Relationships between tissue levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), mRNAs and toxicity in the developing male Wistar(Han) rat

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ABSTRACT

We compared the effects of a single acute dose, or chronic fetal exposure, to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the male reproductive system of the Wistar(Han) rat. Tissue samples were taken from dams on GD16 and GD21, and from offspring on PND70 and 120. Steady state concentration of TCDD was demonstrated in the chronic study: body burdens were comparable in both studies. Fetal TCDD concentrations were comparable after acute and chronic exposure, and demonstrate more potent toxicity after chronic versus acute dosing. In maternal liver, cytochrome P450 (CYP)1A1 and CYP1A2 RNA were induced. In fetus, there was induction of both CYP1A1 and CYP1A2 RNA at medium and high doses, but inadequate evidence for induction at low dose in either study. The low level induction of CYP1A1 RNA at low dose in fetus argues against AhR activation in fetus as a mechanism of toxicity of TCDD in causing delay in balanopreputial separation, and the greater induction of CYP1A1 RNA in PND70 offspring liver suggests that lactational transfer of TCDD is crucial to this toxicity. These data characterise the maternal and fetal disposition of TCDD, induction of CYP1A1 RNA as a measure of AhR activation, and suggest that lactational transfer of TCDD determines the difference in delay in balanopreputial separation between the two studies.

Keywords: Dioxin, Sperm, developmental, toxicity, balanopreputial separation, puberty.

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous toxin, and a prototypical representative of a series of chemicals which effect toxicity through a common mechanism, binding to the Ah Receptor (Poland and Knutson 1982). Much investigation has focussed on the toxicity of TCDD, on the basis that other chemical congeners will show the same toxicity as TCDD, but with altered potency determined by their relative agonism of the Ah Receptor, and pharmacokinetics (Haws *et al.* 2006; Van den Berg *et al.* 2006). We have investigated the effects of TCDD on the developing fetus after acute (Bell *et al.* 2007a) or chronic (Bell *et al.* 2007b) exposure, and have shown that maternal exposure to TCDD causes a delay in BPS in male offspring, which is more pronounced after chronic (vs. acute) exposure to TCDD. However, understanding of such data on the basis of administered dose is limited, and elucidation of the tissue concentration and the biological effects caused by that concentration of TCDD is required for understanding and extrapolation from these data.

In many studies, TCDD is given as a single acute dose, whereas exposure of the human population to TCDD arises from chronic exposure through the diet and from lactational transfer (COT 2001)(Fries 1995). The pharmacokinetics of TCDD are complex with multiple uptake and elimination phases (Weber *et al.* 1993), and thus the disposition of TCDD at 24 hours after a single dose on GD15 is likely to vary from steady state. There is induction of metabolism of TCDD during chronic exposure (Fries and Marrow 1975), and it takes ~13 weeks to attain steady state levels of tissue TCDD in the rat (Rose *et al.* 1976). This is important to understanding reported effects of TCDD that have a narrow temporal window of susceptibility (Ohsako *et al.* 2002), between GD15 and GD18. Indeed, there are clear differences in disposition at GD16 between acute and chronic dosing regimes that give similar concentration in liver tissue (Hurst *et al.* 2000a; Hurst *et al.* 2000b). Thus it is possible that the dosing regimen (*i.e*. acute versus chronic dosing protocol) may be a determinant of sensitivity for the toxic effects of TCDD.

We have directly compared the developmental toxicity of TCDD after chronic administration(Bell *et al.* 2007b)with acute administration of a single dose on Gestational Day 15 (GD15) (Bell *et al.* 2007a): surprisingly chronic administration yielded delay in balanopreputial separation (BPS) at all three dose groups, whereas only the highest dose group

yielded a delay in BPS in the acute dose study ; this finding was unexpected, since the chronic dosing study was designed to give similar body burdens of TCDD to those seen in the acute dose study. This experimental system is complicated by the post-parturition delivery of TCDD via milk (Korte *et al.* 1990; Moore *et al.* 1976; Nau *et al.* 1986). The majority of TCDD in offspring of dosed dams arises from lactational transfer of TCDD (Hurst *et al.* 2000a; Li *et al.* 1995), and there is evidence that chronic dosing protocols can result in higher TCDD delivery to the offspring (Korte *et al.* 1992). Indeed, it has been shown that lactational transfer of TCDD is responsible for the thyroid toxicity seen in Holtzmann rats (Nishimura *et al.* 2003; Nishimura *et al.* 2005; Nishimura *et al.* 2006). There are multiple explanations for the greater potency of TCDD for inducing delay in BPS by chronic (as opposed to acute) dosing, and these include (1) that there is a higher concentration of TCDD in the fetus after chronic versus acute dosing (2) that there is a window of sensitivity of the fetus to TCDD's effect before GD15 and (3) that there is greater lactational transfer of TCDD to the pups after lactational dosing, and that the lactational transfer of TCDD results in the delay in BPS. It is therefore important to measure the concentration of TCDD in relevant organs, and to determine the biological effects of TCDD using a suitable marker gene, such as CYP1A1 (Vandenheuvel *et al.* 1994).

In a direct comparison of the effects of acute and chronic administration of TCDD (Bell *et al.* 2007a, 2007b), we have shown that a single acute dose of TCDD to the pregnant CRL:WI(Han) rat on GD15 causes a delay in BPS in offspring after a dose of 1000 ng TCDD kg^{-1}, whereas chronic administration of 2.4 ng TCDD kg^{-1} day^{-1} yielded a significant delay in puberty in offspring. In order to relate the administered dose of TCDD with the retained tissue levels of TCDD, and consequently the biological effects seen in these studies, we have undertaken analysis of rat tissues for concentration of TCDD. RT-PCR analysis was undertaken as a sensitive means of measuring biological effects of TCDD exposure, *i.e.* activation of the Ah Receptor, by looking at induction of sensitive marker genes. The CYP1A1 gene has been shown to be highly responsive to TCDD (Vandenheuvel *et al.* 1994), and the CYP1A2 gene was examined because of its inducibility by TCDD and its role in hepatic sequestration of TCDD (Poland *et al.* 1989a; Poland *et al.* 1989b).

MATERIALS AND METHODS

Materials.

TCDD was obtained from Cambridge Isotope laboratories, Mass, USA, and purity (99%) was verified by High Resolution-Mass Spectrometry. All other chemicals were also of the highest quality available and were checked for contamination by dioxins and PCBs as appropriate.

Animal Study.

The animal studies were performed at Covance (Harrogate, UK), and were GLP-compliant; the full dataset is published in (Bell *et al.* 2007a, 2007b). CRL:WI(Han) rats were provided food (SQC rat and mouse breeder diet No. 3, expanded; Special Diets Services Ltd., Witham) and water *ad libitum*, and were housed singly (the parental generation post-pairing), or in groups of five for the parental generation pre-pairing and the F_1 generation. Briefly, the dosing schedules are shown schematically in Fig. 1, and were as follows.

Acute. Animals of 16-18 weeks of age (204-294g) were time mated, with the day after mating designated as day 0 of gestation (GD0), delivered to Covance by GD 9, and assigned to treatment groups on GD 12 using a randomisation procedure based on body weight. 75 animals were treated with control vehicle (corn oil) by oral gavage, and 55 animals with 50, 200 and 1000 ng TCDD kg^{-1} bodyweight on GD15; the concentration of TCDD in the dosing vehicle was verified by GC-MS (104.6-106.1% of target concentration). 25 vehicle-treated rats and 15 TCDD-treated rats were killed on GD16 and GD21 for tissue sampling prior to TCDD analysis and mRNA analysis; the remaining females were allowed to litter and rear their offspring until weaning (PND21). For the control animals, tissues were pooled from five animals, and five samples of pooled tissue were analysed. In the TCDD-treated groups, blood samples were pooled from five animals, and three pools analysed; adipose and liver tissue from five individual animals were analysed, except for the high dose group fat on GD16, where six samples were analysed. Fetuses from five individual animals were pooled and analysed. Where possible, blood, fat, fetus and liver were analysed from the same animal; animals were selected randomly for sampling.

Chronic. Animals of 5-6 weeks of age (100-146g) were assigned to treatment groups using a randomisation procedure based on body weight. Animals (75, 65, 65 and 65 respectively) were provided with diet containing 0, 28, 93 and 530 ng TCDD kg^{-1} diet (the TCDD was dissolved in acetone, mixed with the feed, and then acetone evaporated by air drying) ad libitum. After 12 weeks of treatment of the parental females, one female was housed with one untreated male for up to 15 days, and mating confirmed by a vaginal plug. The concentration of TCDD in the diet was verified by GC-MS. 5 and 10 animals per group were killed in weeks 10 and12 after starting on the diet, and on gestation day 16 and 21, 15 animals from the control group and 10 animals from the treated group were killed; tissue samples from these culls were used for TCDD and RNA analysis. TCDD treatment of the dams (and offspring) was discontinued after parturition. The remaining females were allowed to litter and rear their offspring until weaning (PND21) and killed on PND21. Litters were reduced to a maximum size of eight on PND4, and to five males (where possible) on PND21. For the control and treated animals, adipose, fetus and liver tissues were obtained from five individual animals, and individual tissues were analysed; the exceptions were analysis of six samples in medium dose group, GD21 adipose, and GD16 liver, and GD21 high dose group, for fetus and fat. Blood samples were pooled from five animals, and three pools analysed for all dose groups. Fetuses from five individual animals were pooled and analysed.

