus is exposed to TCDD is an important determinant of prenatal mortality (Peterson *et al.* 1993). A single maternal dose of 24 µg/kg TCDD given on gestation day (GD) 6 significantly increases prenatal mortality in C57BL/6 mice compared to vehicle control, but there is only little or no increase in prenatal mortality when TCDD is administered on day 8 of gestation or later (Couture *et al.* 1990). Similarly, there are critical periods of sensitivity to dioxin-induced developmental toxicity other than lethality. In mice, the critical window of sensitivity for an increased incidence of cleft palate is between GD6-12, and no cleft palate occurs when mice are exposed to TCDD on GD14 (Couture *et al.* 1990). Hydronephrosis on the other hand, can still be produced when TCDD exposure first occurs a few days after birth (Couture-Haws *et al.* 1991). The development of the ventral prostate and epididymis in male rat offspring is most affected if the maternal exposure takes place between GD15-18, compared to exposures on GD 8 or after birth (Bjerke and Peterson 1994; Gray *et al.* 1995; Ohsako *et al.* 2002).

1.5 Biochemical and toxic endpoints of dioxins

In laboratory animals, TCDD causes a wide spectrum of toxic responses including lethality, wasting, liver toxicity, metabolic disruption, enzyme induction, immune impairment, gastrointestinal and urinary tract lesions, endocrine disruption, reproductive toxicity, teratogenesis and cancer (Birnbaum 1994; Birnbaum and Tuomisto 2000; Bock 1993; IARC 1987; Pohjanvirta and Tuomisto 1994). Developmental effects (development of immune, nervous, and reproductive systems) appear to be the most sensitive adverse effects of TCDD exposure. The effects of TCDD on tooth development, bone quality and mechanical strength as well as on bile pigments have been recently studied in our laboratory (Jämsä *et al.* 2001; Kattainen *et al.* 2001; Miettinen *et al. submitted*; Niittynen *et al.* 2003; Viluksela *et al.* 2002).

There appear to be only a few reports of acute or chronic toxic responses in humans from background dioxin body burdens generated with normal dietary habits. The Finnish background exposure via mother's milk was shown to increase mineralization defects of first permanent teeth in children (Alaluusua *et al.* 1993).

By contrast, many epidemiological studies have reported various adverse effects following accidentally or occupationally high dioxin exposure (reviewed by Greene *et al.* 2003; Kogevinas 2001; Mukerjee 1998; Sterling and Arundel 1986). The human health effects associated with high dioxin exposure include behavioral alterations (Lai *et al.* 2002) and skin disease (Guo *et al.* 1999). Different types of cancer are also associated to dioxin exposure (Birnbaum and Fenton 2003; Huff *et al.* 1994; IARC 1997; Yu *et al.* 2000), although contradictory data was obtained in recent study in Finland (Tuomisto *et al*., 2004).

There are only limited data on reproductive and developmental effects in humans exposed to dioxins. Maternal exposure to dioxins has been associated with miscarriages, and stillbirths (Constable and Hatch 1985). Also an increase in abnormal sperm number has been reported in man (Guo *et al.* 2000). Paternal exposure to dioxins has been associated with change in boy-to-girl sex ratio (Mocarelli *et al.* 2000).

However, it is important to notice that these populations have been exposed to 10-1000 -times higher concentrations of TCDD than the general population. In addition, it is important to note that humans are typically exposed to mixtures of chemicals, whereas laboratory animals have been mainly exposed to purified dioxins. Especially in occupationally exposed cohorts dioxins have been characteristic impurities of main chemicals, such as chlorophenols and chlorophenoxyacids, and much higher exposure to the main chemicals may be a significant confounding factor. These facts may explain why the results of epidemiological studies appear to be inconsistent for most effects other than dermatological effects (Kogevinas 2001).

More details on some biochemical and toxic endpoints of dioxins based on animal studies are presented below.

1.5.1 Acute lethality

There are many characteristic features in TCDD-induced lethality. TCDD is extremely toxic to some animal species and strains, therefore, it is sometimes considered as the most potent and potentially lethal, man-made chemical. Interspecies and intraspecies differences in the acute lethality of TCDD are over 1000-fold as discussed above (Table 2). Furthermore, typical for TCDD toxicity is the delayed manifestation of lethality after acute exposure. The time lapse before death extends from one week up to 8 week from TCDD exposure (Pohjanvirta and Tuomisto 1994). Although many years have been spent in studying the ultimate mechanism of TCDD–induced lethality, it is still poorly understood.

1.5.2 Wasting syndrome

The wasting syndrome is characterized by a dramatic body weight loss, mainly due to hypophagia (Kelling *et al.* 1985). However, in rats parenteral feeding prevented the weight loss, but the rats still displayed similar latencies before death, as did their *ad libitum* –fed partners (Seefeld *et al.* 1984; Gasiewicz *et al.* 1980). It was concluded that wasting might be one cause of lethality, but not the only one. Depending on the dose, the decrease in feed intake can be dramatic and irreversible, or only temporary (Pohjanvirta *et al.* 1993; Seefeld *et al.* 1984). The mechanism of this quite unusual and drastic body weight loss is still uncertain.

1.5.3 Thymus atrophy

Thymus atrophy is one of the most characteristic effects of TCDD exposure and occurs in most animal species studied (Pohjanvirta and Tuomisto 1994). In rats, thymus atrophy occurs at acutely toxic doses slightly lower than those resulting in body weight loss, but higher than those already inducing CYP1A1 activity. The exact mechanism by which TCDD induces thymus atrophy remains unclear. The possible hypotheses are: 1) TCDD causes thymus atrophy by destroying committed prethymocytes (Fine *et al.* 1989, 1990), 2) TCDD inhibits the intrathymic T cell development (Kerkvliet and Brauner 1990), 3) TCDD kills immature thymocytes by initiating apoptosis (Kamath *et al.* 1997; McConkey *et al.* 1988). A most interesting and recent study showed that $p27^{Kip1}$ gene is involved in the inhibition of thymocyte proliferation (Kolluri *et al.* 1999). The induction of Kip1 by TCDD in cultures of fetal thymus glands inhibits thymocyte proliferation. This effect of TCDD is largely absent in the thymic glands from Kip1-deficient mice (Kolluri *et al.* 1999).

1.5.4 Typical responses of selected biochemical markers to TCDD

EROD induction

CYP1A1 is a phase I xenobiotic metabolizing enzyme. Induction of CYP1A1 enzyme activity reflects increased transcription of the CYPIA1 gene and the gene is regulated by

AHR (Okey *et al.* 1994; Whitlock 1999). In the rat, CYP1A1 preferentially catalyzes the *O*-dealkylation of 7-ethoxyresorufin. Thus, the activity of CYP1A1 is usually measured as ethoxyresorufin-*O*-deethylase (EROD) activity (Parkinson 1996). Induction of the CYP1A1 enzyme is an extremely sensitive marker for exposure to TCDD and other PCDDs. It has been possible to detect increased CYP1A1 mRNA levels following a single dose of as low as 1 ng/kg TCDD in rats (vanden Heuvel *et al.* 1994).

Serum bilirubin

Bilirubin is formed in the catabolism of heme molecules in erythrocytes, myoglobin, cytochromes and peroxidases (Bissell 1975). Serum bilirubin is greatly and rapidly (within one day) elevated in rats after TCDD exposure (Unkila *et al.* 1994; Pohjanvirta *et al.* 1995). Both total and conjugated bilirubins are increased, but their ratio is not affected implying that the increase in serum bilirubin is due to increased formation of bilirubin, and not due to decreased clearance.

Aspartate aminotransferase (ASAT)

Serum ASAT activity is dose-dependently increased after TCDD treatment. In TCDD-treated (50 µg/kg) L-E rats the increase was up to five-fold while H/W rats showed no increase (Pohjanvirta *et al.* 1995). Striated muscle, myocardium and liver are the main sources of ASAT and it is normally little present in plasma, bile, cerebrospinal fluid and saliva (Panteghini 1990).

Free fatty acids (FFAs)

When energy is needed, triglycerides stored in fat cells are hydrolyzed to glycerol and FFAs by a specific hormone-sensitive tissue lipase. Mobilization of FFAs also occurs in response to hypoglycemia or low levels of insulin in the blood. FFA mobilization in the body may not be the primary response to TCDD exposure, but is more likely an adaptive physiological response to the wasting syndrome. Serum concentration of FFAs was increased in L-E rats compared to *ad libitum* –fed controls at a lethal dose of 50 µg/kg (Pohjanvirta *et al.* 1989).

Thyroid homeostasis

Thyroid hormones are indispensable for growth, development and sexual maturation in mammals. A deficiency of thyroid hormones results in decreased metabolic rate, alterations in growth and development, disturbances in water and electrolyte balance, altered functions of the central nervous system, skeletal muscles, and cardiovascular system, and changes in lipid metabolism (Sterling 1979a, b).

In rats, TCDD induces the hepatic UDP-glucuronosyltrasferase (UGT) isozyme that catalyzes the glucuronidation of thyroxine (T_4), thereby increasing its elimination (Bastomsky 1977; Curran and DeGroot 1991; Schuur *et al.* 1997). TCDD reduced serum T_4 concentrations dose-dependently, and the reduction was seen already within 24 hours

(Pohjanvirta *et al.* 1989). After four days the decrease was significant at a dose of 5 µg/kg in both L-E and H/W rats. Pair-feeding studies revealed that decreased feed intake and hypophagia might partly contribute to the reduction in serum T_4 (Gorski *et al.* 1988).

The decrease in serum T_4 concentration in rats is reproducible from one study to another, but the effect of TCDD on serum T_3 concentration seems to be highly variable among different studies ranging from no change (Gorski and Rozman 1987; Rozman and Greim 1986), to a decrease (Potter *et al.* 1986), or an increase (Bastomsky 1977).

1.5.5 Male reproduction

Adult exposure

When TCDD is administered to sexually mature laboratory animals, it is known to reduce testis weight, cause abnormal testicular morphology, decrease spermatogenesis, and impair male reproductive performance (Chahoud *et al.* 1989, 1992; reviewed by Peterson *et al.* 1993). These effects may result, at least in part, from the reduction in plasma testosterone and 5α-dihydrotestosterone (5α-DHT) levels. A decrease in circulating androgens was observed in highly exposed adult rats within 24 hours (Moore *et al.* 1985, 1989), and the pituitary-gonadal axis was involved (Moore et al. 1991). The ED50 for the androgenic deficiency characterized by decreases in plasma androgen concentration and accessory sex organ weights is approximately 15 µg/kg TCDD (Moore *et al.* 1985).

Perinatal exposure

In utero and lactational exposure to TCDD during organogenesis results in multiple effects on the developing offspring (Birnbaum and Tuomisto 2000). Increased attention has been paid to these effects, since they are elicited at very low doses. Among the key findings are the male reproductive effects (Peterson *et al.* 1993; Roman and Peterson 1998).

Normal male gonad and reproductive duct system development in the mouse. Gender is established at the time of conception mainly by sex chromosome constitution, which controls the subsequent differentiation of the gonads, the internal genital duct system and external genitalia (Jameson *et al.* 2003). A gonadal primordium is first detected at GD11, but the fetal gender is still indistinguishable. Around GD13 the developing gonads show first evidence of sexual dimorphism, and Leydig cells within the fetal testes differentiate and, synthesize and secrete testosterone (Staack *et al.* 2003).

