Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the developing male Wistar(Han) rat II: chronic dosing causes developmental delay

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ABSTRACT

We have investigated whether fetal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes defects in the male reproductive system of the rat, using chronically exposed rats to ensure continuous exposure of the fetus. 5-6 week old rats were exposed to control diet, or diet containing TCDD, to attain an average dose of 2.4, 8 and 46 ng TCDD kg^{-1} day^{-1} for twelve weeks, whereupon the rats were mated, and allowed to litter; rats were switched to control diet after parturition. Male offspring were allowed to develop until kills on PND70 (25 per group), or PND120 (all remaining animals). Offspring from the high dose group showed an increase in total litter loss, and the number of animals alive on post-natal day (PND) 4 in the high dose group was ~26% less than control. The high and medium dose offspring showed decreased weights at various ages. Balano-preputial separation was significantly delayed in all three dose groups, compared to control. There were no significant effects of maternal treatment when the offspring were subjected to a functional observational battery, or learning tests, with the exception that the high dose group showed a deficit in motor activity. 20 rats per group were mated to females, and there were no significant effects of maternal treatment on the fertility of these rats, nor on the F_1 or F_2 sex ratio. Sperm parameters at PND70 and 120 showed no significant effect of maternal treatment, with the exception that there was an increase in the proportion of abnormal sperm in the high dose group at PND70; this is associated with the developmental delay in puberty in this dose group. There were no remarkable findings of maternal treatment on organ weights, with the exception that testis weights were reduced by ~10% at PND70 (but not PND120), and although the experiment was sufficiently powered to detect small changes, ventral prostate weight was not reduced. There were no significant effects of maternal treatment upon histopathological comparison of high dose and control group organs. These data confirm that developmental exposure to TCDD shows no potent effect on adult sperm parameters or accessory sexual organs, but show that delay in BPS occurs after exposure to low doses of TCDD, and this is dependent upon whether TCDD is administered acutely or chronically.

Keywords: Dioxin, Sperm, developmental toxicity

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous toxin, and 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). There have been reports that one of the most potent toxic effects of TCDD occurs after exposure of the developing rat fetus by dosing of the pregnant dam on GD15, leading to a spectrum of effects in the reproductive system of the male offspring, principally decreased sperm count in the cauda epididymis, but including decreased weight of the seminal vesicles, prostate and epididymis (Faqi *et al.* 1998; Gray *et al.* 1995; Gray *et al.* 1997a; Mably *et al.* 1992a; Mably *et al.* 1992b; Mably *et al.* 1992c). These effects are remarkably potent, with statistically significant effects after a single maternal dose of 64 ng TCDD kg^{-1} bodyweight. In view of the consistent reports of developmental effects of TCDD on male epididymal sperm counts from three laboratories, these data have been used to set a Tolerable Daily Intake for TCDD and related compounds by the UK Committee on Toxicity (COT 2001), the WHO (JECFA 2001) and the EU Scientific Committee on Food (SCF 2001).

However, the inability of low doses of developmental TCDD exposure to cause a decrease in offspring epididymal sperm counts is a finding common to later studies (Ikeda *et al.* 2005; Ohsako *et al.* 2001; Ohsako *et al.* 2002; Simanainen *et al.* 2004; Wilker *et al.* 1996; Yonemoto *et al.* 2005). Sperm counts are a highly variable endpoint (*e.g.* (Ashby *et al.* 2003)), yet several studies use small group size, and manual sperm counting in concert with a non-blinded analysis. In order to resolve the discordant results in the literature, studies should be implemented using GLP methodology and large group sizes to increase the statistical power and reliability of the analysis, with the explicit prior aim of measuring epididymal sperm levels.