Thereafter, males were then maintained untreated, until killed (25 per group) at PND70, and all remaining animals at PND120. Although kill days are referred to as PND70 and 120, the number of animals involved required that the kills were conducted during post-natal weeks 10 and 17.

TCDD analysis.

Tissue TCDD levels were determined on GD16 and 21, since there was a prior expectation that GD16 would reflect a period of sensitivity of the fetus to TCDD (Gray *et al.* 1995; Ohsako *et al.* 2002), and GD21 would reflect on accumulation of TCDD in the fetus through pregnancy. The maternal liver and adipose tissue were sampled, as TCDD is known to accumulate in these two organs, and fetal tissue was taken as the presumed site of action of the TCDD. Samples were stored frozen until analysed. Adipose tissue and liver samples were analysed individually and fetus samples from individual females were combined, but the volumes of blood samples

were too low for individual analysis, and were pooled. The tissue samples were homogenised (pulping for fetus or liver, chopping for adipose), and an aliquot taken for analysis. Sample aliquots were fortified with ^{13}Carbon labeled dioxins, and exhaustively extracted using mixed solvents. The extracts were initially purified by acid hydrolysis, fractionated on activated carbon and further purified using adsorption chromatography, on alumina. The eluent was concentrated under nitrogen and sensitivity standardised for measurement using additional ^{13}Carbon labeled dioxins. TCDD was measured using high resolution gas chromatography with high resolution mass spectrometric detection at a resolution of ~10000 (defined at 10% of peak height). Instrument performance was monitored during the measurement interval by the use of a calibrant (perfluorokerosene) lock mass and ions corresponding to native and [^{13}C]-labelled dioxins were recorded. Data was processed using Masslynx^{TM} and Microsoft Excel software to provide tissue concentration data. The analytical data met published acceptance criteria (Ambidge *et al.* 1990) for dioxins. The method used is accredited to the ISO17025 standard and has been validated and published after peer review (Fernandes *et al.* 2004). Each batch of samples analysed incorporated a full reagent blank, and analytical results were validated by the analysis of an in-batch reference material (RM) (Maier *et al.* 1995), for which results were compared with certified or assigned data. The contribution from the batch blanks was found to be negligible and quality standards were always met or exceeded.

Essentially the same procedure was used for total polychlorinated dibenzo-p-dioxin and furan World Health Organisation TCDD toxic equivalent (WHO-TEQ) (Van den Berg *et al.* 1998) measurements where these were performed. The ^{13}Carbon labelled mix described above provides internal standardisation for all 17 laterally-substituted PCDDs and PCDFs that contribute to the WHO-TEQ (Van den Berg *et al.* 1998) and ions corresponding to these compounds as well as native PCDDs and PCDFs were recorded and measured using the same instrument parameters as used for TCDD measurement. WHO-TEQ values were calculated by multiplying the measured concentration of each of the 17 compounds by the appropriate WHO TEF (Van den Berg *et al.* 1998) and summed to give S WHO-TEQ .The methodology, including quality criteria is described in (Fernandes *et al.* 2004).

The tissue TCDD concentration from one sample (Acute study GD16 fat from animal 200, high dose group) had a TCDD concentration at ~1% of the value of other adipose tissues from the group, and was held to be anomalous. A further determination was performed on adipose tissue from animal 199, and this data was used instead. Four samples were re-checked; for liver and fetus, duplicate determinations were within 5% of the original value. For adipose tissue, where the sample is chopped, duplicates were 115 and 130% of the first determinations.

Calculation of tissue TCDD burden. The average tissue weights (for liver, fetus) from the appropriate kill point were determined by weighing, and adipose tissue was estimated from the data of (Bailey *et al.* 1980). The average weights were used to determine the mean tissue burden, and the standard deviation is shown as the corresponding standard deviation from the TCDD determination. The whole body burden is calculated from summation of the adipose, liver and fetus TCDD burden, and an indicative estimate of variation, which is the mean multiplied by the the coefficient of variation of the TCDD analyses in the acute (36%) and chronic (27%) studies, respectively, is shown. The apparent half-life of TCDD in the chronic study was calculated on the basis that animals were at steady-state body burden of TCDD; under this assumption, TCDD excretion equals the rate of TCDD consumption, which is known. Apparent half-life is then deduced by solving $C_t=C_0e^{-kt}$. The proportion of TCDD dose disposing to tissues was calculated as follows. The average TCDD concentration in a given tissue at high dose was set as 100%, and the average concentration at low and medium doses was expressed as a percentage of the high dose concentration. The medium and low doses were expressed as a percentage of the maximum dose (*i.e.* 5 and 20% for the acute study, 5.2 and 17.3% for the chronic study). The tissue concentration (as a percentage of maximum concentration) was divided by the dose (as a percentage of maximum dose) to give a ratio, which represents the relative proportion of TCDD dose disposing to a given tissue with dose.

RNA and RT-PCR analysis. Total RNA was extracted from liver or fetus using the Absolutely RNA Miniprep Kit (Stratagene). 20-25mg of frozen tissue was transferred to 0.4ml of lysis buffer and homogenized at room temperature using a mini-homogenizer (Kontes Pellet Pestle). The resulting homogenates were stored at -80°C, prior to isolation and treatment with DNase I according to manufacturer's instruction. The purified RNA was stored at -80°C. The quality of the RNA was assessed by electrophoresis on 1% denaturing agarose gel based on the integrity

of the 28S and 18S bands after ethidium bromide staining. Total RNA was determined by measurement of a fluorescent RNA-binding probe, RiboGreen (Molecular Probes) according to the manufacturer's instructions. The RiboGreen assay was carried out using a ribosomal RNA (rRNA from E. coli) standard curve. Fluorescence was measured using excitation at 485 nm and emission at 510 nm. First-strand cDNA was synthesised using StrataScript QPCR cDNA Synthesis Kit (Stratagene) according to the manufacturer's instruction. 2 μg of total RNA was reverse-transcribed using oligo (dT) and random primersand the resulting products were stored at -20°C. For each batch of cDNA preparation, a no-RT control reaction was set up by omitting reverse transcriptase from the reaction. The concentration of the synthesised cDNA was determined by the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Inc.) according to manufacturer's protocol, using a plate reader (Victor) with excitation at 485 nm and emission at 510 nm. The cDNA sequences of the rat CYP1A1, CYP1A2, AhR, ß-ACTIN and GAPDH were obtained from GeneBank, and the corresponding primers are listed in Table 1. To avoid amplification of contaminating genomic DNA, one of the primers or the probe was placed at the junction between two exons. For example, CYP1A1 forward crosses the junction of exons 4 and 5; and the probe crosses exon 5 and 6, whereas reverse primer is from exon 6. TaqMan probes were dual labelled with fluorescent tag at the 5'end and fluorescence quencher Black-Hole at the 3' end. The specificity of CYP1A1 primers was confirmed by sequencing its RT-PCR amplicon. Real-time PCR (qPCR) was performed using Mx3005P instrument (Stratagene). Initial characterisation showed little or no primer-dimer or genomic amplification were detected and all amplicons were at the size expected. Multiplex real-time PCR was performed using Brilliant Multiplex QPCR Master Mix (Stratagene) according to manufacturer's protocol with slight modification. Briefly, multiplex PCRs were set up in a total volume of 12.5 μl of buffer solution containing the following: 6.25 μl of 2x master mix; 100-300nM of starter probes; 100-300nM of each 5' and 3' of starter primer pairs for CYP1A1, AhR and ß-actin (combination 1) or for CYP1A2 and ß-actin (combination 2); and 5ng of cDNA. The conditions for the multiplex real-time PCR reactions were: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 20 seconds and 58°C for 90 seconds. All PCR reactions were performed in duplicate. Negative controls were processed in the same manner, except that the no-RT product was used or the template was omitted. In order to determine the real-time PCR efficiencies of both targets and reference genes, a standard curve

was generated using 5-fold serial dilution of the cDNA produced from a TCDD treated rat liver. The PCR efficiencies varied from 90%-115% (data not shown). In each experiment, an independent control sample was run side by side with the samples, and the C_t was used as a calibrator to determine the relative quantification of a gene in each sample assayed, and samples were normalised to input cDNA quantity. Amplification efficiency was used to determine copy number (Pfaffl 2001):

$$\text{Change of copy number} = E^{\Delta Ct\ (\text{control-sample})}$$

Where E is the pre-determined PCR efficiency for each transcript; ΔCt (control-sample) is the C_t of the external control transcript minus sample. All data were presented as percent of the control group.

Statistical analysis.

TCDD concentrations in tissues were summarised as mean ± standard deviation. Differences in TCDD concentrations between dose groups were analysed by ANOVA on the log scale. The RNA levels were compared by ANOVA of the logs of the values, followed by t-test to compare means with a common control; $P<0.05$ was considered significant. Because of the small numbers of samples, no allowance was made for litter differences.

RESULTS

All dioxin analysis was performed with negative control (reagent blanks) and positive control (certified reference materials) samples; all of which were within acceptable limits. Limits of detection varied between analyses and analytes, but were approximately 0.03 ng TCDD kg^{-1} of analyte, depending on available sample weight and the levels of TCDD observed in reagent blanks. Measurement uncertainty was determined at an analyte concentration of 1 ng kg^{-1} to be approximately 20%. The experimental schedule for the acute and chronic dosing protocols is outlined in Fig. 1.