Testosterone production stimulates the development of the Wolffian ducts to form the epididymis, vas deference, and seminal vesicles. The upper portion of the Wolffian duct in the region of the testis differentiates into the epididymis at approximately GD18, the middle portion forms the vas deference, and the caudal portion initiates the seminal vesicles on GD16-17. (Staack *et al.* 2003)

Prostate development begins as an epithelial budding from the urogenital sinus (UGS) at approximately GD15-17. The buds emerge in a characteristic pattern that reflects the future lobar organization of the glands; the ventral buds, anterior buds, and dorsolateral buds (Cunha, 1972; Cunha *et al.* 1987b; Timms *et al.* 1994; Lin *et al.* 2003). Branching morphogenesis of the prostate lobes continues post-natally and 90% of the ductal branching is complete by day 10 of neonatal life (Sugimura *et al.* 1986).

TCDD effects on male reproductive development. Permanent adverse effects found in male pups are delayed puberty, altered mating behavior, and decreased sperm counts (Faqi *et al.* 1998; Gray *et al.* 1997; Mably *et al.* 1992a,b,c). Recently, Hamm and coworkers (2003) demonstrated that exposure of rats to a mixture of dioxins, furans, and non-coplanar PCBs resulted in a spectrum of male reproductive system effects similar to those of TCDD. However, due to lower tissue concentrations of certain congeners, two to three times higher doses than expected were required for comparable effects (Chen *et al.* 2001).

The most sensitive and reproducible effects have been seen on sperm numbers (Roman and Peterson 1998); generally the epididymal sperm numbers are affected more than the daily sperm production. The lowest maternal dose that reduced daily sperm production in 120-day-old Holzman rats was 64 ng/kg on GD15 (Mably *et al.* 1992c). Gray and coworkers reported significant decreases in ejaculated sperm numbers in adult Long-Evans hooded rats after a maternal dose of 50 ng/kg on GD15 (Gray *et al.* 1997).

Additionally, weights of accessory sex organs are dose-dependently decreased by TCDD. TCDD interferes with the development of ventral, dorsolateral and anterior prostate (coagulating gland), as well as with seminal vesicle growth and morphogenesis (Bjerke *et al.* 1994b; Gray *et al.* 1997; Hamm *et al.* 2000; Ko *et al.* 2002; Mably *et al.* 1992a; Roman *et al.* 1995; Wilker *et al.* 1996). In Holtzman rats the reduction of ventral prostate weight is observed at the maternal dose of 64 ng/kg on GD15 (Mably *et al.* 1992a). However, when expressed relative to the body weight (measured as organ weight/body weight), 160 ng/kg was the lowest dose with significant reduction in ventral prostate weight.

The sexual behavior of male rats is also altered after *in utero* and lactational exposure to TCDD. The time for mounting receptive females was extended, achieving intromission was more difficult and more thrusts were needed to achieve ejaculation (Mably *et al.* 1992b; Gray *et al.* 1995). Feminization of sexual behavior of male rats was also reported (Bjerke *et al.* 1994a; Mably *et al.* 1992b). However, this change was not observed in Long-Evans hooded rats (Gray *et al.* 1995).

Despite the resistance to acute lethality of TCDD, the developing male reproductive system of the hamster is sensitive to *in utero* and lactational TCDD exposure (Gray *et al.* 1995). The exposure of Syrian hamsters on GD11 to 2 µg/kg TCDD resulted in delayed puberty, and permanently decreased epididymal and ejaculated sperm counts. On the contrary, mice

are less sensitive to TCDD-induced toxicity of developmental reproductive system than are rats or hamsters (Theobald and Peterson 1997). Generally, the rat is the most sensitive mammalian species to developmental male reproductive effects of TCDD; the sensitivity of hamsters is about 1/10 that of the rat (Gray *et al.* 1995), and the sensitivity of mice is about 1/500 that of the rat (Theobald and Peterson 1997).

The effects of TCDD on developing male reproductive system resemble perinatal androgen deficiency. However, the effects on androgen homeostasis in male pups are not congruent. Some studies report a decrease in circulating androgen levels in the male offspring at birth and in young adults (Mably *et al.* 1992a; Bjerke and Peterson 1994). However, many studies with a similar dosing protocol have not been able to replicate these findings (Chen *et al.* 1993; Cooke *et al.* 1998; Gray *et al.* 1997; Roman *et al.* 1995). Furthermore, decreased expression levels of androgen receptor mRNA during puberty were reported in ventral prostates of Holtzman rats exposed *in utero* and lactationally (Ohsako *et al.* 2001).

In the rat, the critical window for male reproductive effects is shown to be around GD15, for example, the beginning of sexual differentiation (Ohsako *et al.* 2002). Hurst and coworkers (Hurst *et al.* 1998; 2000) demonstrated that the concentration of TCDD in the fetal tissue at the critical window is the important factor producing the toxic effects. Statistically significant effects on sperm counts were seen after a dose of 50 ng/kg TCDD on GD15, which results in fetal body burden of only 5.3 pg/g on GD16. At that time the concentrations in the maternal tissue producing adverse effects in experimental animals are nearly at the range of human background exposure to dioxin-like compounds.

1.6 Risk assessment of dioxins

Due to continuing concerns about dioxin-related health risks, a number of authorities have assessed the risks of dioxins worldwide over past 20 years (reviewed by Larsen *et al.* 2000). Strategies for risk assessment have changed during the past years, and different authorities have used different approaches. Therefore, significant procedural differences exist among different agencies and scientific organizations. Recently, most regulatory agencies, except the USEPA, have set their tolerable daily intake level for dioxins into the range of 1-4 pg TEQ/kg/day (Hays and Aylward 2003) (Table 3). The USEPA has concluded that the safe doses are two to three orders of magnitude lower, 0.001-0.01 pg/kg/day (USEPA 2000). The USEPA has used a linear, no-threshold dose-response extrapolation based on carcinogenicity, while the other international agencies use risk extrapolation models that assume an existence of a threshold dose and they use developmental defects as the critical endpoint of toxicity. The tolerable daily intake value has a high political and public health importance. In most European and North American countries the background dioxin exposure is between 1 and 2 pg/kg/day, which is about 100-1000-times above the USEPA tolerable human risk esposure daily intake level.

The differences in risk assessments arise from the still existing uncertainties in dioxin toxicity. The great difference in sensitivity to dioxins among various species, and the uncertainty of human sensitivity exerts difficulties in species extrapolation. Also the elimination half-lives varies greatly between humans and rodents. As mentioned, in humans the elimination half-life is about seven years (Poiger and Schlatter 1986), while in rats the half-life of TCDD is only three to four weeks (Geyer *et al.* 2002). Furthermore, the half-life of TCDD in infants may be far less than in adults. Recently, a half-life of TCDD in newborns was determined to be 0.42 years only (Kreuzer *et al.* 1997; LaKind and Filser 1999). Uncertainty (or safety) factors are used to compensate for intra- and inter-species dynamic and kinetic variation, to protect certain sensitive groups with high exposure, such as breast-fed infants, and to compensate for the possible gaps of knowledge. It is also recommended that the use of body burden as a dose metric rather than daily dose would better recognize the effect of intra-species kinetic variation (DeVito *et al.* 1995).

Table 3: Comparison of recent derivations of tolerable intake levels.

Organization	Endpoints	LOAEL[a] (ng/kg)	Uncertainty factors	TDI [b] (pg/kg/day)
WHO, 1998 (van Leeuwen *et al.* 2000)	Rat offspring - male reproductive system effects - immunosupression	28-73 (maternal body burden)	10 (composite)	1-4
USEPA, 2000 (USEPA 2000)	Range of effects (BMD modeling for ED_{01})[c]	10-50	250-12500 (implied)	0.001-0.01 (implied)
ECSCF, 2001 (ECSCF 2001)	Rat offspring - male reproductive effects - accelerated eye opening - decreased anogenital distance	(maternal body burden) 40 80 20 (NOAEL)[d]	9.6 3.2	2
ATSDR[e], 1998 (ATSDR 1998)	Monkey offspring - neurobehavioral effects	0.12 per day intake in feed (maternal)	90	1

[a] LOAEL = lowest observed adverse effect level

[b] TDI = tolerable daily intake

[c] Benchmark dose modeling for 1% effective dose that would increase the lifetime risk of cancer mortality with 1%

[d] NOAEL = no adverse effect level

e ATSDR = agency for toxic substances and disease registry (USA)

The question of most sensitive effect to be used as the basis of risk assessment is also unanswered. Cancer was generally used as the critical effect risk assessment performed before 1998 (Hays and Aylward 2003). TCDD has been shown to be carcinogenic in

experimental animals (Dragan and Schrenk 2000; Kociba *et al.* 1978), but so far, epidemiological studies have been unable to reliably prove TCDD as a human carcinogen. However, based on the possibility of carcinogenicity in humans and strong animal carcinogenicity data, IARC has also concluded that TCDD is a human carcinogen (IARC group 1; IARC 1997). Nowadays there is a growing concern over non-cancer effects of dioxins, especially the effects on the developing immune, nervous, and reproductive systems. The recent risk assessments other than USEPA have focused on these non-cancer effects of dioxins, and thereby changed the most essential effect group from adults to infants.

The present work aims to provide further information for the complex questions in dioxin risk assessment.

2 Aims of the study

The toxicity of dioxins is characterized by large inter- and intraspecies differences. The differences in sensitivity seem to be dependent on genetic factors, but these factors and their roles as determinants of dioxin sensitivity are only partly understood. Differences in some, but not all endpoints of toxicity complicate the species-to-species extrapolation. Recent toxicity studies in experimental animals and epidemiological studies in humans have shown that very low doses of dioxins may result in developmental defects during the sensitive periods of life. However, some key questions remain unanswered: What are the sensitive periods for different low-dose endpoints? How are these endpoints affected by genetic factors? The general aim of this thesis was to find answers to these questions.

The specific aims in each study were as follows:

1. To find out if the unusual lethal potency of HxCDD in H/W rats can be shown in short-term non-lethal endpoints of dioxin toxicity and, furthermore, to characterize the resistance of H/W rats to four PCDDs in the light of these endpoints. (I)

2. To describe the roles of rat H/W-type resistance alleles *Ahr*^{hw} and *B*^{hw} in short-term non-lethal endpoints of dioxin toxicity. (II)

3. To test, if the sensitivity to male rat reproductive defects after *in utero* and lactational TCDD exposure is dependent on the resistance alleles *Ahr*^{hw} and *B*^{hw}. (III)

4. To test, if the sensitivity to male rat reproductive defects after TCDD exposure in adulthood is dependent on the resistance alleles *Ahr*^{hw} and *B*^{hw}. (IV)

5. To investigate the role of AHR in the normal development of the male mouse accessory sex organs, as well as in the effects of *in utero* and lactational TCDD exposure on the development of these organs. (V)

6. To study the critical time window of sensitivity for various aspects of the development of male mouse accessory sex organs and, consequently, to give insights into the mechanisms by which TCDD exerts its effects. (VI)

7. To investigate the time-course of postnatal development of resistance to TCDD, and to differentiate the roles of the resistance alleles *Ahr*^{hw} and *B*^{hw} in the resistance development in rat. (VII)

3 Materials and methods

3.1 Animals and animal husbandry

The rat strains and lines, and mouse strains used in the thesis are shown in table 4. For LD50 information see table 2.

Rat studies (I-IV)

L-E and H/W rats, as well as line A, B and C rats were obtained from the breeding colony of the National Public Health Institute (Kuopio, Finland) maintained in a specific pathogen free (SPF) barrier unit. The animals are regularly subjected to health surveillance consisting of serological and bacteriological screening as suggested by FELASA (FELASA 1996). These surveys indicated that the animals were free of typical rodent pathogens. Tuomisto and coworkers (1999) have described the breeding protocol used to develop rat lines A, B and C.