In the accompanying paper, we have used a robust design to show that a single acute dose of TCDD to the pregnant CRL:WI(Han) rat on GD15 fails to decrease epididymal sperm levels in male offspring, but that TCDD is a potent toxin that induces lethality and a delay in balano-preputial separation (BPS) in offspring. A limitation of this study is that the TCDD was given as a single acute dose; however it is possible that there could be a narrow temporal window of susceptibility to these effects, and that no effect was seen since the dose missed the window of susceptibility. Therefore, a study was undertaken with chronic maternal dosing of TCDD to ensure continuous exposure of the fetus to TCDD; this chronic exposure to TCDD is also more representative of human patterns of exposure to TCDD 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. Indeed, it has been reported that 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, detailed pharmacokinetic studies show that there are clear differences in disposition at GD16 between acute and chronic doses that give similar TCDD concentration in liver (Hurst *et al.* 2000a; Hurst *et al.* 2000b). Thus it is possible that the mode of administration (*i.e*. acute versus chronic dosing protocol) may be a determinant of sensitivity of the developing fetus to TCDD.

We have therefore undertaken to repeat our previous study, but using a chronic dosing protocol instead of a single acute dose of TCDD. This experiment serves both to compare against our previous work using an acute dose of TCDD, and additionally to compare the effect of acute versus chronic administration on toxicological endpoints. Delay in puberty is an adverse effect, and occurs after very low doses of TCDD during development in rats.

MATERIALS AND METHODS

Materials.

TCDD was obtained from Cambridge Isotope laboratories, Mass, USA, and purity (99% v/v) was verified by HR-MS. All other chemicals were of the highest quality available.

Animal Study.

The animal studies were performed at Covance (Harrogate, UK), and were GLP-compliant; the full report on this study is published as supplementary material. CRL:WI(Han) rats were housed at a temperature of 19-25ûC, with two brief excursions with the lowest to 16.4ûC. Animals 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 F1 generation, with a 12 hour light/ darkness cycle. 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 rats, respectively) were provided with diet containing 0 (acetone alone), 28, 93 and 530 ng TCDD kg^{-1} diet (the TCDD was dissolved in acetone) ad libitum: food intake per cage was measured weekly. Exposure to TCDD in the diet was maintained for a 12 week acclimitisation period, and during mating and pregnancy, and dams were switched to control diet after parturition. 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. 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 (DRB et al., manuscript in preparation). 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. Males were then maintained untreated, until killed (25 per group, one per litter) 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. During post-natal weeks 12 and 13, twenty animals from each group were tested for learning ability (swimming maze), motor activity (every two minutes for thirty minutes), and in week 13, a functional observation battery. During post-natal week 16, twenty males per group were paired with untreated virgin females for up to seven days; mated females were killed on GD16, and examined for terminal body weight, pregnancy status, number of corpora lutea, number and intrauterine position of implantations, which were subdivided into live embryos, and early and late intrauterine deaths, and sex of embryos.

Necropsy and seminology.

At necropsy, animals were weighed and weights of seminal vesicles (with coagulating glands), brain, epididymes (total), liver, ventral prostate, thymus, spleen, kidneys and testes recorded. Testis, epididymis, liver, thymus and prostate from control and high dose groups were fixed,

embedded, sectioned at 5µm, stained with haemotoxylin and eosin, and examined by a pathologist. Sperm counts and viability were assessed from one epididymis from each male killed in post-natal weeks 10 and 17, and samples examined microscopically for morphology. Briefly, the cauda epididymis is dissected free, and the mid-distal cauda pierced two/three times with a scalpel blade. The cauda is placed into 5mls of phosphate buffered saline containing 0.57% (w/v) BSA, preheated to 37ûC. The left testis of males was frozen, pending enumeration of homogenisation resistant spermatids. Sperm number, motility and velocity were recorded by CASA (Computer Assisted Sperm Analysis) with a Hamilton-Thorne TOX-IVOS instrument examining n=10 fields per sample. 500 sperm per animal were examined microscopically and the number of morphologically abnormal sperm was recorded to give the % abnormal sperm.

TCDD analysis.

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, 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.

Statistical analysis.

Data were first analysed at Covance, as they were collected, with their standard statistical package. Continuous outcomes were analysed using one-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA), after log transformation where necessary. Pairwise comparisons with control were made using Dunnett's test and a linear trend test was applied. Data measured as proportions of animals were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. These tests were interpreted with one sided risk for increased incidence with increasing dose.