Acute study

TCDD was adminstered as a single dose on GD15, and the concentration of TCDD at GD16 and 21 in maternal and fetal tissues is shown in Table 3. The coefficient of variation in these samples was 36 % of mean values (Table 3); the variation seen in these biological samples was consistently higher than that seen with reference samples (data not shown). However, upon repeated assay of liver or fetus samples, the reproducibility was within 10% (AF, SW, MR, unpublished data), suggesting that the observed variability is due to inter-animal differences in pharmacokinetics of TCDD. The TCDD analytical method was sufficiently sensitive to accurately quantify TCDD levels in control tissues (Table 3). The concentration of TCDD in control tissues was above the limit of quantitation, and was at least forty times below the levels in the corresponding tissue from the lowest dose group, 50 ng TCDD kg^{-1}. ANOVA demonstrated that there was a statistically significant difference between dose groups in the level of TCDD for all tissues (data not shown).

GD16

Adipose tissue and liver showed higher concentrations of TCDD than fetus or blood, and although the ratio of TCDD concentrations in liver: adipose tissue was approximately equal in control animals, the relative concentration of TCDD in liver increased with increasing dose (Table 3). Estimation of the total body burden of TCDD as the sum of adipose, liver and fetus (Table 5), shows that ~38.3, 43.8 and 42.6% of the administered TCDD has been taken up into these organs at low, medium and high dose, respectively.

GD21

At GD21, total body burden of TCDD was 39.5, 33.2 and 37.5% of the administered dose, respectively (Table 5). Compared with GD16 data, liver concentrations had reduced, and adipose tissue concentrations had doubled (Table 3). Although fetal concentrations of TCDD had remained at ~90% of the levels on GD16, the increase in fetal mass resulted in fetal TCDD burden increasing by ~eight-fold between GD16 and 21.

Chronic study

The coefficient of variation for TCDD determinations on tissue samples in this study was 27%. TCDD concentrations in control animal tissues were comparable with those seen in the acute study (Table 4), and were substantially less than those in the lowest dose group; however some blood and fetus samples showed marginally higher levels or limits of detection due to the prevailing measurement conditions such as the weight of available sample and the concurrent solvent blanks. In order to be certain that control tissue levels of dioxin and dioxin-like compounds were below the level of the TCDD-treated groups, the levels of TCDD toxic equivalents (TEQ) (Van den Berg *et al.* 1998) were determined (Table 2). These data demonstrate that the level of TEQ in the control rat liver and adipose tissue is orders of magnitude below the levels of TCDD seen in the lowest dose group. The chronic study dose levels were designed to give approximately the same hepatic level of TCDD as seen in the acute study; the hepatic levels of TCDD in the chronic study were bracketed by the levels of hepatic TCDD at GD16 and 21 in the acute study (Table 3). The chronic dosing study was designed to achieve steady-state burdens of TCDD with a 12 week feeding period, before mating of the animals. Comparison of tissue concentrations of TCDD in animals in week 10, 12 and GD16 and 21, shows that both adipose tissue and liver TCDD concentrations were at equilibrium (Table 4); however, body weight increases over this time period, and since the body fat was calculated on a metric where fat increases with body weight (Bailey *et al.* 1980), this resulted in a higher total body burden of TCDD, expressed both as an amount, and in ng kg^{-1} (Table 5). ANOVA demonstrated that there was a statistically significant difference between dose groups in the level of TCDD for all tissues (data not shown), with the exception of GD16 blood, where the difference between low and medium dose groups fell short of statistical significance.

There was a reduction in liver mass of ~14% between GD16 and 21 ((Bell *et al.* 2007b)) whilst hepatic TCDD concentration remained unchanged (Table 4); thus redistribution of the hepatic TCDD as a result of the decrease in liver mass accounted for ~5% of total body burden of TCDD. The fetal TCDD concentrations had ~doubled by comparison to GD16, and with increased fetal mass, the total burden of TCDD in the fetal compartment had risen by ~20-fold compared to GD16 (Table 4).

Fetal disposition of TCDD and relationship with BPS

The ratio of fetal TCDD concentration to maternal body concentration was plotted against maternal body concentration for both acute and chronic studies (Fig. 2A). At GD16, this ratio was two-three fold lower after chronic administration of TCDD, compared with acute administration. At GD21, the rising concentration of fetal TCDD had brought the ratios closer together, although the acute ratios were still up to 50% higher than in the chronic study. These data showed that acute dosing leads to a higher fetal TCDD at earlier stages of pregnancy than chronic dosing, and confirms prior observations in Long-Evans rats (Aylward *et al.* 2005; Hurst *et al.* 2000a; Hurst *et al.* 2000b). Fetal TCDD concentrations from the acute and chronic studies were plotted against the delay in puberty (day of balanopreputial separation) in each study (Bell *et al.* 2007a, 2007b) (Fig. 2). Although each individual study supported a quasi-linear relationship between delay in BPS and fetal TCDD concentration, the comparison of the two studies showed that there was no overall relationship between fetal TCDD concentration and delay in BPS, at either GD16 or GD21. These data are evidence that fetal TCDD concentration at GD16 to 21 was not related to delay in BPS. Although the delay in puberty (BPS) for individual animals did not show any relationship with body weight at the PND21 or 42 (Bell *et al.* 2007a, 2007b), a plot of delay in BPS (presented as the hazards ratio) against group reduction in weight on PND4 (Fig. 2C) showed that there was an r^2 of ~0.9 between delay in BPS and weight reduction relative to the control group on PND4.

RT-PCR analysis

RT-PCR was established with primers and an internal fluorescent probe, the Taqman methodology, for enhanced specificity (Table 1); the primer probe sets were validated to be resistant to contamination by genomic DNA (data not shown). Assays were run in the presence

of a no template control, which routinely produced C_t values of >40, demonstrating an absence of contaminating cDNA or cross-reacting genomic DNA.

Fig. 3 shows the analysis of β-actin, AhR, CYP1A2 and CYP1A1 RNA from maternal liver at GD16 and 21 from the two studies: CYP1A1 and CYP1A2 are graphed as a function of hepatic TCDD concentration. β-actin RNA was relatively constant with treatment, although the treated samples from the acute GD21 series showed a minor deviation from control, with values from 1.5 to 1.9-fold of control (Fig. 3A), and these values showed no dose-related statistically significant differences from control by ANOVA. The rest of the TCDD-treated samples show β-actin RNA values close to 100% of control. The AhR RNA was not significantly perturbed by acute or chronic treatment with TCDD under the conditions which we assayed (Fig. 3B). Fig. 3C shows that there was statistically significant induction of CYP1A2 RNA and that, in the acute study, the GD16 values for induction of CYP1A2 were lower than the values for GD21, in spite of the higher TCDD concentrations at GD16; whereas in the chronic study, the CYP1A2 values for GD16 and GD21 were much closer, and the TCDD concentrations were similar. The threshold-doses for induction of CYP1A2 were distinct in the two studies, with low dose (50 ng kg^{-1}) giving a ~3-fold induction on GD21 in the acute study, but high dose (46 ng kg^{-1} day^{-1}) required for a ~4-fold induction in the chronic dosing study, *i.e.* a liver concentration of TCDD that is ~30-fold higher to achieve similar induction of CYP1A2. CYP1A1 was a much more sensitive indicator of treatment with TCDD, with statistically significant induction, and the lowest exposure groups giving >10-fold induction of CYP1A1 (Fig. 3D), at ~ 100-300 ng TCDD kg^{-1} liver. In the acute study, the CYP1A1 levels at GD16 were consistently lower than GD21 values, in spite of the hepatic TCDD concentrations being 3-5 fold higher at GD16 than at GD21. There was a similar trend to lower CYP1A1 RNA at GD16 than GD21 in the chronic study, but hepatic TCDD concentrations were approximately equal. Given that TCDD was administered on GD15, it is likely that the GD16 values in the acute study did not represent an organ at equilibrium in the TCDD induction response, but the comparison between GD16 and 21 values in the chronic study should have been at equilibrium. However, it is clear from considering liver weights that the liver was enlarged at GD16 (Bell *et al.* 2007a, 2007b; Weitman *et al.* 1986), but that the liver weight has decreased

by ~15% on GD21; thus it is likely that the physiological functionality of the liver may be different at these two times.

Fetal RNA analysis used samples of RNA from total fetus at GD16, but fetal liver from GD21; note that TCDD analysis was on fetus at both timepoints. Fig. 4A and B shows that β-actin RNA and AhR RNA stayed relatively constant with no statistically significant effect of TCDD dosing, under both dosing regimes. However, while the GD16 total fetus samples showed no effect of TCDD on CYP1A2, the GD21 fetal liver samples showed a strong induction effect, with >20-fold induction of CYP1A2 at medium dose, rising to >1000 fold induction at high dose; thus CYP1A2 was more inducible in fetal liver (compared to maternal liver), and there was little difference between chronic and acute dosing schedules. The CYP1A1 RNA also showed statistically significant and high inducibility, with total fetus showing >100-fold induction on GD16 in acute and chronic studies (tissue concentrations of 45 and 27 ng TCDD kg^{-1}, respectively). At GD21 in fetal liver, high levels of induction of CYP1A1 were detectable at the medium dose level, and the high dose induction was >1000 fold. However, induction of CYP1A1 RNA in fetal liver was not statistically significant at low dose level after either chronic or acute administration of TCDD (but note that n is small). There was clear evidence for induction of CYP1A1 RNA in fetus at the medium dose in both acute and chronic studies, showing that TCDD had activated the AhR signalling pathway in the fetus at these higher dose levels.

Fig. 4E compares the induction of CYP1A1 RNA in maternal liver and fetal tissue, as a function of tissue TCDD concentration; this shows that fetal tissue had reached comparable levels of fold-induction as maternal liver, at lower concentrations of TCDD in the tissue.