Non-pregnant rats were housed in stainless steel wire-bottom cages, 5-6 rats per cage. Pregnant and lactating females (III, IV) were housed singly in plastic cages that had wire-mesh covers. Heat-treated aspen chips (Tapvei Co., Kaavi, Finland) were used as bedding and nesting material. Following weaning, the pups were housed with same-sex littermates similarly to pregnant females.

Rats received commercial rat chow (R36; Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The ambient temperature in animal room was 20±2°C, and the relative humidity 45±10%. The rats were kept under a photoperiodic cycle of 12 h light/ 12 h dark in an air-conditioned animal room.

All rat procedures were conducted by protocols that were approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government.

Mouse studies (V, VI)

AHRKO mice were generated as described by Schmidt and coworkers (1996) and generously provided by Dr. Christopher A. Bradfield after being backcrossed to C57BL/6J mice for six generations. Colonies of these mice, and those of C57BL/6J mice, were maintained at the University of Wisconsin, School of Pharmacy animal facility (Madison, WI, USA). The mice were backcrossed to C57BL/6J mice for three to five additional generations. Dosing comparable percentages of dams with TCDD at each stage of backcrossing minimized contributions of different genetic backgrounds to differences between treatments.

Mice were kept in a temperature-controlled room (24 ± 1°C) with 35 ± 4% relative humidity, lighted from 0600 to 1800 hours. Mice were housed in clear plastic cages with heat-treated chipped aspen bedding. Feed (5015 Mouse Diet, PMI Nutrition International, Brentwood, MO, USA) and tap water were available *ad libitum*.

All mouse procedures were conducted under protocols approved by the University of Wisconsin Animal Care and Use Committee.

Table 4: Description of rats and mice used in the study.

Animal	Strain	*Ahr* allele	*B* allele	TCDD acute lethality
Rat	H/W	$Ahr^{hw/hw}$	$B^{hw/hw}$	Resistant
	A	$Ahr^{hw/hw}$	$B^{wt/wt}$	Resistant
	B	$Ahr^{wt/wt}$	$B^{hw/hw}$	Semi-resistant
	C	$Ahr^{wt/wt}$	$B^{wt/wt}$	Sensitive
	L-E	$Ahr^{wt/wt}$	$B^{wt/wt}$	Sensitive
Mouse	C57BL/6J	$Ahr^{b/b}$	-	Sensitive
	AHRKO	$Ahr^{-/-}$	-	Resistant

- Not known

3.2 Chemicals

TCDD, PeCDD, HxCDD and HpCDD were purchased from the UFA-Oil Institute (Ufa, Russia); they were over 99% pure as confirmed by gas chromatography-mass spectrometry (Vartiainen *et al.* 1995; Viluksela *et al.* 1998b).

In rat studies the PCDDs were weighed and dissolved in diethyl ether. A small volume of diethyl ether was mixed with corn oil and the ether was let to evaporate. Dosing solutions were mixed in a magnetic stirrer and sonicated for 20 minutes before dosing (I-IV, VII). For mouse studies TCDD was dissolved in acetone and an adjusted volume of acetone was mixed with corn oil to get a dissolved mixture of 95% corn oil/ 5% acetone.

Other chemicals and equipment used are given in the original publications. (I-VII)

3.3 Methods

3.3.1 Mating and weaning

Adult (12-15 week-old) female rats in estrus were mated with untreated males from 9 to 12 a.m. and vaginal smears were collected and examined for the presence of sperm. The day of sperm detection in vaginal smears was considered as GD0. The offspring were weaned at the age of 28 days. (III)

Heterozygous ($Ahr^{+/-}$) female mice at the age of 13-17 weeks were paired overnight with $Ahr^{+/-}$ males. The next day was considered as GD0. The offspring's were weaned at the age of 21 days. (V,VI)

3.3.2 Uterine implantation sites

At the time of weaning the dams were killed and the number of uterine implantation sites was counted after staining the uteri with an ammonium sulfide solution (10%, v/v). For each litter, the percentage of postimplantation loss was calculated by the following formula:

$$100 \times (a - b)/a \quad [2]$$

where a is the total number of implantation sites, and b is the number of pups born. (III)

3.3.3 AHRKO mouse genotyping

Genotyping was done by polymerase chain reaction (PCR) analysis of ear punch tissue taken at 10-16 days of age, as previously described (Benedict *et al.* 2000). Shortly, tissue was lyzed with proteinase K at 65°C for 30 min with a 15 s vortexing after 15 and 30 min incubation times. PCR was carried out from the lysate using the following primers: 1) Neo F TTGGGTGGAGAGGCTATTCG, 2) Neo R CCATTTTCCACCATGATATTCG, 3) AhrI3F TCTTGGGCTCGATCTTGTGTC and 4) TTGACTTAATTCCTTCAGCGG. The conditions for PCR were as follows: 94°C, 2 min initial denaturation, 35 cycles of 94°C for 5 s, 60°C for 30 s, and 72° for 1 min, with a final extension at 72°C for 2 min. (V, VI)

3.3.4 Real-time reverse transcription- polymerase chain reaction (RT-PCR) for mRNA quantification

Total RNA was isolated from prostate lobes using RNeasy Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Oligo-dT primed first-strand cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) in 20 µl reactions containing 500 ng total RNA, following the manufacturer's instructions. Final reverse transcription reactions were diluted to 100:l for storage and for real-time LightCycler (Roche Molecular Biochemicals, Indianapolis, IN) quantitative PCR analysis. The exact PCR and LightCycler protocols are described in detail in the original publication (V). Primer sequences, product size, annealing temperatures, and gene descriptions are shown in Table 5. (V, VI)

Table 5: Primer sequences, product size, annealing temperatures and gene descriptions used in quantitative Real-Time reverse transcription-PCR analysis.

Gene (GeneBank accession no.)	**Primer sequences**	**Temp (°C)**	**Size***	**Description**
Androgen receptor (emb X59590)	AATCTGGATGTGGAGAGAGC AGAGAACAGAACACTAGCGC	60	166	Regulates patterns of gene expression in response to testosterone and dihydrotestosterone.
Cytokeratin 8 (emb X12789)	ACCAGGAGCTTATTAACGTC AGGAGCTCATTCCGTAGCTG	65	204	Marker for fully structural cytodifferentiation of luminal epithelial cells (Fuchs 1988).
Cyclophilin (emb X52803)	ATCACGGCCGATGACGAGCC TCTCTCCGTAGATCCACCTGC	65	217	Commonly used normalization control, mRNA expressed in all cell types (Weisinger *et al.* 1999).
MP25 (emb X06246)	AGAGCCCAGAATGTCCTGGG TTATCACGTGCTCTCCGTCC	65	214	Androgen-dependent secretory glycoprotein produced by mouse ventral prostate (Mills *et al.* 1987).
Probasin (emb AF005204)	TTACATGTCGCATAGTACCTG GGACAGAGACATGAAAGAGAAA	60	219	Androgen-dependent secretory protein produced by dorsolateral and anterior prostate (Johnson *et al.* 2000).
PSP94 (emb X16642)	TGCCACCATGGAAGCTTGGC TAGCGTTGGTACAGCAGGTG	65	224	Prostate secretory protein of 94 amino acids; protein primarily produced by lateral prostate (Xuan *et al.* 1999).
Renin 1 (gb U89840)	ACTCGGTGACTGTGGGTGG AGGTGGGAACCCCTGTTGTAG	65	220	Androgen-dependent protein highly expressed in mouse anterior prostate (Fabian *et al.* 1993).
SVS II (dbj AK020661)	AAACAGAGGAAGACTTATCCC AGGCGAGTCCTTGATATTGATC	60	237	Seminal vesicle secretion II protein; protein specific to seminal vesicle (Lundwall 1996).

*Size, product size (base pairs).

3.3.5 Daily sperm production and cauda epididymal sperm count

The frozen testes and cauda epididymides were homogenized for two minutes using Ultra Turrax homogenizer (model T25 basic, IKA-WERKE GMBH & CO, Germany) in 0.9 % saline, containing 0.05% Triton X-100 and 0.01% thimerosal. Homogenization resistant sperm heads were counted using hemocytometer. (III,IV)

3.3.6 Testis morphometry

The left testes were fixed in bouin's solution, containing saturated picric acid, formaldehyde and glacial acetic acid (15:3:1; v/v), and cleared in 70% ethanol. Sections were cut (5 µm) and stained with periodic acid-Schiff-haematoxylin (PAS-H). For all control and highest TCDD dose animals, one section from one testis was examined. The diameter of 10 stage VII/VIII seminiferous tubules was analyzed with computer assisted light microscope using Stereo-Investigator and NeuroExplorer (MicroBrightField, VT) – softwares. The numbers of Sertoli cells, spermatogonia, preleptotene spermatocytes,

pachytene spermatocytes, and round spermatids were analyzed from five stage VII/VIII seminiferous tubules using light microscope. (IV)

3.3.7 Biochemical assays and dental inspection

Androgen assays. For rat studies (III, IV) testosterone was measured from diethyl ether extracts of serum samples by time-resolved fluoroimmunoassay (DELFIA, Wallac Ltd., Turku, Finland). In the mouse study (V) serum testosterone and 5α-androstane-3α,17ß-diol (3α-diol) concentrations were determined from anhydrous ethyl ether extracts of serum samples by radioimmunoassays (RIA), according to the protocol of the antibody supplier (Endocrine Sciences, Calabasas Hills, CA, USA).

Ethoxyresorufin-O-deethylase (EROD) activity. EROD activity in liver S9 fraction was assayed fluorometrically according to Kennedy and Jones (1994) with slight modifications. (I, II)

Protein concentration. Protein concentrations were determined by the Bradford (1976) method. (I, II)

Serum total T4 concentration. Serum total thyroxine (T4) concentrations were measured using a coated tube T4 kit (Orion Diagnostica, Turku, Finland). The radioactivity was counted using gammacounter (1272 CliniGamma Gammacounter, Wallac Oy, Turku, Finland). (I)

Serum ASAT activity. ASAT activity assay was based on kinetic measurement using UV photometric detection, and it followed the Scandinavian Committee on Enzymes (S.C.E.) of the Scandinavian Society for Clinical Chemistry and Clinical Physiology recommendations (1974). (I, II)

FFA concentration. Serum FFA concentrations were analyzed according to the method of Shimizu and coworkers (1980). (I, II)

Total bilirubin concentration. Serum total bilirubin was measured using a modified diazo ultramicromethod of Walters and Gerarde (1970). All these assays were performed with the Kone Specific selective chemistry analyzer (Kone Instruments, Espoo, Finland). (I, II)

Tooth examination. Lingual attrition surfaces of lower incisors were examined for the presence and severity of pulpal perforations (Alaluusua *et al.* 1993) using a stereomicroscope. Severity of pulpal perforation was scored semi-quantitatively using a scale 0-3 (0, no perforation; 1, initial perforation; 2, perforation; 3, perforation with pulpal hemorrhage). (I, II)

3.3.8 Efficacy and potency

Efficacy (or relative efficacy) describes the magnitude of effect and it was defined as a relative change of the effect from the control level: (Emax-Emin)/Emin. Absolute efficacy was defined as an absolute change: Emax-Emin. Efficacy ratio was defined as the relative efficacy in one rat line divided by that in the reference line (usually line C). Sometimes absolute efficacy ratio was calculated by using absolute efficacies. Potency was defined as the inverse of ED50. (I, II)

3.4 Statistical analyses and curve fitting

Rat studies (I-IV, VII)

Treatment groups were compared by one-way analysis of variance (ANOVA), and the least significant difference (LSD) test was used for *post hoc* comparisons. In the case of non-homogeneous variances (according to Bartlett´s test, $P < 0.01$), the Kruskal-Wallis ANOVA was used, followed by the distribution-free multiple comparison test (I) or Mann-Whitney U test (II-IV, VII). *P* values less than 0.05 were considered significant.