Further analyses of selected variables carried out in the package GenStat (Payne 2004) and included terms for random variation between litters. F_1 body weights were analysed by a mixed model ANOVA, with a one-way treatment group structure, and a normally-distributed random term for litters. In most analyses litter effects were significant, with the effect that estimated standard errors were larger than in the simple ANOVA model. Comparisons between treated groups and control used Williams' test (Williams 1972). Early body weights were analysed with a two-way (dose group × day) ANOVA mixed model with random litter effects. From PND21 onwards, when the pups were individually identified in the data, a repeated measures model was applied, with random terms for litter and pup differences. Time to BPS was analysed by a proportional hazards mixed model with a random term for litter effects (Lee *et al.* 2006), and with body weight as a covariate.

RESULTS

Dietary levels of TCDD were targeted to achieve comparable levels of TCDD in the liver to that seen in GD16 liver during the acute study (Bell et al, 2007a) (Bell *et al*, manuscipt in preparation), extrapolating from previous comparisons of TCDD tissue burden using acute and chronic dosing regimes (Hurst *et al.* 2000a; Hurst *et al.* 2000b); the diet was made up at 28, 93 and 530 ng TCDD kg^{-1}, and assuming a food intake of 10% of body weight per day, a dose of 2.8, 9.3 and 53 ng TCDD kg^{-1} day^{-1}. The concentration of TCDD in the diet was verified by measuring TCDD in ten 25 gramme aliquots of low dose diet; the determined concentration was 29 ng TCDD kg^{-1}, and the coefficient of variation was 1.5%. Feed stability was also tested; after a two month interval, four samples of the same diet was found to contain 30 ng TCDD kg^{-1}, with a coefficient of variation of 4.2%. Duplicate feed samples (25g) were tested on two occasions, yielding the following TCDD concentrations (all in ng TCDD kg^{-1})- control 0.6, 0.1; low dose 30, 24; medium dose 68, 102; high dose 560, 371.

Parental health

One animal in the high dose group died on day 2 of lactation, but all other animals survived. Females in the high dose group gained slightly more weight in the first five weeks of the pre-pairing period than controls, but over the whole of the pre-pairing period (and at all other times), there was no significant difference in weight gain between groups. During the pre-pairing period, the high dose group ate more food than controls, but there was no difference in food intake between groups during the lactation period (see Supplementary Data). The average dose based on nominal feed concentration, and average food intake, was 2.4, 8 and 46 ng TCDD kg^{-1} day^{-1}.

Littering and offspring

In all groups, the pre-coital time was two- three days, and the mating, fertility and fecundity indices were similar in all groups. In the animals killed on GD16, there was a significant dose response relationship to show fewer implanatations and fetuses with TCDD dose, but there were no significant pairwise comparisons with control, and the pooled GD16 and GD21 data showed no significant effect of TCDD dose on the uterine/ implantation data (see Supplementary Data). At littering, three females in the control group, four females in the low and medium dose group, and eight females in the high group showed total litter loss (Table 1). The number of pups alive on day 1, expressed as a ratio to the number of pups born, was significantly decreased in the high dose group, and the number of pups surviving between days 1 and 4 (as a ratio of number of pups alive day 1) was also statistically significantly reduced in the high dose group.

F_1 body weight gain and BPS

There was a dose-related reduction in mean pup body weight on PND1, and this remained so throughout the lactation period (Fig. 1). The decrease in body weight, relative to control, was

most marked at PND4, and was apparent in all three dose groups. Males in the low and medium dose groups had similar weight to control at PND21, but showed a dose-related trend to being lighter than the control group until PND120. The high dose group males were lighter than controls at PND21, and gained less weight than controls over the course of the study, with a marked reduction in weight gain immediately after weaning in the high dose group offspring. Balano-preputial separation (BPS) was significantly delayed in all three treatment groups (by 1.8, 1.9 and 4.4 days for low medium and high dose respectively), and this was dose dependent (Fig. 2). The delay in BPS was analysed by fitting a proportional hazards model to the incidence rates, and the rate ratios relative to control were 54% (95% Confidence Interval 38-75%), 49% (35-69%) and 26% (17-38%) for the low, medium and high dose groups, respectively. The day of BPS was analysed with body weight on PND21 or PND42 as covariates, and with a random effect for litter differences; while the effects of body weight fell short of significance, there was highly significant between-litter variation. However, the adjustments to the mean BPS were minimal, and the treated groups remained significantly different from control; thus the body weight at PND21 or 42 does not affect delay in BPS.