RT-PCR analysis of RNAs in the PND70 rats showed that β-actin RNA (Fig. 5A), and AhR RNA (Fig. 5B), were not statistically significantly perturbed by maternal treatment. By contrast, CYP1A1 RNA levels were statistically significantly induced in medium and high dose groups from the acute study, but at all three treatment groups in the chronic study; morever, the fold-induction of CYP1A1 RNA was higher in the chronic study, compared to the acute study, for all three dose groups (Fig. 5C).

DISCUSSION

Analysis of TCDD concentrations using high-resolution Mass Spectrometry, with internal isotope-labelled standards, brings high specificity, sensitivity and quality control methods to the determination of TCDD concentrations. This method enables detection of TCDD in samples from control animals, and thereby allows comparison of the TCDD burden in control and in treated animals, thereby showing that there were at least forty-fold higher concentrations of TCDD in the adipose or liver of the lowest dose group of the treated animals than in the controls (Table 3, Table 4). Moreover, we undertook a direct determination of the WHO-TEQ burden, and Table 2 also shows that the levels of WHO-TEQ in the control animals from the chronic study are considerably lower than the TCDD levels in the lowest dose group. This clear separation of TCDD concentration in control, versus treated, tissues, is reflected in the marked induction of hepatic CYP1A1 RNA in the low dose groups, compared to control (Fig. 3), at GD16 or 21, in both studies. This shows that a biological effect of TCDD, which is dependent upon activation of the AhR by ligand, is orders of magnitude above comparable levels in control animals in the low dose groups.

The chronic dosing study (Bell *et al.* 2007b) was designed by extrapolation from (Hurst *et al.* 2000a; Hurst *et al.* 2000b) to achieve comparable concentrations of hepatic TCDD to those obtained on GD16 in the acute dose study (Table 3)(Bell *et al.* 2007a). Table 4 shows that the concentrations of hepatic TCDD obtained are approximately 50% of those obtained in the acute study, and cover a ~ten-fold range in total body burden of TCDD (Table 5). Thus the dosing regimen yielded an appropriate range of tissue concentrations of TCDD for comparability to the acute study. An important criterion for the chronic dosing regimen was that the tissue concentrations of TCDD should be at equilibrium, and the dosing period of twelve weeks prior to mating was based on (Rose *et al.* 1976). Table 4 shows that the concentration of TCDD in the liver and adipose tissue does not change appreciably from week 10 of dosing through GD21 (*i.e.* ~five weeks later), thus demonstrating that the tissue concentrations are at steady state. However, there are differences in total body burden of TCDD over this period, whether expressed as an amount, or as a concentration (Table 5). This is due to the increase in body mass and in relative organ mass of the animals over this time, and due to pregnancy; a limitation of our study is that the estimates for adipose tissue mass (Bailey

et al. 1980) were based on data from Sprague-Dawley rats, and may not accurately represent the adipose depot in pregnant CRL:WI(Han) rats.

There are complications arising from the physiological consequences of pregnancy; for example, the food intake of pregnant rats increases by ~20%, resulting in a greater dose of TCDD when expressed on a ng kg^{-1} basis (Bell *et al.* 2007a, 2007b). A further issue is that the liver mass decreased by ~14% between GD16 and 21 (Bell *et al.* 2007a, 2007b; Weitman *et al.* 1986); the hepatic TCDD concentration remains unchanged during this period (Table 4), so this must result in ~5% of the total body burden of TCDD redistributing during this period. There is some evidence for altered hepatic functionality between GD16 and GD21, insofar as GD21 CYP1A1 levels are consistently higher than GD16 levels (Fig. 3D), even though TCDD concentrations are approximately equal; it is not clear what the basis of this effect is.

Disposition of TCDD into the fetus shows dose-dependency, with a greater proportion of the dose reaching the fetus at lower doses of TCDD. It has been shown that both AhR and CYP1A2 null mice show enhanced disposition of TCDD to the fetus (Dragin *et al.* 2006; Thomae *et al.* 2004), showing that this phenomenon is dependent on both AhR and also the CYP1A2 gene. Fig. 2A shows that the dosing regimen is a variable; after acute dosing with TCDD, the ratio of TCDD concentration in fetus: dam remains constant between GD16 and 21. However, this is a different pattern from the chronic dosing regimen, where GD16 ratios are ~half the level at GD21. Acute and chronic dosing regimens thus result in dose- and time-dependent differences in the proportion of TCDD dose reaching the fetus. The absolute concentration of TCDD in the fetus doubled between GD16 and 21 in the chronic study (Table 4), whilst staying constant in the acute study (Table 3); this is particularly notable since fetal mass increases by ~ten-fold during this period, and suggests that there is disposition of TCDD from lipid reserves into the fetus during this period of fetal growth.

The absolute fetal concentrations of TCDD in treated animals are >30-fold below the corresponding level in maternal liver, but are twenty-fold greater than the concentration of TCDD in control fetus. CYP1A1 and CYP1A2 RNA are not significantly induced at low dose in either study, but this is not due to fetal tissue being refractory to induction of CYP1A/2 RNA; a plot of CYP1A1 RNA versus tissue TCDD concentration shows that equivalent fold

induction of CYP1A1 RNA is obtained at approximately thirty-fold lower TCDD concentrations in fetus/ fetal liver, as compared to maternal liver (Fig. 4E). The higher apparent potency of TCDD in fetus/ fetal liver, as compared to adult liver, may be due to a lower proportion of free TCDD in adult liver, as a consequence of greater biochemical sequestration of TCDD by CYP1A2 in adult liver (Poland *et al.* 1989a; Poland *et al.* 1989b); the absolute levels of CYP1A2 RNA are ~10^4 lower in fetal liver than in maternal liver (Fig. 4, supplementary data). (Vandenheuvel *et al.* 1994) have shown that a hepatic concentration of 2.5-21 ng TCDD kg^{-1} represents the limit of detection for induction of CYP1A1 RNA, consistent with our data, and (Kawakami *et al.* 2006) have found a similar threshhold for tissue TCDD concentration to induce CYP1A1 RNA in fetal liver on GD20. Both CYP1A1 and CYP1A2 are highly inducible (~ 10^3-fold) in fetal liver, whereas CYP1A2 shows much lower induction (ten-fold) in maternal liver; this could be due to the lower basal levels of CYP1A2 in fetal, as compared to maternal, liver. Whilst fetal TCDD concentrations are sufficient to activate AhR-mediated gene transcription of CYP1A1 and CYP1A2 in total fetus (GD16) or fetal liver (GD21) at medium and high doses, the evidence for AhR-mediated gene activation (CYP1A1/2) at low dose does not attain statistical significance; moreover, given that the acute study only showed toxicity (lethality, delay in BPS) at high dose, whereas the chronic study led to delay in puberty in all three dose groups, the pattern of AhR mediated gene activation in fetus or fetal liver at GD16 and GD21 fails to correlate with these endpoints. The induction of CYP1A1 is regarded as one of the most sensitive endpoints of AhR activation (Vandenheuvel *et al.* 1994), and so the failure to detect significant and marked induction of CYP1A1 RNA in fetus at low dose levels argues against a toxic effect of TCDD mediated by AhR either between GD16 and GD21, or before GD16; however, it is not possible to exclude the possibilies either that there is a tissue within the fetus which accumulates TCDD to higher levels, or that there is an AhR responsive gene which is more sensitive to TCDD levels than CYP1A1, and which mediates toxicity.

Acute administration of TCDD on GD15 has much less effect on delay of BPS than chronic administration of TCDD (Bell *et al.* 2007a, 2007b) at doses that yield comparable concentrations of TCDD either in the fetus (Table 3, Table 4), or in total maternal body burden (Table 5), and this finding demonstrates that TCDD is a more potent developmental toxin after

chronic, as opposed to acute, administration. A plot of fetal TCDD against delay in puberty seen in the acute or chronic studies fails to correlate simply at either GD16 or 21, *e.g.* Fig. 2B. This finding shows that the greater potency of TCDD after chronic administration is not due to enhanced disposition of TCDD to the fetus between GD15-21, and suggests that if the offspring have a window of enhanced susceptibility to TCDD, it is either prior to GD15 or after GD21. The fact that fetal CYP1A1 RNA is barely perturbed at low dose in fetus in both studies (Fig. 4) strongly suggests that the AhR has not been sufficiently activated to mediate a physiologically relevant response at GD16-21. However, a plot of relative decrease in bodyweight on PND4 versus delay in BPS (Fig. 2C) shows a correlation (r^2~0.9) between these two variables. It is possible that the decrease in bodyweight, and subsequent delay in BPS, is due to lactational transfer of comparatively large amounts of TCDD. The disposition of TCDD from the diet into breast tissue (and milk) may not be accurately reflected in the abdominal adipose depot concentrations of TCDD, since dietary fats (and presumably, dietary TCDD) can be directly taken up by the mammary gland (Neville and Picciano 1997); thus when comparing the dose of TCDD administered by lactation between the chronic and acute studies, this may not be simply reflected in the liver and abdominal adipose depot concentrations. This is important, since lactational transfer of TCDD accounts for the majority of pup TCDD after an acute dose of TCDD on GD18 (Li *et al.* 1995) or GD15 (Nishimura *et al.* 2005), and a chronic dosing regimen can achieve high concentrations of TCDD in the offspring (Hurst *et al.* 2000a; Korte *et al.* 1992). Further, TCDD causes hypothyroidism and hydronephrosis in F_1 rats via lactational transfer of TCDD (Nishimura *et al.* 2003; Nishimura *et al.* 2005; Nishimura *et al.* 2006). Thus it is not clear whether acute and chronic TCDD dosing regimens result in a different lactational delivery of TCDD to the offspring.