Dose-response curves (I, II) were fitted assuming a shape of cumulative normal distribution after logarithmic dose transformation and using the least square method with sequential quadratic programming (SPSS 10.0 statistical program, SPSS Inc., Chicago, IL, USA). The regression analysis was performed for all strains and all endpoints separately using the following formula:

$$E_i(d) = Emin + (Emax - Emin) * \Phi((d-\mu_i)/\sigma_i) \quad [3]$$

where $E_i(d)$ is the observed effect at d = log(dose), Emin is the control effect, Emax is the maximal effect, μ_i is log($ED50_i$), σ_i is log(GSD_i) (GSD denoting for geometric standard deviation), Φ is the cumulative standard normal distribution and $_i$ is the index for the four congeners (I). The following restrictions were used for input values: ED_{50} 0.01 – 3000 µg/kg; GSD >1.02; GSD < 50 (only II); Emin$\geq$ 0; Emax $\geq$ 0.

For study II a probability that one estimate is smaller than the other was calculated by randomly sampling values from the likelihood distributions of the two estimates. The difference between strains was considered statistically significant if this probability was smaller than 0.025 or greater than 0.975.

The data (II) were also modeled using nonlinear regression with a modified hyperbolic curve, the Hill equation (DeVito *et al.* 1997; Holford and Sheiner 1981).

$$E(D) = Emin + ((Emax-Emin) * D^n)/ED_{50}^n + D^n) \quad [4]$$

Where E(D) is the observed effect at D = dose, Emin is the control effect, Emax is the maximal effect, ED_{50} = effective dose 50%, and n = Hill coefficient, the shaping factor.

Mouse studies (V, VI)

Analyses were conducted with the litter as the experimental unit. Treatment groups were compared by one-way analysis of variance (ANOVA), and the least significant difference (LSD) test was used for *post hoc* comparisons. In the case of non-homogeneous variances (according to Levene´s test), Kruskal-Wallis ANOVA and the median test were used, followed by the distribution-free multiple comparison test as the *post-hoc* test. *P* values less than 0.05 were considered significant.

3.5 Experimental designs

The summary of the experimental design for each study is presented in Table 6.

(I) Structure-activity relationship and endpoint categorization

Five to six adult H/W and L-E rats were used per dose group. Rats were given a single oral dose of one PCDD (TCDD: 0.03 – 100 µg/kg; PeCDD: 0.1 – 300 µg/kg; HxCDD: 0.3 – 300 µg/kg; HpCDD: 0.3 – 3000 µg/kg) or corn oil as a control (4 ml/kg). There were separate control groups for each congener. On day 8 after exposure the rats were decapitated with a guillotine. Liver and thymus were weighed and EROD activity was analyzed from liver samples stored at –80°C. Serum samples were stored at -80°C. Serum ASAT activity, and serum FFA, T4 and bilirubin concentrations were analyzed. Mandibles were stored in 10% neutral buffered formalin and studied for incisor defects.

(II) Role of resistance alleles in acute non-lethal TCDD endpoint categorization

Five to six adult line A, B and C rats were used per dose group. Rats were given a single oral dose of TCDD in corn oil, the TCDD doses being at approximate three-fold intervals between 0.03-3000, 0.03-1000 and 0.03-100 µg/kg body weight in line A, B and C rats, respectively. Control animals received corn oil vehicle (4 ml/kg). On day 8 postexposure, rats were decapitated with a guillotine, and the analyses were carried out as in structure-activity relationship study (I), except that serum T4 concentration was not analyzed.

(III) Role of resistance alleles in the effect of perinatal TCDD exposure on male reproductive system

The pregnant rats were dosed by gavage, with a single dose of 0.03-1 µg/kg TCDD for dose-response study or 1 µg/kg TCDD for time-course study. Control animals received an equivalent volume of corn oil vehicle (4 ml/kg). Anogenital distance (AGD) and crown-rump length (CRL) was measured on postnatal day (PND) 1 and 4. Body weights of dams and offspring were determined weekly. Pups were weaned on PND28. For the dose-response study all male pups were terminated on PND70 and for time-course study one or

two male pups from each litter were terminated at different ages, PND14, 21, 28, 35 and 49. Weight of testes, right epididymis (and cauda epididymis), ventral-, dorsolateral- and anterior prostate, seminal vesicles and thymus were determined. Sperm counts were analyzed from cauda epididymis and testis, and testosterone levels from serum. Additionally, for PND35 terminated male pups the weight of lungs, heart, kidneys, liver, salivary glands and spleen was determined.

(IV) Role of resistance alleles in the effect of adult TCDD exposure on male reproductive system

Five to six adult line A, B and C rats were used per dose group. Rats were given a single oral dose of TCDD in corn oil, the TCDD doses being at approximate three-fold intervals between 0.1-1000, 0.1-300 and 0.1-30 µg/kg body weight in line A, B, and C rats, respectively. Control animals received corn oil vehicle (4 ml/kg). On day 17 postexposure, rats were anesthetized with CO_2, and blood was collected with heart puncture. Serum was separated and stored at –80°C until later analyses. Weight of testes, right epididymis (and cauda epididymis), ventral prostate and seminal vesicles (emptied) were determined. Sperm counts were analyzed from right testis and cauda epididymis, and testosterone levels from serum.

(V) The development of accessory sex organs and the role of AHR

Pregnant mice were given a single oral dose of TCDD (5 µg/kg) or vehicle (95% corn oil/5% acetone, 5 ml/kg) on GD13. Pups were weaned on PND21. Mice were euthanized by CO_2 overdose on PND35 or 90. Accessory sex organs were either frozen for mRNA analyses, or placed overnight in Z-5 fixative (Anatech Ltd., Battle Creek, MI) for histopathological analyses. The abundance of MP-25, probasin, renin-1, PSP94, SVS II, androgen receptor, cytokeratin-8, and cyclophilin mRNA were analyzed by real-time reverse-trancriptase PCR from the separate prostate lobes. The results are presented separately for AHRKO and wild-type mice.

(VI) Critical window for accessory sex organ defects after TCDD exposure

Pregnant mice were given a single oral dose of TCDD (5 µg/kg) or vehicle (95% corn oil/5% acetone, 5 ml/kg) on GD13. At birth, pups were fostered to dams from the same treatment group or cross-fostered to dams from the opposite treatment group. Additional dams were given a single oral dose of TCDD (5 µg/kg) on GD16; they were allowed to keep their own pups. The analyses were carried out as in the AHR dependence study (IV), except that PSP94 and SVS II mRNA levels were not analyzed. Additionally, all results presented are from the wild-type mice.

(VII) Postnatal development of resistance to high-dose effects of TCDD

A pilot study was carried out in TCDD-resistant H/W rats. Newborn H/W litters were dosed i.p., 4 ml/kg, with a single dose of 1000 µg/kg TCDD or corn oil vehicle at the age of 5, 7, 9, 11 and 14 days and survival of the pups was monitored for 42 days.

In the main study newborn line A litters were given a single i.p. dose (4 ml/kg) of TCDD or corn oil vehicle at the age of 2, 5, 8, 11, 14 or 17 days. Line B offspring were dosed at the age of 5, 14, 28, 42 or 56 days. Line A pups received 1000 µg/kg TCDD, and line B male and female pups 200 or 100 µg /kg TCDD, respectively. Offspring were weaned at the age of 28 days. The condition and mortality were monitored for 42 days postexposure, and the animals were weighed once a week for the whole duration of the experiment. All rats were subjected to macroscopic examination after death or euthanasia. The weights of the kidneys were recorded and the severity of hydronephrosis was evaluated semi-quantitatively by assessing the renal pelvic dilatation. A subgroup of 6 male rats from each dosing group was randomly selected from survivors on day 42 postexposure. These rats were followed up to 135 days, while the other rats were euthanized on day 42.

An additional group of line A litters was dosed at the age of 5 days according to the main study protocol. The pups were euthanized eight days after dosing by decapitation and thymus, testes, kidneys and livers were weighed. Liver samples were preserved in 10% neutral buffered formalin for histological examination. In addition, the presence of hydronephrosis was macroscopically scored as described above.

Table 6: Experimental designs.

Study	Animal species, strain and sex	Dose (µg/kg)	Age at dosing	Exposure/termination of the study	Object
I	Rat; H/W and L-E females	TCDD: 0.03-100 µg PeCDD: 0.1-300 µg HxCDD: 0.3-300 µg HpCDD: 0.3-3000 µg	Adult; 13-15 weeks	Acute, single dose/ 8 days postexposure	Dose-response analysis of acute non-lethal toxic endpoints.
II	Rat; Line A, B, and C females	TCDD, Line A: 0.03-3000 µg Line B: 0.03-1000 µg Line C: 0.03-100 µg	Adult; 8-12 weeks	Acute, single dose / 8 days postexposure	Dose-response analysis of acute non-lethal toxic endpoints.
III	Rat; Line A, B, and C males	TCDD 0.03-1 µg	Maternal dosing on GD15	*In utero* and lactational/ male pups sacrificed at PND70 or at PND14, 21, 28, 35, 49	Dose- and time-response analysis of male reproductive endpoints.
IV	Rat; Line A, B, and C males	TCDD, Line A: 0.1-1000 µg Line B: 0.1-300 µg Line C: 0.1-30 µg	Adult; 12-16 weeks	Acute, single dose / 17 days postexposure	Dose-response analysis of male reproductive endpoints.
V	Mouse; AHRKO ; $Ahr^{-/-}$ and $Ahr^{+/+}$ males	TCDD 5 µg	Maternal dosing on GD13	*In utero* and lactational/ male pups sacrificed at PND35 or 90.	Prostate growth and functional development.
VI	Mouse; AHRKO ; $Ahr^{+/+}$ males	TCDD 5 µg	Maternal dosing on GD13 or GD 16. Cross-fostering (GD13).	*In utero* and lactational, *in utero* only or lactational only/ male pups sacrificed at PND35.	TCDD sensitive periods in prostate growth and functional development.
VII	Rat; H/W, Line A, and line B males and females	H/W: 1000 µg Line A: 1000 µg Line B; female 100 µg male 200 µg	H/W: days 5-14; Line A: d 2-17; Line B: d 5-56 of age.	Acute ,single dose / 42 days postexposure; 6 or less 42-day survivors monitored for longer period	Development of resistance to acute lethality.

4 Results

4.1 Resistance alleles and AHR as modifiers of TCDD toxicity

4.1.1 The exceptional relative sensitivity of H/W rats to higher chlorinated dioxins is limited to acute lethality (I)

Dose-responses for short-term (8 days postexposure) non-lethal TCDD responses (body weight loss, thymus atrophy, serum bilirubin, and liver EROD activity) followed the normal rank order of potency in both L-E and H/W rats (I: Fig. 1-2). TCDD was the most potent congener followed by PeCDD, HxCDD and HpCDD. There was no clear dose-response in H/W rats for serum ASAT activity or FFA levels at the dose levels used. However, there was no sign of exceptional potency of higher chlorinated dioxins either (I: Fig. 3).