Learning and Motor Activity

There were no adverse effects of maternal treatment on learning and memory in the swimming maze, nor in the performance in the functional observational battery. However, the offspring of animals in the high dose group were less active than the controls ($P<0.05$) when subject to a test of motor activity overy thirty minutes (see Supplementary Data).

Analysis of Reproductive Capacity of F1 Males

20 F_1 males were mated during post-natal week 16, and the median pre-coital time was 2 to 3 days in all groups. The uterine/ implantation data were similar in all groups, and there was no significant difference in the proportion of male offspring between groups (Table 2).

In the F_1 males killed in post-natal week 10, epididymal sperm counts were not significantly different between dose groups, nor were parameters describing sperm motility (Table 3). However, the proportion of abnormal sperm was significantly elevated in the high dose group, compared to control. The mean number of spermatids in the high dose group was 14% lower than control, and this difference was statistically significant. However, in the males killed in post-natal week 17, mean seminology data were unaffected by maternal treatment. Neither epididymal sperm levels, nor testicular spermatids, were affected by treatment (Table 3). Moreover, comparison of the control epididymal sperm levels with our previous data from CRL:WI(Han) rats showed that the absolute value of sperm counts were similar between the two studies (Fig. 3), confirming the consistency of these estimations.

Body Weight and Pathology

Terminal body weights were not significantly different between the four groups at PND70, but were significantly decreased in the high (6.9%) dose group, relative to control, at PND120; a 5.5% decrease in the medium dose group was just outside statistical significance at $P<0.05$. At

PND70, the high dose group testes were lighter than control as both an absolute and ratio to body weight (~12 and 8%, respectively). Spleen weight was significantly elevated (by 8%) in the high dose group animals at PND70, and was elevated (by 1.3-3.4%) in all three dose groups at PND120. At PND120, kidneys of the low and medium dose group were statistically significantly greater than control (~2%), ventral prostate of the medium dose group was greater than control (9.4%), liver to body weight ratios from the low and high dose group were significantly greater than control (~4.5%), and absolute brain weight was less than control in the medium dose group (~2.2%), and greater than control as a ratio to body weight in the medium and high dose groups. Histological examination of organs revealed solely minor findings that were consistent with the usual pattern of findings in animals of this strain and age; there were no findings that were associated with maternal TCDD treatment.

DISCUSSION

TCDD was administered in the diet, at a constant amount per kg of diet; the TCDD was stable in the diet, and there was batch to batch variation of up to 30% in TCDD concentration. Dietary administration of TCDD in this manner results in variation in the administered dose on a dose kg^{-1} basis, as animals eat more food as a fraction of bodyweight when they are younger (see Supplementary data); thus maternal dietary intake of TCDD was ~150% of average at the start of the study, and ~70% of average at the end of gestation. Over the course of the study, average food intake was slightly less than the nominal 10% kg^{-1} day^{-1}, and hence the administered dose was ~10% less than planned. A further source of variation arises from the fact that animals were housed in groups of five animals; it is not clear that all rats consumed equal amounts of food. Analysis of tissue TCDD concentrations and mRNA levels confirm that TCDD was adequately dosed, and will be reported in detail elsewhere (Bell *et al.*, manuscript in preparation).