To examine the possibility that lactational transfer of TCDD to the pups was different under chronic and acute dosing regimens, we examined the levels of CYP1A1 in liver RNA of rats at PND70 (Fig. 5). This experimental design is inherently limited, since the pups were principally dosed through lactation, which finished on PND21; the time from PND21 to PND70 is three-fold the apparent whole body half life of TCDD in the dams, without considering the dilution of TCDD caused by the ~900% growth in body mass between PND21 and 70. Hence TCDD concentrations would be expected to be considerably lower than at puberty, and the

comparison between groups would be complicated by any dose-dependent decrease in half-life. There was no reliable correlation between day of puberty and CYP1A1 RNA abundance in either the acute or chronic study (data not shown). However, the induction of CYP1A1 RNA was significantly elevated above control values in all three dose groups in the chronic dosing study, whereas only the highest dose group showed an elevation above control in the acute dosing study (Fig. 5C). Since the induction of CYP1A1 is both AhR- and TCDD-dependent, this suggests that the pups in the chronic dosing study received a higher dose of TCDD lactationally than the pups in the acute dosing study.

Although we have restricted our analysis in this paper principally to explaining the biology of delay in BPS, the information in Table 3 and Table 4 also provides insight into maternal pharmacokinetics of TCDD, which is determinative of the fetal effects. The Supplementary Data contains analysis of the pharmacokinetics, and shows that TCDD disposition is dependent on dose, and whether dosing is acute or chronic; that the proportion of the TCDD dose reaching extrahepatic tissues increases with decreasing dose; and that the apparent half-life of TCDD is dose-dependent.

In summary, we have characterised the concentration of TCDD and marker genes in tissue samples from acute and chronic dosing studies. Our data confirm that TCDD was adequately dosed, that the chronic study animals were at steady state, and that the tissue TCDD concentrations in the chronic study were comparable to those in the acute dosing study. Whereas maternal CYP1A1 RNA is highly induced at all dose levels, the concentration of TCDD in fetus is insufficient to induce fetal CYP1A1 at the low dose group in either study, suggesting that activation of the AhR in fetus is insufficient to account for the subsequent delay in puberty. In agreement with a role for lactational transfer of TCDD in delay in puberty, hepatic CYP1A1 RNA in PND70 males from the chronic study were at higher levels than the corresponding group from the acute study, showing that there was greater transfer of TCDD to pups during lactation in the chronic study. These results illustrate the complexity of pharmacokinetic and biological responses to TCDD, and are key to understanding, and inter-species extrapolation, of the toxicity of TCDD.

SUPPLEMENTARY DATA

Individual animal data is provided as an appendix. Analysis of maternal pharmacokinetics is analysed separately in the supplementary data.

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FIGURES

FIG. 1. Cartoon of TCDD dosing regimes.

The dosing schemes are taken from (Bell *et al.* 2007a, 2007b). In the chronic dosing protocol, the time of dosing is marked by an underline showing when the dams were dosed, whereas in the acute study protocol, the time of dosing is marked by an arrow. The cartoon is not to scale.

FIG. 2. Role of fetal TCDD concentration.

A. Dose dependency of fetal disposition in acute and chronic dosing. The fetal TCDD concentration was divided by the total maternal concentration, and plotted against maternal body concentration. Data points from the acute study are circles on a solid line, whereas the chronic study is represented by squares on a dashed line. GD16 points are in black, and GD21 data points are in white. Results are presented as mean ± SD; note that the SD is shown both for the ratios, and for TCDD concentration. B. Correlation between fetal TCDD concentration and delay in BPS. Fetal TCDD concentration at GD16 (black symbols) or GD21 (white symbols) was plotted against days of developmental delay compared to control, after dosing with TCDD by acute (circles) or chronic (squares) dosing. Values are shown as mean and SD; note that the SD is shown for both parameters. C. Delay in Balano-Preputial Separation (BPS, in days) is shown as the percentage decrease in the incidence rate of BPS (Bell *et al.* 2007a, 2007b), and plotted against the relative reduction in body weight on PND4. The relative reduction in body weight on PND 4 is derived by subtracting the natural log of the weight of the appropriate treatment group from the corresponding control group; an increasing value shows a greater weight reduction in a treated group. Acute study data points are circles, and chronic study data points are squares. Linear regression was undertaken with SigmaPlot, and the line of best fit is shown.

FIG. 3. RT-PCR analysis of maternal liver.

RT-PCR for β-actin (A), AhR (B), CYP1A2 (C) and CYP1A1 (D) was performed as described in materials and methods, on n=5 samples per group. A, B, Cand D are presented as % of control, as mean ± SD. A and B are plotted against dose group, whereas C and D are plotted against the group mean TCDD concentration, and have error bars both for RNA level,

and TCDD concentration. Acute dose samples are shown as circles, and chronic study samples are triangles; GD16 samples are shown by black symbols, and GD21 samples as white symbols.

FIG. 4. RT-PCR analysis of fetal samples.

Total fetus RNA (GD16), or fetal liver RNA (GD21), was analysed by RT-PCR for β-actin (A), AhR (B), CYP1A2 (C) and CYP1A1 (D) was performed as described in materials and methods. A, B C and D are presented as % of control. A and B are plotted against dose group, whereas C and D are plotted against the group mean TCDD concentration, and have error bars both for RNA level, and TCDD concentration. Acute dose samples are shown as circles, and chronic study samples are triangles; GD16 samples are shown by black symbols, and GD21 samples as white symbols. E. Comparison of CYP1A1 induction and tissue TCDD concentration. CYP1A1 RNA is plotted as percent of control on a log scale, and TCDD concentration was determined as described in materials and methods. Maternal values are with black symbols, whereas fetal samples are white; chronic GD21 samples are circles, and acute GD16 samples are squares. Samples are mean ± S.D.

FIG. 5. RT-PCR analysis of F1 PND70 liver samples.

Liver RNA was prepared from n=20 or 21 PND70 liver samples per group from the acute (circle) or chronic (triangle) studies, and analysed by RT-PCR for β-actin (A), AhR (B), and CYP1A1 (C), as described in materials and methods. Samples are mean ± S.D.

TABLES

TABLE 1: Sequence of oligonucleotides used in qPCR

Gene name primer	Sequence	Genbank Accession number	Label
CYP1A1		X00469	
Primer (f)	CCACAGCACCATAAGAGATACAAG		
Primer (r)	CCGGAACTCGTTTGGATCAC		
Probe	ATAGTTCCTGGTCATGGTTAACCTGCCAC		FAM-BH1
CYP1A2		K02422	
Primer (f)	ACC ATC CCC CAC AGT ACA A		
Primer (r)	GTT GAC CTG CCA CTG GTT TA		
Probe	ACATTCCCAAGGAGCGCTGCAT		CY5-BH3
AhR		Af082124	
Primer (f)	GCAGCTTATTCTGGGCTACA		
Primer (r)	CATGCCACTTTCTCCAGTCTTA		
Probe	TATCAGTTTATCCACGCCGCTGACATG		HEX-BH1
β-actin		V01217	
Primer (f)	CTGACAGGATGCAGAAGGAG		
Primer (r)	GATAGAGCCACCAATCCACA		
Probe	CAAGATCATTGCTCCTCCTGAGCG		ROX-BH2
GAPDH		Ab017801	
Primer (f)	GTATCGGACGCCTGGTTAC		
Primer (r)	ACTGGAACATGTAGACCATGTAGTT		
Probe	TTGCCATCAACGACCCCTTCA		ROX-BH2

The sequence of primers used for qPCR are shown from 5' to 3'. Forward (f) and reverse (r) primers, and probe sequence, are indicated. The genbank accession number from which these sequences are derived is indicated, and the gene name is given. GAPDH is glyceraldehyde 3-phosphate dehydrogenase. The labels for the probe are abbreviated; BHx represents Black Hole quencher 1-3; FAM iscarboxy fluorescein; CY5 is the Cy5 dye; Hex is hexachlorofluorescein; ROX is 5(6)-Carboxy-X-rhodamine.

TABLE 2: Dioxin WHO-TEQ in control CRL:WI(Han) rats

	Tissue WHO-TEQ concentration (ng kg^{-1})	
	GD16	GD21
Adipose	1.93±0.199	1.7±0.319
Fetus	0.28±0.079	0.08±0
Liver	0.44±0.106	0.43±0.081

Animals of 5-6 weeks of age were provided with diet that had been treated with acetone control vehicle (see supplementary materials) ad libitum, *i.e.* this is the control group. After 12 weeks of treatment, animals were mated and mating confirmed by a vaginal plug. 15 animals per group were killed on GD16 and GD21 (as indicated in the Table), and tissues were analysed from individual animals (n=5). Results are presented as mean±Standard Deviation.