Overall the relative potency (REP) estimates for TCDD, PeCDD, HxCDD and HpCDD were in fairly good agreement with the current WHO-TEF values in both L-E and H/W rats (I: Table 4; Fig. 5), especially for body weight change, thymus atrophy, EROD induction, and increases in serum bilirubin levels.

The dose-response data together with the REP values indicate that the exceptional relative sensitivity of H/W rats to HxCDD and other higher chlorinated dioxins is limited to the effects that result in acute lethality.

4.1.2 The efficacy ratio of 0.5 classifies the effects into type I and type II categories (I, II)

Instead of potency, the efficacy expressed as a relative change ((Emax – Emin)/Emin) turned out to be the most important factor for classification of non-lethal short-term TCDD toxic effects into two different categories, type I and type II endpoints. The results with line A, B and C rats showed that the Ahr^{hw} is the most important factor decreasing the TCDD efficacy, and the mechanistic difference between type I and II endpoints is linked to the Ahr^{hw} allele and thereby to the altered AHR transactivation domain. In contrast, the B^{hw} allele has only a minor effect on efficacy when judged based on most endpoints.

Type II endpoints. H/W rats showed lower efficacy than L-E rats for TCDD-induced body weight loss, serum ASAT activity, and serum concentrations of total bilirubin, fatty acids and thyroxine (I: Fig. 2 and 3). For these endpoints the efficacy in H/W rats was less than half of that in L-E rats (I: Table 4), and they were thus categorized as type II endpoints (efficacy ratio < 0.5). The results with line A and C rats confirmed the validity of this classification criterion. The efficacy ratios between line A and line C rats for these

endpoints were also less than 0.5 (II: Table 2). Serum thyroxine concentration was not analyzed in line A, B and C rats.

Type I endpoints. The efficacy for TCDD-induced thymus weight change, EROD activity, and incisor tooth defects was similar in H/W and L-E rats, as well as in line A and line C rats (I: Fig 1; Table 4, II: Fig 1; Table 2). These effects were categorized into type I endpoints with more than 0.5 fold efficacy ratio between the resistant and sensitive rats.

4.1.3 TCDD-induced decrease in sperm numbers is modified by the rat resistance alleles (III, IV)

In utero and lactational TCDD exposure (III)

TCDD consistently and statistically significantly decreased the ventral prostate weight (III: Table 2), as well as anterior and dorsolateral prostate weights. Also sperm counts, especially the cauda epididymal sperm count was a sensitive endpoint, being consistently decreased after *in utero* and lactational TCDD exposure (III: Fig. 1).

Up to the age of 49 days the TCDD effects on male reproductive endpoints appeared quite similar between the rat lines. At the age of 70 days the decrease in daily sperm production and cauda epididymal sperm number seemed to me more pronounced in line B and C rats than in line A rats, indicating that decrease in sperm numbers is modified by the resistance alleles. The resistance alleles did not profoundly affect the sensitivity of the development of male reproductive organ to *in utero* and lactational TCDD exposure.

TCDD exposure delayed the puberty-related increase in serum testosterone levels, which were most severely depressed on day 49. Afterwards the levels approached the control levels.

Adult TCDD exposure (IV)

Decreases in sperm parameters and ventral prostate weight were the most sensitive endpoints observed after adult TCDD exposure. The effects were seen at doses that also decreased the body weight, i.e. 10 µg/kg TCDD and higher in line A, B and C rats (IV; Figs. 1, 3, 5). Serum testosterone levels were also decreased at the highest TCDD doses (IV; Fig. 2). Testis weight was not affected and only a marginal decrease was observed in epididymis, cauda epididymis and seminal vesicle weights.

The rat lines did not show clear sensitivity differences for the TCDD-induced decrease in androgenic status. However, the resistance alleles did increase the resistance to the TCDD-induced decrease in sperm numbers.

The highest dose of TCDD showed a tendency to decrease the number or Sertoli cells, round spermatids, as well as pachytene- and preleptotene spermatocytes per tubulus in each

rat line (IV; Fig. 4), and there were no sensitivity differences between the rat lines. The number of spermatogonia per tubulus was not affected.

4.1.4 Most effects of TCDD on growth and functional development of male accessory sex organs in mice are AHR-dependent (V)

The effects of TCDD on the growth and functional development of accessory sex organs, analyzed on PND35 and 90, are summarized in Table 7. In general, the influence of TCDD on growth and functional development of prostate lobes was dependent on the AHR. The only exceptions from AHR dependency were the decreased luminal epithelial cell specific gene expression in ventral and dorsolateral prostates, as well as decreased seminal vesicle weight (V: Fig. 7), as these effects were observed also in TCDD-exposed AHRKO mice.

Serum androgen concentrations (V: Fig. 8), or androgen receptor expression (V: Figs. 2,4,6) were not significantly affected by *Ahr* null mutation and/or TCDD exposure.

Interestingly, TCDD exposure seemed to cause some re-specification of gene expression in wild-type mouse ventral and lateral prostate lobes. In ventral prostate, the lateral prostate specific PSP94 mRNA expression was increased by TCDD exposure (V: Fig. 2). In addition, in certain TCDD-exposed wild-type and vehicle exposed knockout dorsolateral prostates, the seminal vesicle specific SVS II mRNA was produced at least at a level that was an order of magnitude greater than the highest level seen in any wild-type mouse exposed to the vehicle (V: Fig. 6).

The *Ahr* null mutation plays a role in the development of the accessory sex organs, though these effects are organ specific. The *Ahr* null mutation restrained the growth of the dorsolateral prostate and delayed that of the anterior prostate (V: Fig. 3, 5). However, gene expression and histology appeared normal. Also seminal vesicles were smaller in AHRKO mice (V: Fig. 7), even though the histology appeared normal. No effect of the *Ahr* null mutation was seen on the ventral prostate development.

Table 7: Effects of TCDD on the growth and functional development of accessory sex organs analyzed on PND35 and 90.

		AHRWT + TCDD	AHRKO + CO	AHRKO + TCDD
Ventral prostate	Absolute weight	↓ [a]	- [b]	-
	Relative weight	↓	-	-
	MP25 mRNA			
	- relative to cyclophilin	↓	-	-
	- relative to CK8	↓	-	↓
	CK8 relative to cyclophilin	↓	-	-
	Histology	-	-	-
Dorsolateral prostate	Absolute weight	↓	↓ only PND35	-
	Relative weight	↓	↓	-
	Probasin mRNA			
	- relative to cyclophilin	-	-	-
	- relative to CK8	↓	-	↓
	CK8 relative to cyclophilin	-	-	-
	Histology	-	-	-
Anterior prostate	Absolute weight	↓	↓; only PND35	-
	Relative weight	↓	↓; only PND35	-
	Renin-1 mRNA			
	- relative to cyclophilin	↓	-	-
	- relative to CK8	↓	-	-
	CK8 relative to cyclophilin	-	-	-
	Histology	-	-	-
Seminal vesicle	Absolute weight	↓	↓	↑; PND35 ↓; PND90
	Relative weight	↓	↓	↑
	Histology	-	-	-

[a] Statistically significant ($p<0.05$) decrease (↓) or increase (↑) compared to wild type vehicle control.

[b] No statistically significant effect compared to wild type vehicle control.

4.2 Developmental stage as a determinant of TCDD toxicity

4.2.1 The developing male rat reproductive system is more susceptible than the adult reproductive system (III, IV)

The effects of TCDD on daily sperm production, cauda epididymal sperm reserve and prostate weight were observed after the adults were given a dose of 10 µg/kg TCDD or higher (IV). *In utero* and lactational TCDD exposure induced similar effects on sperm parameters and prostate development after a maternal TCDD dose of only 0.3 µg/kg.

Therefore, the male rat reproductive system is clearly more sensitive to TCDD during perinatal development than in the adulthood.

4.2.2 The critical time window of TCDD sensitivity is prostate lobe specific in mice (VI)

The effect of TCDD on ventral prostate growth (measured as weight of organ/body weight) was mostly due to *in utero* exposure (VI: Fig. 1). Dorsolateral prostate and anterior prostate growth was inhibited by both *in utero* and lactational TCDD exposure (VI: Fig. 2,3). However, a combined *in utero* and lactational exposure was necessary for inhibition of seminal vesicle growth (V: Fig. 4). Delaying the start of TCDD exposure from GD13 to GD16 resulted in a smaller but significant reduction of ventral and anterior prostate weight (VI: Fig. 1,3). However, the delay of TCDD exposure had no effect on the growth of dorsolateral prostate or seminal vesicle (VI: Fig. 2,4).

The effect of TCDD on functional cytodifferentiation in different prostate lobes was measured as androgen-dependent gene expression per cell; MP25 for ventral prostate, probasin for dorsolateral prostate, and renin-1 for anterior prostate. The effect on ventral prostate secretory cytodifferentiation was mostly due to *in utero* exposure, and that on dorsolateral prostate approximately equally due to *in utero* and lactational exposure (VI: Fig. 2, 3). The functional cytodifferentation of anterior prostate was inhibited regardless of the timing of TCDD exposure (VI: Fig.3).

Structural cytodifferentiation was measured as a proportion of fully differentiated luminal epithelial cells compared to all cells (cytokeratin 8:cyclophilin). In the ventral prostate, the delay of the start of TCDD exposure from GD13 to GD16 significantly increased the cytokeration 8:cyclophilin ratio (VI: Fig.1), indicating that the proportion of structurally fully differentiated luminal epithelial cells was affected by the timing of TCDD exposure between GD13-16. Structural cytodifferentiation was not affected in dorsolateral or anterior prostate development (VI: Fig. 2, 3).

Altogether, the time during which the four organs are the most vulnerable to TCDD varies from one organ to another. The most vulnerable periods during the growth and functional development of accessory sex organs are presented in Table 8.

Table 8: Critical windows of sensitivity of growth and differentiation of accessory sex organs in mice.

Accessory sex organ	Growth	Structural cytodifferentiation	Functional cytodifferentiation	Developmental stages affected
Ventral prostate	GD13 – 16	GD13 - 19	GD13 - 16	Early bud stage and budding
Dorsolateral prostate	GD16 – postnatal	Not affected	GD16 - postnatal	Later prenatal processes [a]
Anterior prostate	GD13 – 16	Not affected	Postnatal	Multiple processes (prenatally and postnatally)
Seminal vesicle	GD16 – postnatal	- [b]	-	After rudiments appear

[a] Bud formation, bud elongation and/or duct formation.

[b] Not examined.

4.2.3 The time-course for development of resistance to mortality is different in line A and in line B rats (VII)

Line A rats were fairly resistant to TCDD already two days after birth, and the full resistance to acute lethality developed during the first week after birth for both males and females. Line B rats did not attain complete resistance until at the age of 28 days in females and 42 days in males. (VII: Fig. 1)

Line A males that survived through the 42-day period still died on average 80 days after TCDD exposure (VII: Fig. 2; Table 1). Necropsies revealed that in addition to wasting most rats suffered from severe liver damage and hydrothorax.

Body weight gain was decreased in both line A and B rats and settled below the control level. The difference in body weight seen shortly after exposure remained fairly constant to the end of the observation period (VII: Fig 3).

Hydronephrosis was observed with high frequency and severity in line A and B rats exposed to TCDD as late as during the first two weeks of life (VII: Fig. 4). The time of sensitivity to hydronephrosis of both line A and B rats appeared to be similar.