There was no evidence of direct maternal toxicity of the TCDD, although the high dose group ate more food than controls during the pre-pairing period. However, the high dose group had ~8% fewer pups alive on day 1 (as a ratio of pups born), and the number surviving from PND1 to PND 4 was ~18% lower than control. Thus the TCDD treatment in the high dose group reduced pup numbers by ~26% by PND4. This effect of TCDD on increased pup lethality is consistent with other work (Bjerke and Peterson 1994; Bjerke *et al.* 1994; Gray *et al.* 1995; Gray *et al.* 1997a; Mably *et al.* 1992c; Roman *et al.* 1995; Roman *et al.* 1998; Sommer *et al.* 1996), and with the accompanying study in the CRL:WI(Han) rat (Bell *et al.* 2007). Whereas there were ~15% fewer pups alive on PND 4 after an acute maternal dose of 1000 ng TCDD kg^{-1} (Bell *et al.* 2007), there were 26% fewer pups alive on PND4 after a chronic maternal dose of 46 ng TCDD kg^{-1} day^{-1} (Table 1). Moreover, these data conceal a much stronger effect on post-natal lethality after chronic administration of TCDD, since the acute dose study lethality consisted of 12% fewer pups born, and ~3% of pups dying after birth and before PND4, consistent with a higher peak concentration of TCDD *in utero* arising from the single acute gavage dose by comparison with the chronic dosing regime. It is of interest that this post-natal lethality is coincident with a severe depression in weight in the treated groups (Fig. 1). The substantial post-natal lethality confirms the extraordinary potency of TCDD as a developmental toxicant.

In agreement with previous results in the same (Bell *et al.* 2007) and other rat strains (Bjerke and Peterson 1994; Bjerke *et al.* 1994; Faqi *et al.* 1998; Gray *et al.* 1997a; Roman *et al.* 1995; Roman *et al.* 1998; Sommer *et al.* 1996; Yonemoto *et al.* 2005), maternal TCDD treatment led to a delay in BPS (Fig. 2). However, whereas an acute dose of 1000 ng TCDD kg^{-1} led to a significant average delay in puberty of 2.8 days (Bell *et al.* 2007), chronic doses of 2.4, 8 and 46 ng TCDD kg^{-1} day^{-1} led to average delays of 1.8, 1.9 and 4.4 days, respectively (Fig. 2). The more potent effect of TCDD after chronic dosing is unlikely to be attributable to an unlikely sampling of the control group that resulted in an artefactually low control value, since our previous study found a mean day of BPS in the control group of PND45.8 ((Bell *et al.*

2007)), as against PND45.4 in this study (Supplementary data, Fig. 2), and these two values are not significantly different from each other. Thus developmental delay in puberty is the most sensitive adverse effect of TCDD in this study.

The greater sensitivity of rats to chronic, as opposed to acute, dosing of TCDD is unexpected. Our data shows that acute exposure on GD15 gives a markedly less sensitive effect of TCDD on BPS compared to chronic exposure (Bell *et al.* 2007), (Fig. 2); thus it is likely that gestational days 15-21 are not a period when the fetus is especially sensitive to this effect of TCDD. It is not clear when chronic maternal exposure to TCDD exerts the toxic effect in offspring leading to delayed BPS; it could be during gestation, or it could result from lactational transfer of TCDD to the offspring. There are differences in disposition arising from acute versus chronic exposure to TCDD, and so it would follow that a high dose acute exposure may well be uninformative for determining whether intrauterine or lactational transfer of TCDD is important during low dose chronic exposure. Lactational transfer of TCDD accounts for the majority (>90%) 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). Indeed, it has been shown that high maternal doses of TCDD cause hypothyroidism and hydronephrosis in F_1 rats via lactational transfer of TCDD (Nishimura *et al.* 2003; Nishimura *et al.* 2005; Nishimura *et al.* 2006). Further, the ability of TCDD to directly suppress testosterone levels in adult rats is well established (Kleeman *et al.* 1990; Mandal *et al.* 2001; Moore *et al.* 1985). We therefore propose that the effect of chronic administration of TCDD to the dam resulting in lethality and delayed BPS in the offspring is mediated via lactational transfer of TCDD.

There were no significant effects of maternal TCDD treatment on learning and memory endpoints, with the exception that the offspring from high dose group animals were less active than the controls in a test of motor activity over thirty minutes (Supplementary data). This is in general agreement with the lack of effect seen on these endpoints in the acute dose study; however, the effect at high dose is associated with a >25% incidence of pup lethality, and it is not clear whether this is a non-specific effect associated with the high dose of TCDD.