TABLE 3
TCDD concentration in maternal tissue and fetus after a single dose on GD15

	Tissue TCDD concentration (ng kg^{-1}) [ng per tissue]			
Dose of TCDD (ng kg^{-1})	0	50	200	1000
GD16				
Adipose	0.65±0.27 [0.014±0.006]	108±44 [2.33±0.95]	338±83 [7.18±1.77]	1570±450 [34.6±9.95]
Blood	0.024±0.005	2.03±0.36	4.47±0.45	44.2±14.5
Fetus	0.04±0.02 [0.00021±0.00009]	3.3±1.2 [0.017±0.006]	8.9±1.6 [0.042±0.007]	51.4±14 [0.291±0.079]
Liver	0.66±0.38 [0.008±0.005]	256±116 [3.06±1.38]	1300±437 [15.5±5.21]	6880±2340 [87.3±29.7]
GD21				
Adipose	5.2±5.6 [0.14±0.15]	212±93 [5.52±2.43]	685±111 [17.9±2.91]	3640±1060 [93.2±27]
Blood	0.05±0.03	0.62±0.15	2.5±0.18	13.8±1.49
Fetus	0.04±0.02 [0.002±0.0009]	2.07±0.89 [0.11±0.046]	7.74±1.91 [0.36±0.089]	45.2±12.7 [2.46±0.69]
Liver	0.41±0.14 [0.004±0.001]	75.4±53.4 [0.77±0.54]	310±257 [3.38±2.81]	2400±573 [25.2±6]

Animals of 16-18 weeks of age were time mated, with the day after mating designated as day 0 of gestation (GD0). 75 animals were treated with control vehicle (corn oil) by oral gavage, and 55 animals with 50, 200 and 1000 ng TCDD kg^{-1} bodyweight on GD15; the concentration of TCDD in the dosing vehicle was verified by GC-MS (104.6-106.1% of target concentration). 25 vehicle-treated rats and 15 TCDD-treated rats were killed on GD16 and GD21 for tissue sampling prior to TCDD analysis. For the control animals, tissues were pooled from five animals, and five samples of pooled tissue were analysed. In the TCDD-treated groups, blood samples were pooled from five animals, and three pools analysed; adipose and liver tissue from five individual animals were analysed. The adipose, blood and liver are maternal tissues. Fetuses from five individual animals were pooled and analysed. The exceptions, where six animals per group are analysed, are set out in Materials and Methods. Liver and fetus weight were directly determined, and adipose tissue assumed to be 10% of body weight, in the calculation of amount of TCDD per tissue. Results are presented as mean ± Standard Deviation.

TABLE 4
TCDD concentration in maternal tissue and fetus after chronic dosing

	Tissue TCDD concentration (ng kg^{-1}) [ng per tissue]			
Dose of TCDD (ng kg^{-1} day^{-1})	0	2.4	8	46
Week 10				
Adipose	NP	363±82 [4.99±1.13]	832±235 [11.1±3.14]	2730±1180 [40.9±17.7]
Liver	NP	173±48 [1.17±0.32]	569±63 [3.96±0.44]	4050±1070 [30.3±8.04]
Week 12				
Adipose	NP	411±57 [6.23±0.86]	827±161 [12.4±4.21]	2830±950 [42.7±14.4]
Liver	NP	162±50 [1.21±0.038]	680±124 [4.77±0.04]	5010±935 [40.3±0.41]
GD16				
Adipose	1.66±0.15 [0.038±0.0035]	394±146 [8.09±3]	647±131 [13.1±2.65]	2790±1170 [56.4±23.7]
Blood	0.25±0.26	1.52±0.22	1.91±0.49	7.61±2.2
Fetus	0.1±0.03 [0.0006±0.0002]	2.27±0.63 [0.013±0.0035]	4.65±0.29 [0.023±0.0015]	17.3±5.75 [0.072±0.024]
Liver	0.2±0.06 [0.0023±0.0007]	156±48 [1.75±0.54]	665±224 [7.46±2.51]	3980±770 [46.1±8.93]
GD21				
Adipose	1.45±0.32 [0.0417±0.0039]	470±74 [11.7±1.83]	862±401 [22.2±10.3]	2590±1300 [68.3±34.3]
Blood	0.06±0.01	1.34±0.19	2.9±0.99	8.82±4.22
Fetus	0.03±0 [0.001±0]	4.79±0.92 [0.28±0.055]	9.08±2.7 [0.51±0.151]	26.9±11.6 [1.5±0.65]
Liver	0.21±0.05 [0.002±0.0005]	154±49 [1.39±0.44]	700±160 [6.77±1.54]	4050±813 [40.9±8.21]

Animals of 5-6 weeks of age (100-146g) were provided with diet containing 0, 28, 93 and 530 ng TCDD kg^{-1} diet (the TCDD was dissolved in acetone) ad libitum. After 12 weeks of treatment of the P females, one female was housed with one untreated male for up to 15 days, and mating confirmed by a vaginal plug. 5 and 10 animals per group (treated groups only) were killed in weeks 10 and12 after starting on the diet, and on gestation day 16 and 21, 15 animals from the control group and 10 animals from the treated group were killed; tissue samples from these culls were used for TCDD analysis For the control and treated animals, adipose, fetus and liver tissues were obtained from five individual animals, and individual tissues were analysed. Blood samples were pooled from five animals, and three pools analysed for all dose groups. Fetuses from five individual animals were pooled and analysed. The exceptions, where six animals per group are analysed, are set out in Materials and Methods. Liver and fetus weight were directly determined, and adipose tissue assumed to be 10% of body weight, in the calculation of amount of TCDD per tissue. Results are presented as mean ± Standard Deviation. NP= no analysis performed

TABLE 5
Estimated body burden of TCDD

	Body burden ng ($ng\ kg^{-1}$)			
TCDD dose group	Control	Low	Medium	High
Acute study				
GD16	0.022±0.01 (0.078±0.024)	5.41±1.7 (18.8±5.91)	22.7±7.14 (80±25.2)	122±38.5 (418±132)
GD21	0.14±0.046 (0.44±0.14)	6.41±2.01 (19.6±6.18)	21.7±6.83 (66.3±20.9)	121±38 (375±118)
Chronic study				
Week 10	NP	6.17±1.68 (28.8±7.86)	15.1±4.1 (71.8±19.6)	71.3±19.4 (316±86)
Week 12	NP	7.44±2.03 (32.7±8.92)	17.1±4.67 (76±20.7)	83±22.6 (366±99.7)
GD16	0.041±0.011 (0.14±0.037)	9.85±2.71 (35.4±9.74)	20.6±5.6 (74.9±20.4)	103±28 (373±102)
GD21	0.045±0.012 (0.14±0.039)	13.4±3.68 (42.4±11.6)	29.4±8.08 (91.2±25)	111±30.4 (337±92.6)

Tissues were analysed as described in Tables 1, 2 and in the materials and methods. Fetus and liver were weighed, and adipose tissue was assumed to be 10% of body weight; body burden is assumed to be the sum of liver, fetus and adipose burden for five animals (or pools for control) per group. Results are presented as mean ± an indicative estimate of variation, which is the mean multiplied by the the coefficient of variation of the TCDD analyses in the acute (36%) and chronic (27%) studies, respectively. NP= no analysis performed.