5 Discussion

Although dioxins have been extensively studied for a number of years, there are still remarkable uncertainties in the human risk assessment. Two crucial questions of the dioxin toxicity discussed in the present thesis are: 1) **the role of resistance alleles** in a) the validity of the TEF approach, and in b) TCDD toxicity, especially the male reproductive effects, 2) **the influence of the developmental stage** at the time of TCDD exposure on the outcome.

5.1 The role of resistance alleles and AHR (I-V)

Of the animal models generally used in mechanistic dioxin research, the C57BL6 / DBA mouse model and the AHRKO model have been useful in demonstrating the dependence of a variety of dioxin-induced toxic effects on the AHR signalling pathway as well as in identifying those effects that are not mediated by AHR. On the other hand, our rat model based on the resistance alleles proved to be useful in further characterisation of AHR mediated effects by detecting genotype-dependent differences in sensitivity. Furthermore, based on these sensitivity differences it was possible to divide AHR mediated effects of dioxin into two mechanistically different categories, the type I and type II endpoints. This animal model also indicated that the Ahr^{hw} with mutated transactivation domain is an important from the point of view of sensitivity differences. Although it is still premature to estimate the potential significance of these findings for the human risk assessment, it is highly interesting that also the first data to link genetic variability in the human AHR to sensitivity differences in a dioxin-induced effect suggest that the transactivation domain would be involved (Wong *et al.* 2001).

5.1.1 The validity of TEF approach (I)

Dioxin congener potencies in TCDD-resistant H/W rats are different from those typically expected, because H/W rats are exceptionally sensitive to HxCDD in terms of acute lethality (Pohjanvirta *et al.* 1993). By establishing the structure-activity relationships for different endpoints of toxicity in this thesis it was possible to define the role of the H/W-type resistance alleles in the characteristic non-lethal endpoints of dioxin toxicity. This knowledge provides mechanistic information about the different endpoints, but it also demonstrates the influence of these resistance alleles on the TEF approach.

The structure-activity relationships of PCDDs.

Despite the exceptional relative sensitivity of H/W rats to acute lethality caused by higher chlorinated PCDDs, TCDD was found to be the most potent congener in inducing all studied short-term non-lethal toxic effects, including body weight loss in H/W and L-E rats.

Therefore, it appears that the abnormal potency of higher chlorinated PCDDs is limited to acute lethality and the non-lethal toxic endpoints follow the normal rank order of potency (DeVito *et al.* 1997; Hebert *et al.* 1990; Safe 1990; Weber *et al.* 1992); TCDD being the most potent congener followed by PeCDD, HxCDD and HpCDD.

The results indicate that the exceptional potency of higher chlorinated PCDDs in H/W rats is limited to acute lethality and to the high dose levels that cause lethality. This implies that lethality is mechanistically different from the other, lower dose outcomes of dioxin-induced toxicity and, therefore, HxCDD-induced lethality does not represent a direct manifestation of the typical toxic effects of dioxins. Furhermore, this type of lethality can only be observed in rats that are resistant to the characteristic TCDD toxicity (cf. Pohjanvirta *et al.* 1995), i.e. in H/W and in line A rats. This hypothesis is supported by our recent findings indicating that at very high doses there are clear differences in the outcome of toxicity between HxCDD and TCDD –treated rats (Simanainen *et al.* 2003). All rats that died from HxCDD had an exceptionally pale liver, HxCDD reduced body weight more rapidly than TCDD and, unlike TCDD, HxCDD caused diarrhea. In addition, the mean survival time before death was only 14 days for HxCDD treated rats compared with 33 days for TCDD treated rats. Interestingly, 10 mg/kg HxCDD caused lethality in H/W and line A rats, while the same dose of TCDD did not.

Also two previous studies from our laboratory are in accordance with this interpretation of the present findings. Firstly, based on observations at very high PCDD doses Pohjanvirta *et al.* (1995) were the first to point out that there may be at least two distinct mechanisms of PCDD toxicity. The predominant one is characteristic for TCDD and PeCDD, and the other one is characteristic for HxCDD and HpCDD and can only be detected in animals resistant to the primary mechanism. Secondly, Unkila *et al.* (1998) showed that in H/W rats a high dose of HxCDD (5000 µg/kg 6 days before sampling) resulted in more profound body weight loss and tryptophan homeostatsis disruption compared to the same dose of TCDD.

REP estimations and the current TEF values

The REP estimates for TCDD, PeCDD, HxCDD and HpCDD were found to be consistent with the latest consensus WHO-TEF values (Table 1; I: Fig. 5). Only the REP estimate for PeCDD deviated from the WHO-TEFs, and was closer to the previous TEF of 0.5 than to the current TEF of 1. This is comprehensible considering that the TEF for PeCDD was upgraded to 1 in the last WHO expert meeting based on *in vivo* liver tumor promotion data, giving lower priority to all short-term data (Van den Berg *et al.* 1998).

Deviation of single REP estimates from consensus TEF values reflects the endpoint prioritization dilemma in TEF determination. Each TEF is derived from a range of REP estimates obtained from *in vivo* and *in vitro* studies. A recent study demonstrated that the WHO TEFs for the PCDD/Fs are at the upper bound of the underlying REP distributions (Finley *et al.* 2003). This tends to lead to conservative risk assessment. Finley and

coworkers (2003) suggested that instead of using the point estimate TEFs, the use of REP data distributions would allow more informed evaluation of variability and uncertainty in dioxin risk estimates.

5.1.2 Short-term toxicity and male reproductive toxicity of TCDD (I - V).

The influence of genetic variation on human sensitivity to dioxins is largely unknown, but it is expected to be significant, as the influence of genetic factors clearly influence the variation of TCDD toxicity in experimental animals, e.g. in rats and mice. The roles of genetic factors vary also among different endpoints of dioxin toxicity (Pohjanvirta *et al.* 1988; Tuomisto *et al.* 1999; **I – IV)**.

Criteria for endpoint classification into type I or type II

Previous studies from our laboratory have shown that short-term toxic responses to TCDD were either similar in H/W and L-E rat strains, or H/W rats were more resistant (Pohjanvirta *et al.* 1988; Tuomisto *et al.* 1999). In this study the dose-responses for short-term effects were determined, modeled and quantified in a systematic manner for the first time and thereby it was possible to define the exact criteria for classification of different endpoints (**I**, **II**). The major difference between the rat strains was in the efficacy seen in certain endpoints. Efficacy is description of the magnitude of toxic response induced by TCDD, while potency determine the concentration or a dose of TCDD that produce a toxic response (i.e. ED50). The H/W:L-E efficacy ratio of 0.5 appeared to classify "clear" type I and type II short-term endpoints (Table 9; **I**). Additionally, the studies on line A, B and C rats confirmed the validity of efficacy as a classification criterion for type I and type II endpoints in short-term toxicity studies (**II**).

Table 9: Classification of short-term PCDD endpoints in rats.

	H/W:L-E (line A:lineC) efficacy ratio	Role of resistance alleles, Ahr^{hw} and B^{hw}	Typical endpoints
Type I	> 0.5	No effect	EROD induction, change in thymus weight, incisor tooth defects
Type II	< 0.5	Increase resistance	Lethality, change in body weight, serum ASAT activity, serum bilirubin and FFA level

The role of Ahr^{hw} allele. The Ahr^{hw} proved to be the major factor decreasing TCDD efficacy (**II**), indicating that the mechanistic difference between type I and II effects is linked to the Ahr^{hw} allele. The deletion mutation in Ahr^{hw} is located in the transactivation domain and thus the domains responsible for ligand and DNA binding as well as heterodimerization are intact (Pohjanvirta *et al.* 1998). Therefore, the decreased ability of

the *Ahr*^{hw} to mediate PCDD toxicity in type II endpoints can be localized into steps beyond DNA binding, i.e., into the AHR-dependent gene expression.

The C-terminus of AHR is required for the formation of functional AHR conformation that increases promoter accessibility and facilitates promoter occupancy by different transcription factors (Ko *et al.* 1996; Kronenberg *et al.* 2000). As the promoter sequences and the transcription factor machinery vary among genes, the two types of endpoints imply that the mutation in the *Ahr*^{hw} selectively affects the formation of functional AHR conformation as well as communication between enhancer and promoter, leading to altered expression of different genes.

Another theory involves the possibility of two different AHR-mediated signaling pathways, i.e. the AHR-ARNT-DRE –dependent pathway and the pathways that are independent of AHR transcriptional activation. In fact, the concept of two different AHR mediated signaling pathways has been previously suggested by Matsumura and co-workers (Blankenship and Matsumura, 1997; Dunlap and Matsumura, 2000; Enan and Matsumura, 1996; Matsumura *et al.* 1997). Their data suggest that TCDD cause activation of tyrosine kinases, in particular p60$^{c\text{-}src}$ kinase mediating at least a part of TCDD toxicity. Therefore, it can be hypothesized that type I endpoints like CYP1A1 induction are mediated by AHR-dependent activation of gene expression in the nucleus, i.e. the AHR-ARNT-DRE –pathway, and type II endpoints are mediated by AHR-dependent tyrosine kinase pathway. However, this theory presumes that the latter pathway is functional in all type II endpoints. Previously, Dunlap and coworkers (2002) demonstrated that *c-src* knockout mice that do not express c-src appear to be slightly more resistant to TCDD-induced depletion of glycogen storage and down-regulation of phosphoenolpyruvate carboxykinase (PEPCK), which are changes associated with wasting syndrome (Viluksela *et al.* 1995; Weber *et al.* 1995).

The role of B^{hw} *allele*. The *B*^{hw} allele had only a minor influence on TCDD efficacy, and the classification into either type I or type II endpoints was not clear. Therefore, it is concluded that a mechanism specifically associated to the *Ahr* genotype is necessary for the endpoint classification. However, as the responses in line B rats differ from those in line C rats, the *B* allele is somehow involved in the mechanistic pathway of TCDD toxicity.

As the identity of *B* allele has not yet been determined, it is hypothesized that *B*^{hw} could be a protein participating in the AHR signaling pathway. Despite the fact that ARNT has several different products of alternative splicing of mRNA, there were no differences in relative expression levels of the ARNT variants between H/W and L-E, or among the line A, B and C rats (Korkalainen *et al.* 2003; Korkalainen, unpublished results). The AHRR was also considered as a possible candidate for *B*^{hw}, but recent results suggest that AHRR does not contribute to dioxin sensitivity differences observed in H/W and L-E rats (Korkalainen *et al.* 2004).

Further characterization of type I and type II endpoints

Even though type II endpoints appear to be mainly high-dose effects, the type I and II categorization does not clearly comply with the low/high-dose endpoint classification. For example, the ED50 estimates for two type I endpoints, i.e. liver EROD activity and incisor tooth defects, differed by more than 100-fold. However, it is likely that only some of the effects of TCDD are primarily due to TCDD. Others may be either adaptive physiological responses to biochemical changes, or secondary to some other toxic responses (Pohjanvirta and Tuomisto 1994). Therefore, the diversity of the toxic effects of TCDD is problematic in the analysis of type I and type II endpoints. For example, the biochemical and biological processes behind the induction of EROD activity, incisor tooth defect, and liver toxicity are diverse and involve different numbers of intermediary steps.