Seminological investigations failed to reveal any remarkable findings, with the exception that the % of abnormal sperm was elevated and testicular spermatids were decreased in the high dose group at PND70 (Table 3). However, given the size of the delay in puberty in the high dose group (Fig. 2), this increase in abnormal sperm can be parsimoniously explained by a delay in puberty retarding the start of spermatogenesis, and the consequent delayed onset of spermatogenesis yielding high numbers of abnormal sperm (Creasy 2003). Notably, there was no significant difference in the effects of maternal treatment on epididymal sperm number (Table 3). This data was consistent with the data after acute exposure to TCDD, and direct comparison of the absolute sperm counts in the control groups (Fig. 3) showed that the studies had good consistency. These studies were performed to determine if previous reports of gestational TCDD exposure causing decreases in epididymal sperm levels were reproducible (Faqi *et al.* 1998; Gray *et al.* 1997a; Mably *et al.* 1992a); although our previous single dose study failed to show this effect (Bell *et al.* 2007), it remained possible that this result was due

to a failure to expose the developing fetuses at the correct developmental stage. However, exposure of the fetus with a chronic dosing protocol excludes this possibility for the failure to show a decrease in epididymal sperm levels, and the experiment has a 95% power for detecting a 30% difference in control means at $P<0.05$, based on the data from the control group (Table 3). In agreement with these data, a functional test of mating ability of the F_1 males showed no significant difference in the reproductive function of control or treated animals (Table 2). Although there have been sporadic reports of alterations in sex ratios after parental TCDD exposure (Ikeda *et al.* 2005; Mocarelli *et al.* 2000), these reports are difficult to interpret since they are mechanistically inconsistent (maternal vs. paternal exposure) and there is no precedent for either finding. We therefore set out an explicit, prior hypothesis that TCDD would alter sex ratios in the F_1 or F_2 animals in a well-powered study; our data show no significant effect of TCDD treatment on sex ratio (Table 2), consistent with other animal studies (Rowlands *et al.* 2006). In this experiment, the weight of ventral prostate was directly measured, and this showed no significant difference from control (Table 4). Given the variability of the control animals, our experiment has a ~90% power for detecting a 10% decrease from control values in ventral prostate weight, which would be sufficient to detect the ~40% decrease in prostate weight described by (Mably *et al.* 1992c; Ohsako *et al.* 2001; Ohsako *et al.* 2002).

This study fails to show developmental toxicity of maternal TCDD on F_1 epididymal sperm levels, and this finding is difficult to explain by appealing to strain differences, since, Holtzmann rats (Ikeda *et al.* 2005; Mably *et al.* 1992a; Ohsako *et al.* 2001), Long-Evans rats (Gray *et al.* 1997b; Yonemoto *et al.* 2005), and Wistar/ Wistar(Han) (this study, (Bell *et al.* 2007; Faqi *et al.* 1998)) have all been used, and in each case, the repeat studies showed that developmental exposure to TCDD (at less than 500 ng kg^{-1}) causes no decrease in F_1 epididymal sperm levels. This study used the outbred CRL:WI(Han) rat for comparison with the Bor Wistar strain used by (Faqi *et al.* 1998), since this laboratory has background data for this strain, and this is essential for putting the results into the context of historical control data (Ashby *et al.* 2003). It is of note that a Wistar(Han) line shows resistance to TCDD toxicity (Pohjanvirta *et al.* 1987)(Tuomisto *et al.* 1999). However, susceptibility to acute lethality of TCDD varies between Wistar(Han) substrains (Pohjanvirta and Tuomisto 1990), and Table 1 and (Bell *et al.* 2007) demonstrate that the CRL:WI(Han) rat is sensitive to TCDD-induced lethality as a consequence of maternal exposure. We are currently characterising the molecular basis and prevalence of the AhR (Tuomisto *et al.* 1999) in CRL:WI(Han) and in Wistar strains (DRB, unpublished data).The function of the AhR in mediating TCDD toxicity is paramount, and understanding the role of this rat AhR, and indeed, the human AhR (Connor and Aylward 2006), in mediating toxicity is a key component for risk assessment.