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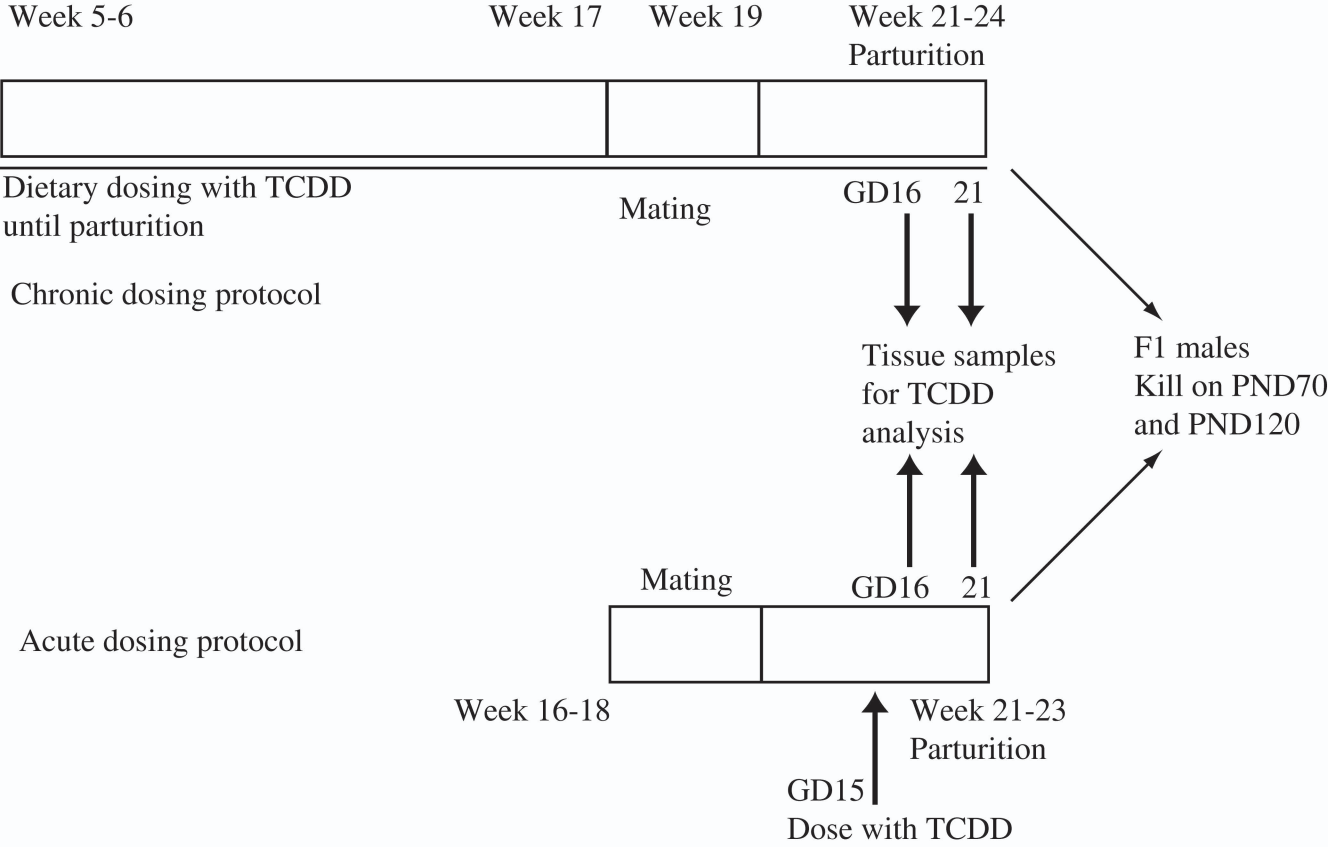
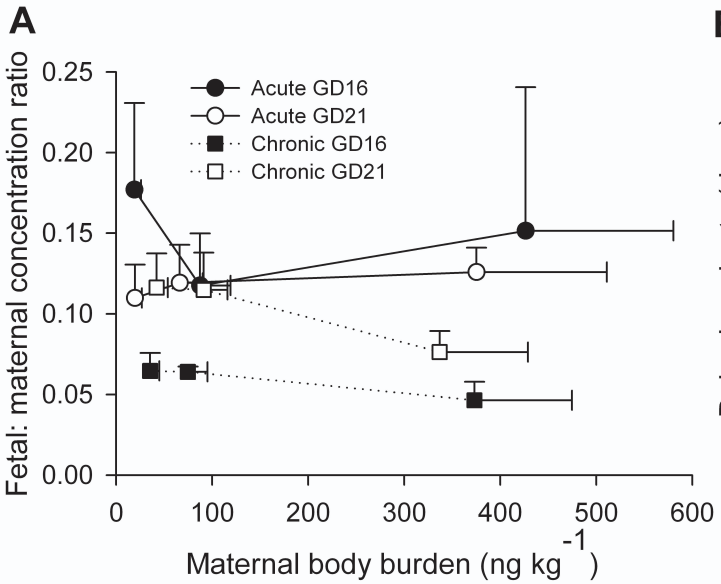
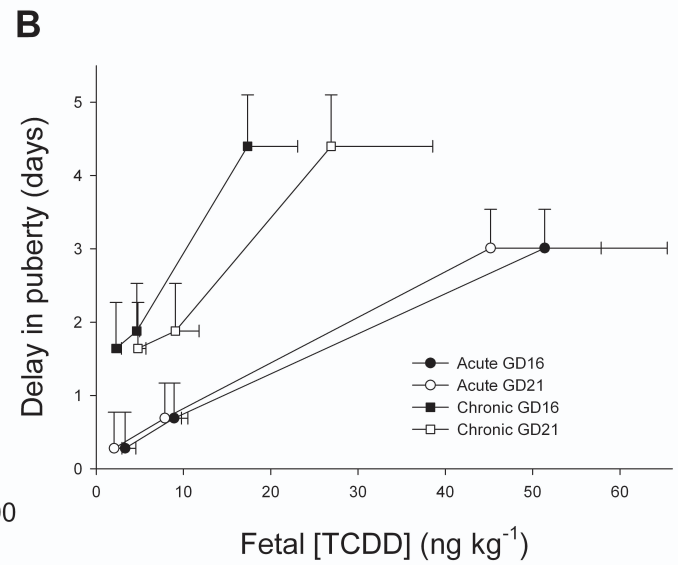
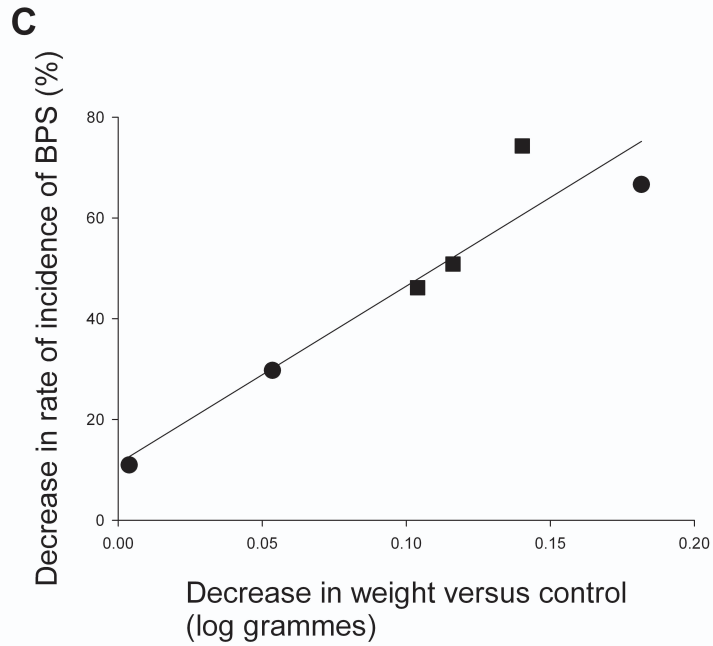
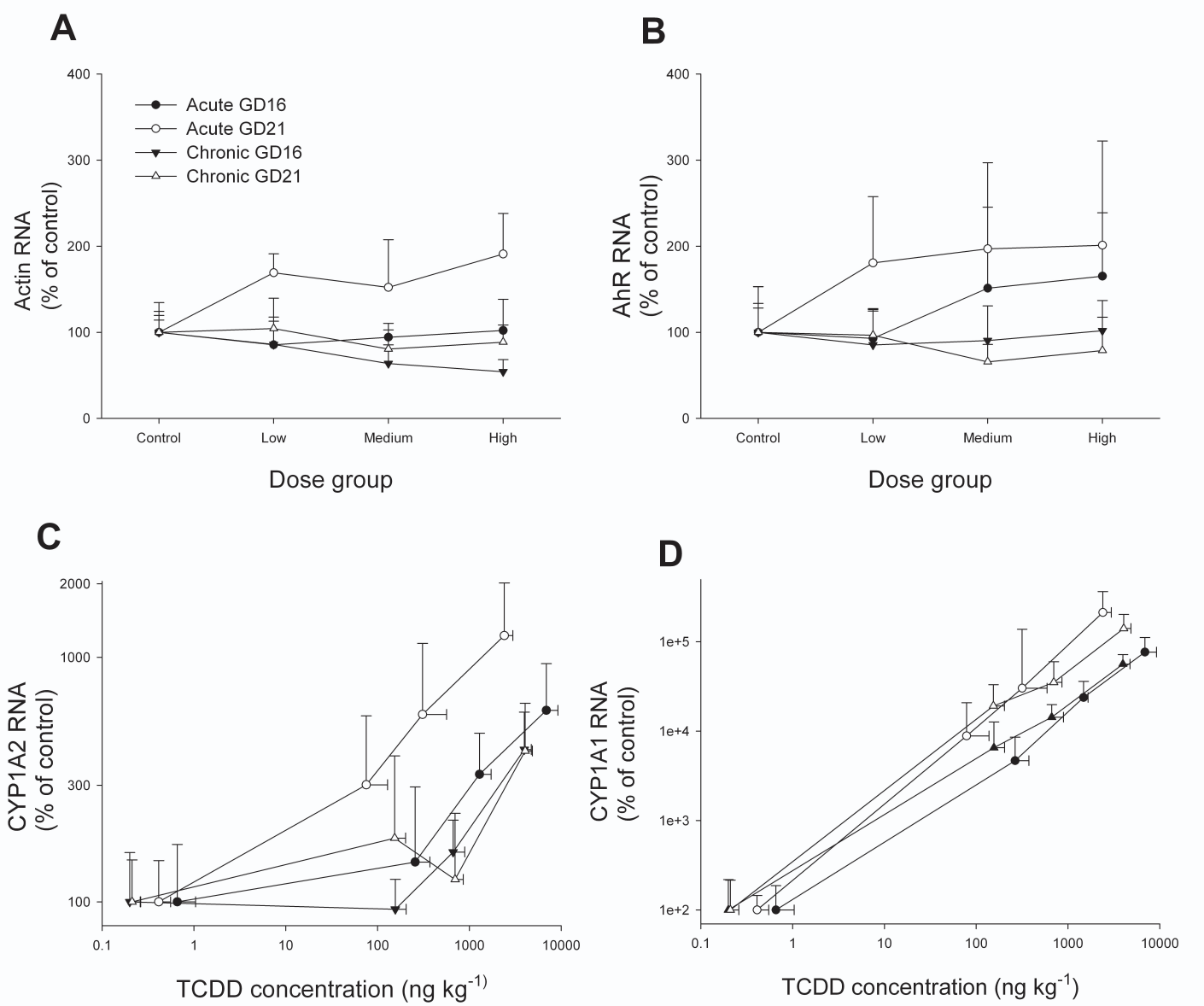
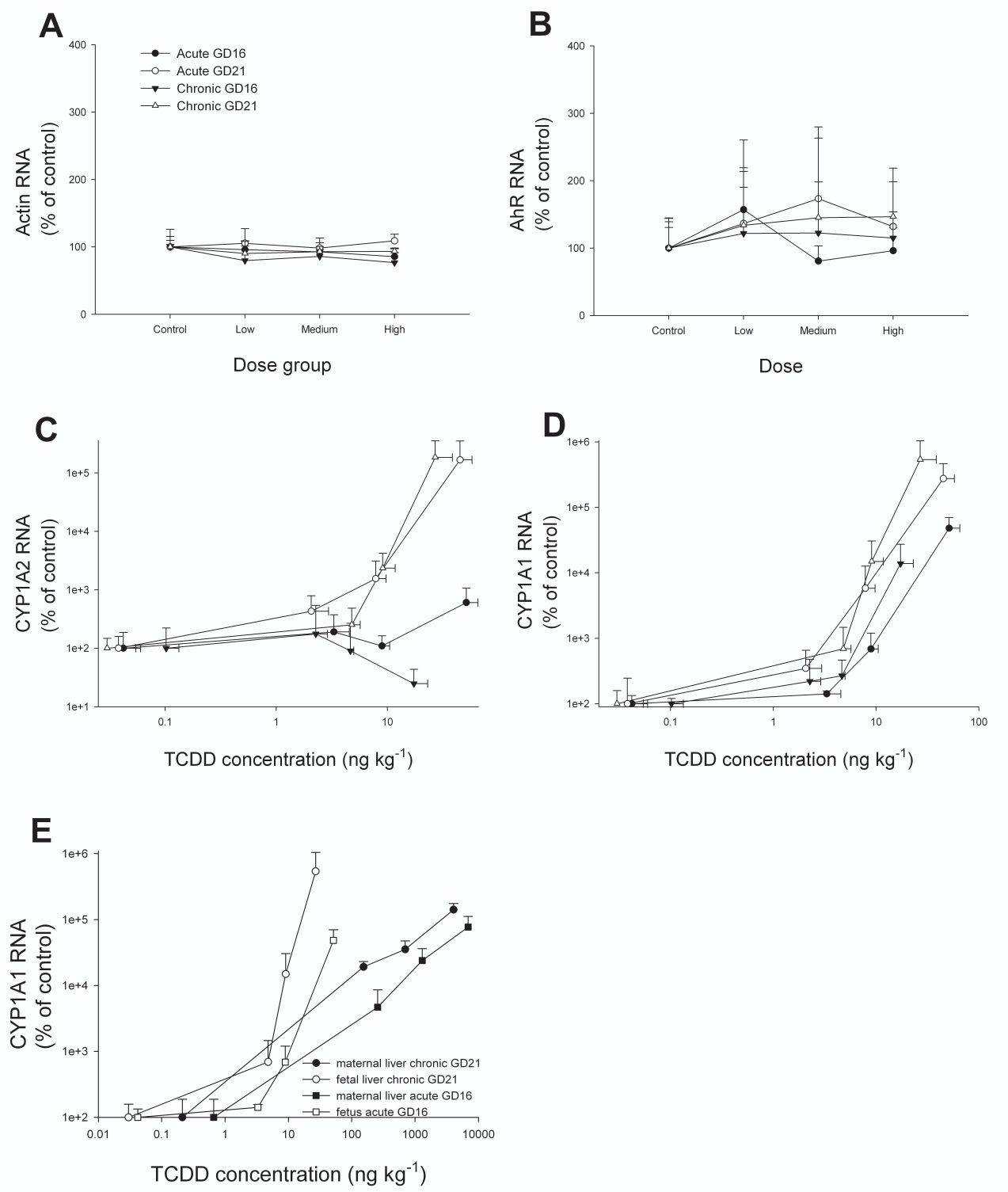