In the case of an endpoint that results from a chain of preceding events, it would be more important to examine the dose-response relationships of the primary insult rather than the final endpoint. However, this is not always possible. For example, long-term multi-step events, such as carcinogenicity, are likely to represent an accumulation of a variety of effects. Therefore, the long-term effects may not follow the classification criteria developed for short-term effects (cf. Viluksela *et al.* 2000). Similarly, after a 20-week exposure to TCDD, the H/W and L-E rats showed little difference in efficacy but about a 100-fold difference in potency for plasma ASAT activity (Viluksela *et al.* 2000), which is a typical type II endpoint based on short-term experiments (**I, II**). This implies that the primary difference is in efficacy, and it is observed first, while toxicity or tolerance manifested after several weeks may modify the effect, leading to secondary differences in potency.

How does endpoint categorization apply to mortality?

The studies by Pohjanvirta and co-workers (Pohjanvirta *et al.* 1993; Unkila *et al.* 1994) and Tuomisto *et al.* (1999) demonstrate that, although the resistance alleles considerably affect the potency for TCDD-induced mortality, the alleles also appear to have an effect on the efficacy (Fig. 4). At high TCDD doses, the mortality did not increase even if the dose was increased (low efficacy). However, the true mortality curve for H/W and line A rats is not known because due to limited solubility of TCDD it is technically not possible to dose these rats with higher doses than 10,000 µg/kg TCDD. Nevertheless, the potency differences in short-term toxicity do not seem to explain, at least alone, the differences seen in acute lethality among the rat lines. Therefore, it can be hypothesized that mortality belongs to type II endpoints with different efficacy in L-E and H/W rats. Lethality is triggered only after a strong induction/activation of the underlying biological mechanism and, due to low efficacy in H/W rats, the lethal processes are not triggered even at high doses.

AHR and resistance alleles in male reproduction

Role of functional AHR. It is generally believed that most, if not all of the toxic effects of TCDD are mediated through AHR. The use of AHRKO mice has enabled the determination of the role of AHR in TCDD toxicity, and also its possible role in the normal development. By using these mice we showed that, in general, the effects of TCDD on growth and functional development of the ventral, dorsolateral and anterior prostate were AHR-dependent and were not observed in AHRKO mice (**V**). On the contrary, TCDD was able to modify the development of the seminal vesicles in AHRKO mice. The biological significance of these effects of TCDD on AHRKO mice may be questionable, but at least they demonstrate that TCDD can also cause AHR-independent effects. The other possibility is that mice express more than one form of AHR, as observed, for example, in zebrafish (Andreasen *et al.* 2002). Consequently, not all forms of AHR might have been eliminated from AHRKO mice. However, there is currently no evidence for the presence of more than one form of AHR in mammals (Hahn 2002).

The AHR was also shown to be necessary for the normal development of the dorsolateral prostate, anterior prostate, and seminal vesicle (**V**). The ventral prostate was most affected by TCDD exposure in wild-type mice, but was not affected by the null mutation. In a previous study it was shown that the development of other male reproductive organs, such as the testis and epididymis, were affected by the AHR null mutation (Lin *et al.* 2001). The role of the AHR in normal male reproductive system development is still unclear. However, it may involve interactions of the AHR with one or more endogenous ligands, or just be due to some unknown function of unliganded AHR.

Role of resistance alleles Ahr^{hw} and B^{hw}. Despite the major role of functional AHR in mediating the effects of TCDD on the development of accessory sex organs (**V**), the H/W-type truncated AHR in line A rats was still able to mediate in a normal way the TCDD-induced defects of the male reproductive organs after adult and perinatal TCDD exposure (**III, IV**).

However, the AHR with a truncated transactivation domain appears to modify the effects of TCDD on sperm parameters (daily sperm production and cauda epididymal sperm number) after adult as well as *in utero* and lactational TCDD exposure. This may also be important for the risk assessment of humans to dioxins as the decrease in sperm parameters is one of the most sensitive endpoints of dioxin toxicity in rats (Roman and Peterson 1998), the effects appear to be permanent (Mably *et al.* 1992c) in rats, and most of the polymorphisms in the human AHR are located on in the transactivation domain (Harper *et al.* 2002). In theory, a mutation in human AHR C-terminal end could result in decreased sensitivity to dioxin-induced male reproductive effects as observed in rats with H/W-type mutated AHR. However, due to the significance of this area in signal transduction, it is also possible that other types of mutations in AHR transactivation domain could result in increased sensitivity, although this was not observed in the present study.

It is clear, however, that despite the minor modifying effect of the resistance alleles on the sensitivity, the overall effects on male reproductive system observed after TCDD exposure are to some extent influenced by the animal species and strain, as well as the dosing regime used (Chahoud *et al.* 1992; Faqi *et al.* 1998; Gray *et al.* 1995; Latchoumycandane *et al.* 2002a; Mably *et al.* 1992a, b, c; McConnell *et al.* 1978; Moore *et al.* 1985; Ohsako *et al.* 2001; Rune *et al.* 1991a, b). Nevertheless, the variation in the sensitivity of species, or strains of the same species, is in general much smaller after perinatal TCDD exposure than in acute lethality (Gray *et al.* 1995; Theobald and Peterson, 1997). With respect to male reproductive defects, no studies have been conducted with TCDD-responsive C57BL/6 and –non-responsive DBA/2 mice to examine the influence of lower binding affinity of AHR on sensitivity to TCDD.

5.2 The influence of developmental stage during TCDD exposure (III-VII)

The developmental stage during dioxin exposure is an important factor in dioxin toxicity, as the most sensitive endpoints of dioxin toxicity seem to be related to developmental processes.

5.2.1 The developing male reproductive system is more susceptible than the adult reproductive system

The sensitivity of the growth and functional development of mouse ventral prostate during the fetal stage is an important observation from the risk assessment point of view, as the transfer of TCDD to offspring is highly limited during the prenatal period. The majority of maternal TCDD body burden is transferred to pups in milk rather than across the placenta (Abbott *et al.* 1989; Nau *et al.* 1986; Weber and Birnbaum, 1985). This is important because the critical period for effects of TCDD on ventral prostate development coincides with the period of placental TCDD exposure, and not with the postnatal period of peak TCDD transfer. In addition, the development of dorsolateral prostate, anterior prostate and seminal vesicle are affected by *in utero* TCDD exposure. Therefore, it is concluded that the development of male accessory sex organs is highly sensitive to TCDD, and adverse effects are observed at the extremely low amounts of TCDD, which cross the placenta.

The studies on A, B and C rats also showed a clear difference in sensitivity between developing and adult male reproductive systems, as demonstrated by the toxic effects on prostate (**III, IV**). In the adult study, a single dose of 30 µg/kg TCDD in line B and C rats, and 1000 µg/kg TCDD in line A rats, significantly decreased the weight of the ventral prostate 17 days postexposure (**IV**). On the other hand, *in utero* and lactational exposure to a single maternal dose of 1 µg/kg TCDD on GD15 significantly reduced the ventral prostate weight in line A, B and C offspring, though the reduction was not permanent (**III**). A single

maternal dose of 1 µg/kg TCDD on GD15 results in fetal TCDD body burden of only 36.4 ng/kg on GD21 (Hurst *et al.* 2000), and the rat prostate development is affected already during the fetal period (Bjerke and Peterson 1994). Thereby, it can be concluded that the growth of ventral prostate in these rat lines is significantly more sensitive to fetal TCDD exposure than to adult exposure, and the sensitivity difference is at least 2-3 orders of magnitude. This rough estimate is fairly close to the previous estimate, indicating that fetal male reproductive defects are observed at doses approximately one hundredth of those of adult defects (Roman and Peterson, 1998).

It is important to note that there are mechanistic differences in the way that the perinatal and adult exposure dosing regimens decrease prostate growth and/or functional development. An important factor associated with male reproductive organ effects after adult TCDD exposure is the precipitous decrease in serum testosterone concentration; male reproductive organ effects are mostly observed at doses that cause these decreases in circulating androgen levels (**IV**). On the contrary, after fetal exposure to a low dose of TCDD, there seems to be a delay in pubertal testosterone production, but as the pups get older the levels are returned close to normal (**III, V**). Therefore, it seems that the primary mechanism behind the male reproductive effects in adulthood and during development is different.

Currently available information (Cooke *et al.* 1998; Faqi *et al.* 1998; Gray *et al.* 1995; Loeffler and Peterson, 1999; Roman *et al.* 1995; Timms *et al.* 2002; **III**) suggests that the *in utero* and lactational TCDD exposure has only a minor or no effect on androgen levels in young adults. The mechanism may be related to the decreased androgen receptor mRNA expression levels observed after *in utero* and lactational TCDD exposure in ventral prostate gland (Ohsako *et al.* 2001), though this was not confirmed in the present study (**V**). Recently, it was also shown with LNCaP prostate cancer cells that there could be an interaction between AHR and androgen receptor signaling pathways (Jana *et al.* 1999; Morrow *et al.* 2004). However, another work using an androgen receptor reporter assay showed that TCDD did not interfere with androgen action in the urogenital sinus (Ko *et al.* 2004). In addition, TCDD was not shown to have antiandrogenic effect on androgen receptor activation in Chinese hamster ovary cells transiently co-transfected with AR and MMTV-luciferace plasmid (Vinggaard *et al.* 2000).

Prostate may also be a target for estrogen action (Cooke *et al.* 1991; Makela *et al.* 2000; Nilsson *et al.* 2001). In fact, the prostate defects are linked to the changes in intrauterine estradiol levels and to the sex of the neighboring fetuses inside the uterus (Timms *et al.* 2002). Additionally, it is also possible that the estrogenic status is changed due to interaction between AHR and estrogen receptor signaling pathways (Kharat and Saatcioglu 1996; Ohtake *et al.* 2003; Safe *et al.* 2000; Wormke *et al.* 2003). However, the essential studies to show the exact role of estrogen in TCDD-induced disturbances in prostate development have not yet been done.

5.2.2 Critical time windows in TCDD-induced male reproductive toxicity and hydronephrosis.

The available data indicate that the male reproductive system is most sensitive to *in utero* and lactational TCDD exposure. For determination of the vulnerable events in organogenesis it is still important to determine the critical windows of sensitivity more accurately within this period of life. However, a long elimination half-life of dioxins makes the exact determination of critical windows difficult. Some organs seem to have quite limited and clear-cut sensitive periods during their development, while others are sensitive throughout the development (Couture *et al.* 1990; Miettinen *et al.* 2002). The present results show that also in ventral, dorsolateral and anterior prostate as well as in seminal vesicle development the most vulnerable stages of development differs (**VI**).

The initial step in prostate development is the outgrowth of epithelial buds from the urogenital sinus into the surrounding mesenchyme (Cunha, 1972; Cunha *et al.* 1987). The first buds arise at different stages of development for different prostate lobes and for seminal vesicles. In mice the anterior prostate buds appear on GD15, followed by dorsolateral buds on GD16 and ventral prostate buds on GD17. The seminal vesicle rudiments develop from the caudal ends of the Wolffian ducts, and first become visible on GD14. The working hypothesis for study **VI** was that the sensitivity differences between different prostate lobes correlate with the developmental stage of bud formation during exposure. However, the results demonstrated that there was no correlation. Ventral prostate development was more inhibited by TCDD than was dorsolateral prostate development and it was affected between GD13 to GD16 before the first ventral buds were visible. On the contrary, dorsolateral prostate was most affected after GD16, when the dorsolateral buds are already developing. The regional differences in temporal/spatial AHR expression in rudiments of different organs or even in the urogenital sinus where the prostate lobes start to develop might explain the different sensitivities of the lobes. However, these expression patterns have not been studied.