In summary, this study and the accompanying paper (Bell *et al.* 2007) demonstrate a consistent parttern of TCDD toxicity in the CRL:WI(Han) rat, including perinatal lethality, weight loss and delay in BPS, but our data show no potent effect of TCDD on F_1 epididymal sperm levels or accessory sexual organ weight, despite having adequate power to detect these effect. Our data identify delay in puberty as the most sensitive adverse effect of maternal TCDD toxicity,

and show that chronic dosing dramatically alters the incidence of this effect. It will be relevant to determine if this effect is mediated via lactational transfer of TCDD to the pups.

SUPPLEMENTARY DATA

The full study report with individual animal data is provided as an appendix.

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FIGURES

FIG. 1. Body weight of F_1 males.

Pregnant dams were dosed on GD15 with 0 (black circle), 2.4 (white inverted triangle), 8(black square) or 46 (white diamond) ng TCDD kg^{-1} bodyweight day^{-1}, and allowed to litter. Offspring from ~25 animals per group were allowed to litter, and pups maintained as described in materials and methods. A. The body weight of male offspring is recorded, and shown as mean (symbol) ± Standard Deviation. Body weights prior to PND 1-21 are not associated with particular animals, but after PND21 are associated with individual animals; a break in the X-axis is shown to indicate this. B. Geometric mean weights were calculated for each group, and the differences on the log scale of treated groups from the control group is plotted. The error bars represent one standard error, based on a pooled variance estimate.

FIG. 2. Balano-preputial separation in F_1 offspring from dams treated with TCDD.

Dams were treated as described in Fig. 1, and BPS in the offspring was determined by daily inspection. The cumulative % of animals undergoing BPS is shown. Dose groups that are significantly different from control (proportional hazards model with adjustment for litter) at $P<0.05$ are indicated by an asterisk.

FIG. 3. Comparison of studies of maternal dosing of TCDD on epididymal sperm levels.

Epididymal sperm levels were determined on PND70 and 120, and data are presented as mean ± Standard Deviation. Data from PND 70 are presented as circles, and data from PND120 are shown with squares. Samples that are statistically significantly different from their concurrent control ($P<0.05$) are marked with an asterisk. Data from this study are shown in white symbols, and data from the acute dosing study (Bell *et al.* 2007) in black symbols.

TABLES

TABLE 1

Group Mean Litter Data

Dose of TCDD (ng kg^{-1} day^{-1})	0	2.4	8	46
Number of females giving birth	27	29	27	27
Mean duration of gestation (days)	22.3±0.5	22.1±0.3	22±0.2	22.3±0.6
Number of dams with total litter loss	3	4	4	8
Mean number of pups born	9.8±2.2	10.8±2.4	10.1±2.4	9.7±3.2
Mean number of pups alive Day 1	9.8±2.2	10.4±2.1	9.9±2.3	9.4±2.9
% male pups Day 1	49.1	47.2	48.5	48.9
Mean number of pups alive Day 4 before culling	9.2±2.2	9.4±2.5	9.3±2.9	8.7±3.1
Mean number of pups culled Day 4	1.5±1.8	1.8±1.7	2±1.6	1.6±1.9
Mean number of pups alive Day 4 after culling	7.7±0.9	7.7±1.4	7.3±1.7	7±1.8
Mean number of pups alive Day 7	7.6±0.9	7.6±1.4	7.3±1.7	6.9±1.8
Mean number of pups alive Day 14	7.5±0.9	7.4±1.5	7.3±1.7	6.9±1.8
Mean number of pups alive Day 21	7.5±0.9	7.4±1.5	7.3±1.7	6.9±1.8

4-5 week old CRL:WI(Han) rats were dosed via the diet with 0, 2.4, 8 or 46 ng TCDD kg^{-1} bodyweight day^{-1} for 12 weeks, and then mated and allowed to litter on the same diet, before switching to control diet after littering. Gestation and littering data were recorded (excluding those animals with total litter loss), and are presented above. Results are presented as mean ± Standard Deviation. *= different from the control group at $P<0.05$.