Week 5-6
Week 17
Week 19
Week 21-24
Parturition
Dietary dosing with TCDD
until parturition
Mating
GD16
21
Chronic dosing protocol
Tissue samples
for TCDD
analysis
F1 males
Kill on PND70
and PND120
Mating
GD16
21
Acute dosing protocol
Week 16-18
Week 21-23
Parturition
GD15
Dose with TCDD

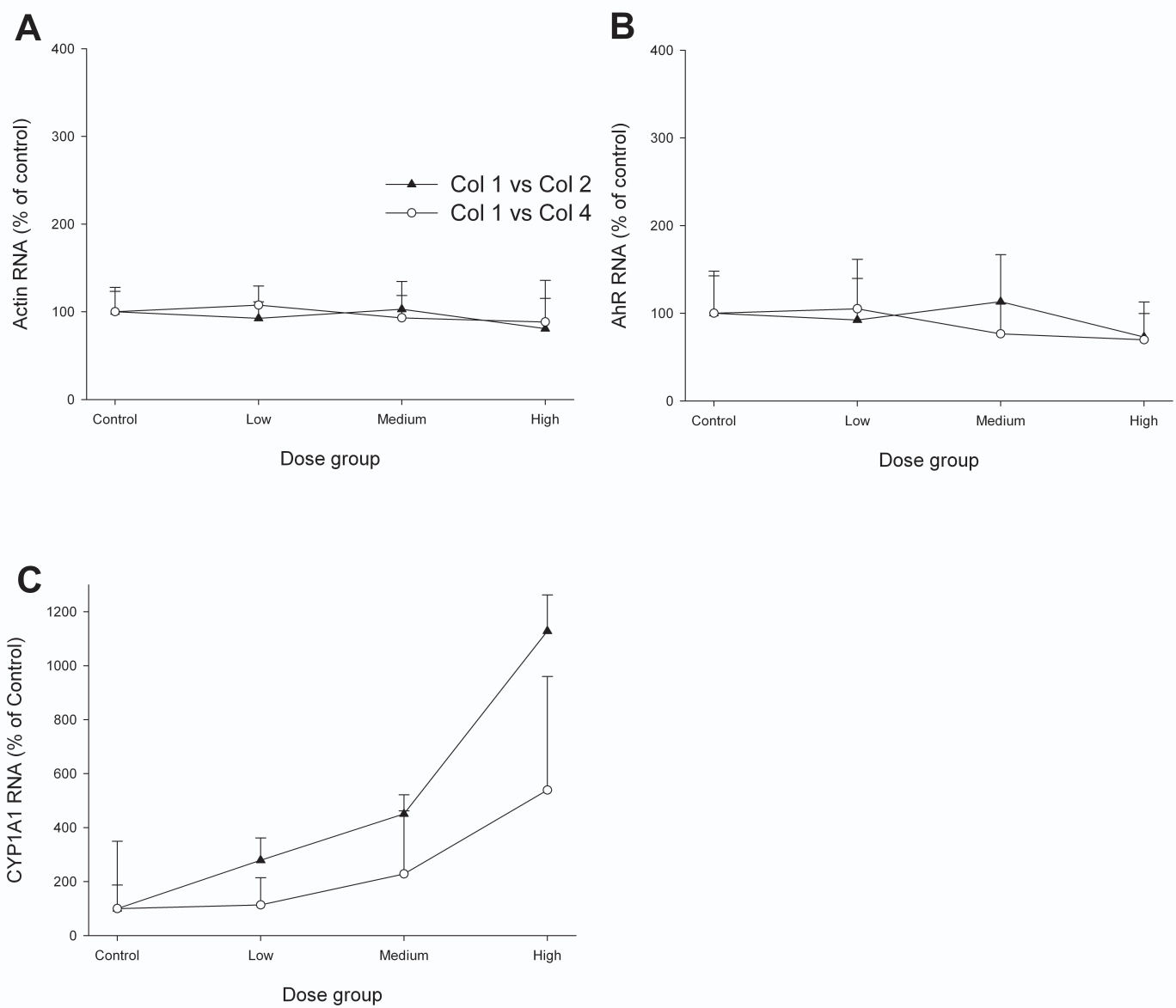

A
Fetal: maternal concentration ratio
0.25
0.20
0.15
0.10
0.05
0.00
Acute GD16
Acute GD21
Chronic GD16
Chronic GD21
0
100
200
300
400
500
600
Maternal body burden (ng kg-1)


B
Delay in puberty (days)
5
4
3
2
1
0
Acute GD16
Acute GD21
Chronic GD16
Chronic GD21
0
10
20
30
40
50
60
Fetal [TCDD] (ng kg-1)


C
Decrease in rate of incidence of BPS (%)
80
60
40
20
0
0.00
0.05
0.10
0.15
0.20
Decrease in weight versus control
(log grammes)


A
B
C
D
Acute GD16
Acute GD21
Chronic GD16
Chronic GD21
Actin RNA (% of control)
AhR RNA (% of control)
CYP1A2 RNA (% of control)
CYP1A1 RNA (% of control)
Control
Low
Medium
High
Dose group
TCDD concentration (ng kg-1)


A
Acute GD16
Acute GD21
Chronic GD16
Chronic GD21
Actin RNA
(% of control)
400
300
200
100
0
Control
Low
Medium
High
Dose group
B
AhR RNA
(% of control)
400
300
200
100
0
Control
Low
Medium
High
Dose
C
CYP1A2 RNA
(% of control)
1e+5
1e+4
1e+3
1e+2
1e+1
0.1
1
10
TCDD concentration (ng kg-1)
D
CYP1A1 RNA
(% of control)
1e+6
1e+5
1e+4
1e+3
1e+2
0.1
1
10
100
TCDD concentration (ng kg-1)
E
CYP1A1 RNA
(% of control)
1e+6
1e+5
1e+4
1e+3
1e+2
maternal liver chronic GD21
fetal liver chronic GD21
maternal liver acute GD16
fetus acute GD16
0.01
0.1
1
10
100
1000
10000
TCDD concentration (ng kg-1)


A
400
300
200
100
0
Actin RNA (% of control)
Col 1 vs Col 2
Col 1 vs Col 4
Control
Low
Medium
High
Dose group
B
400
300
200
100
0
AhR RNA (% of control)
Control
Low
Medium
High
Dose group
C
1200
1000
800
600
400
200
0
CYP1A1 RNA (% of Control)
Control
Low
Medium
High
Dose group