A critical window for TCDD-induced hydronephrosis in C57BL/6N mice has been shown to occur on PND1 (Couture-Haws *et al.* 1991). Hydronephrosis has also been reported in H/W rat fetuses after a maternal dose of 1 or 10 µg/kg TCDD on GD8 (Huuskonen *et al.* 1994). Surprisingly, in the present study hydronephrosis was observed with high frequency and severity in line A and B rats exposed to TCDD as late as the first two weeks of life. Therefore, our data indicate that in rats, the period of sensitivity to hydronephrosis is extended postnatally until at least 17 days of age, if the dose is high enough.

Knowledge of the critical stages in TCDD toxicity is essential in exploring the primary toxic mechanisms of TCDD. The critical stages in prostate development enabled us to focus on the more sensitive time-period in mechanistic studies. Studies done after the publication of the papers on which this thesis is based have elucidated the effects of TCDD on region-

specific urogenital sinus bud formation (Lin *et al.* 2003). In mice, ventral prostate epithelial bud formation was prevented by maternal dose of 5 µg/kg TCDD on GD13. In addition, the number of dorsolateral buds was reduced, and dorsal, lateral and anterior prostate budding was delayed by approximately one day. This early effect of TCDD on bud formation was mediated through AHR. To identify the molecular mechanism, by which TCDD disrupts urogenital sinus development, the new methods termed as "-omic" technologies were applied. The gene expression profiling was done at the RNA (transcriptomics) and protein (proteomics) level. Affymetrix Microarrays were used to measure the region-specific gene expression in the mouse urogenital sinus. Urogenital sinuses were collected from GD16 male fetuses after maternal dose of 5 µg/kg TCDD on GD13 (Lin *et al.* 2002). The genes found differently expressed are involved in neural development, cell adhesion, growth factor signaling pathways as well as in cytoskeletal and extracellular matrix composition. In addition, by using the proteomics tools we have started to examine the changes in mouse urogenital sinus proteome on GD16, after a maternal dose of 5 µg/kg TCDD on GD13 (Karjanlahti *et al.* 2004).

5.2.3 Development of resistance to type II endpoint.

A previous teratogenicity study demonstrated only a slight strain difference in prenatal mortality between H/W and L-E rats. Postimplantation loss, resorptions and late fetal deaths were almost as common in H/W rats at 10 µg/kg TCDD as in L-E rats at 5 µg/kg (Huuskonen *et al.* 1994). However, we demonstrated that TCDD-resistant H/W and line A rats were fairly resistant to TCDD already few days after birth, and the full resistance was achieved in two weeks. These findings suggest that the prenatal sensitivity of these rat strains may be related to maternal and/or placental factors independent of the resistance alleles Ahr^{hw} and B^{hw} that are known to be involved in the determination of resistance of these rats postnatally. Placenta is a likely candidate, as several studies have shown that it is a target of TCDD toxicity (Chen *et al.* 2002; Hassoun *et al.* 1995, 1997; Ishimura *et al.* 2002a, b; Khera 1992).

Interestingly, the time course for resistance development in line B rats differed from that of line A and H/W rats. TCDD resistance in line B rats was attained at the age of 28 days in females and 42 days in males. This time difference indicates that the time-course of resistance development associated with Ahr^{hw} and B^{hw} alleles is different, and reflects that there are differences in pathways mediating resistance between these alleles.

At high TCDD doses, the growth and weight gain was only slightly affected by the age of an offspring during the exposure, and no specific differences between Ahr^{hw} and B^{hw} alleles could be detected. Animals dosed at younger ages did not seem to reach the body weight of the animals dosed at an older age even in 100 days, and neither line A nor line B developed full resistance to the effects of high TCDD doses on body weight. Furthermore, in accordance with the hypothesis of TCDD-triggered dose-dependent decrease in the putative

set-point for body weight (Seefeld *et al.* 1984), the difference in body weight seen shortly after exposure remained fairly constant as the rats aged.

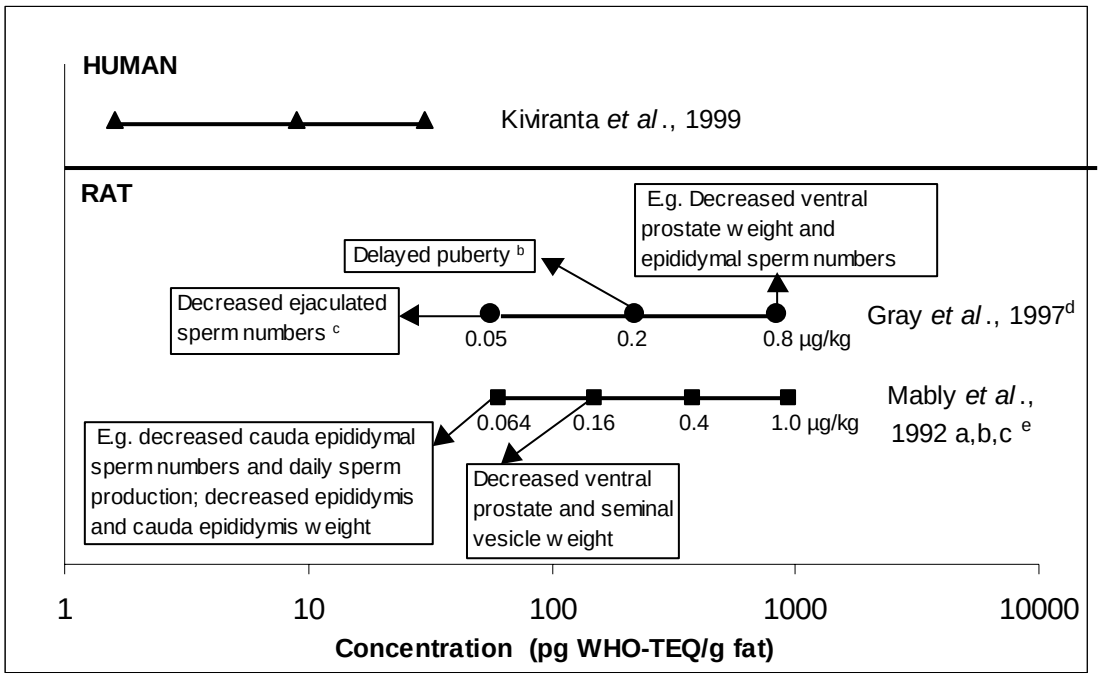
5.3 Implications for dioxin risk assessment

The TEFs are essential in human risk assessment of complex environmental mixtures of dioxins. The short-term non-lethal endpoints of toxicity (at doses of 3000 µg/kg TCDD or less) followed the normal rank order of dioxin sensitivity, and the REP values were in relatively good accordance with the current TEF values (**I**). Results of this thesis also showed that the influence of resistance alleles is endpoint dependent. Therefore, the measured endpoint, as well as the strain (or species) may significantly affect the REP derived from a single study, and may therefore affect the derived TEF estimate, especially for type II endpoints (**I-IV**). This study strongly supports the current use of the TEF concept when REPs from various studies are combined, and indicate that the observed deviations in dioxin structure-activity relationships of acute lethality are not relevant for dioxin risk assessment.

The research on the role of genetic factors in dioxin toxicity has mainly been limited to the dioxin-induced mortality and other high-dose effects, while their significance in the sensitivity to low dose effects, especially to developmental defects has been less studied. The present study demonstrated that, the resistance alleles Ahr^{hw} and B^{hw} do not significantly influence the sensitivity to TCDD-induced male reproductive organ defects after perinatal exposure, although the effects on accessory sex organs are mediated through AHR (**III, V**). However, after *in utero* and lactational TCDD exposure the extent of decrease in sperm parameters was affected by the resistance alleles. In accordance with our findings, other similarly designed studies with a variety of rat strains (and even with other rodent species) have shown that, although the sensitivity to male reproductive defects is to some extent strain specific and, therefore, affected by certain genetic factors, the sensitivity differences are still very small from the risk assessment point of view. In addition, it is apparent that the dosing protocol could have some influence on the observed adverse effects and their sensitivity. Repeated dosing for prolonged periods would more closely resemble the human dioxin exposure than does single dosing but for practical reasons the single dose protocol has been widely used. Overall, in terms of risk assessment it is important to recognize that effects do occur, and the TCDD sensitivity is only slightly affected by resistance alleles. This makes the extrapolation from experimental animal to human more straightforward.

In addition, data of this thesis confirm the earlier findings indicating that the developmental processes, especially during the fetal period, are highly sensitive to TCDD toxicity (**III-VI)**. There is variation in critical periods of certain developmental processes and, therefore, it is important to pay attention to the dosing procedure when assessing the presence and/or

absence of certain defects. Additionally, the differences observed in the critical periods of sensitivity in the development of different male accessory sex organs provide further information on the mechanism behind the defects. The critical windows for male reproductive effects in mouse begin around GD13 to GD16. The corresponding human developmental stage is 5-12 weeks. At that time the maternal tissue concentrations associated with adverse effects in experimental animals are close to the range of human background exposure to dioxin-like compounds (Fig. 5). In general, the study emphasizes the particular importance of the fetus but also nursing infants as primary concern in human risk assessment.



[a] The maternal fat concentrations in rat were estimated based on Hurst *et al*. (2000)

[b] Delayed puberty measured at the age of approximately 40 days.

[c] Decreased ejaculated sperm number measured at the age of 15 months.

[d] Pregnant Long-Evans hooded rats were treated with single dose of 0.05-0.8 µg/kg TCDD on GD15.

[e] Pregnant Holtzman rats were treated with single dose of 0.05-0.8 µg/kg TCDD on GD15.

Fig. 5 Comparison of lipid-based dioxin concentration in mother's milk determined in general population in 1993-1994 and in pregnant rat (gestation day 16) [a], as well as male reproductive defects occurring in pups born to rats exposed at the age of 63 days.

6 Conclusions

1. The exceptional sensitivity of H/W rats to higher chlorinated dioxins is limited to acute lethality.

2. There are two types of endpoints of short-term dioxin toxicity. Type I endpoints (EROD activity, thymus weight, tooth defect) are independent of genotype variation while in type II endpoints (e.g. weight loss, serum bilirubin) the efficacy is suppressed by the resistance alleles, the Ahr^{hw} allele being the most important factor. The efficacy ratio of 0.5 classifies the effects into type I and type II categories.

3. TCDD effects on male reproductive organs after adult or perinatal TCDD exposure are not modified by the resistance alleles and therefore belong to type I endpoints. However, resistance to TCDD-induced decrease in sperm parameters appears to be increased by the resistance alleles.

4. The effects of TCDD on the growth and functional development of male accessory sex organs in mice are mainly AHR-dependent.

5. The developing male reproductive system is more susceptible than the adult reproductive system to the toxic effects of TCDD.

6. The critical time window of TCDD sensitivity is prostate lobe specific. Ventral and anterior prostate growth was most affected during fetal period, while dorsolateral prostate required both fetal and postnatal exposure for maximal effect.

7. The full resistance of H/W and line A rats to mortality develops after birth, and the time-course for the resistance development differs between the resistance alleles Ahr^{hw} and B^{hw}.

8. The period of sensitivity to hydronephrosis is extended postnatally until at least 17 days of age at high doses of TCDD, and the sensitivity does not differ between the resistance alleles Ahr^{hw} and B^{hw}.

9. The results strongly support the current use of TEF concept in dioxin risk assessment. The observed deviations are not relevant for the risk assessment.

10. The study emphasizes the importance of the fetus, in particular, but also nursing infants as primary concern in human risk assessment.

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