TABLE 2

F_1 mating data

Dose of TCDD (ng kg^{-1} day^{-1})	0	2.4	8	46
Number of paired males	20	20	20	20
Number of males inducing pregnancy	18	18	20	19
Mean number of corpora lutea per female	12.9±1.3	12.7±1.7	13.9±2.3	12.7±1.5
Mean number of implantations per female	11.3±2.9	11.1±2.7	12.6±1.8	11.8±2.1
Pre-implantation loss: mean %	11.7±20.9	12.6±18	8.5±11.8	7.5±11.5
Post-implantation loss: mean %	7.3±9.4	6.4±9.6	5.2±10	7.4±7.7
Mean number of embryos per female	10.4±2.7	10.5±2.7	11.9±2.1	10.9±2.1
Sex ratio (% male[n])	48.4 [188]	47.6[189]	52.5[238]	46.9[207]

4-5 week old CRL:WI(Han) rats were dosed via the diet with 0, 2.4, 8 or 46 ng TCDD kg^{-1} bodyweight day^{-1} for 12 weeks, and then mated and allowed to litter on the same diet, before switching to control diet after littering. F_1 males were allowed to survive until post-natal week 16, when 20 animals per group (each from a different litter) were mated with one untreated, virgin female rat (see Materials and Methods). Pregnant females were killed for necropsy on GD16. The sex ratio was based on anatomical differences, and is presented as the % male, with the total number in brackets. Results are presented as mean ± Standard Deviation. *= different from the control group at $P<0.05$

TABLE 3

Seminology Data

Post-Natal Day 70				
Dose of TCDD (ng kg^{-1} day^{-1})	0	2.4	8	46
Number of males examined	25	25	24	25
Mean total epididymal sperm count (10^6/mL)	3.2±2.6	2.9±2.2	4.4±3.3	2.5±2
Mean % motile	89±7	90±5	88±10	84±18
Mean average path velocity (μm/s)	196±22	200±21	198±31	197±32
Mean curvilinear velocity (μm/s)	367±32	378±29	375±44	369±54
Mean straight line velocity (μm/s)	137±16	139±15	139±23	137±20
Mean straightness (%)	68±3	68±2	68±2	68±3
Mean abnormal sperm (%)	3.7±2.1	4.4±2.9	4.3±3.6	5.8±2.8*
Mean total homogenisation resistant spermatid count (10^6/mL)	22.9±3.8	ND	ND	19.7±5.2*
Post-Natal Day 120				
Number of males examined	74	73	67	42
Mean total epididymal sperm count (10^6/mL)	14±7.5	13.3±7.1	14.4±7	14.1±7.2
Mean % motile	90±7	89±8	89±12	90±8
Mean average path velocity (μm/s)	195±25	196±23	201±27	198±25
Mean curvilinear velocity (μm/s)	350±42	355±37	362±45	357±38
Mean straight line velocity (μm/s)	137±19	137±16	142±19	139±18
Mean straightness (%)	68±2	68±2	68±1	68±2
Mean abnormal sperm (%)	2.1±2	2.2±1.8	1.7±1.7	2.6±2.3
Mean total homogenisation resistant spermatid count (10^6/mL)	20±5	ND	ND	19.5±3.8

The F_1 males from dams treated as described in Table 2, were killed at the indicated time. The indicated number of animals were killed on PND70 or 120, and sperm taken from the cauda epididymis for analysis, or homogenisation resistant spermatids from the testis, as described in the Materials and Methods. Results are presented as mean ± Standard Deviation. Samples that are different from control at $P<0.05$ are indicated with a *. ND= not determined.

TABLE 4

Terminal F_1 Body and Organ Weights

	PND 70				PND120			
	Dose of TCDD (ng kg^{-1} day^{-1})				Dose of TCDD (ng kg^{-1} day^{-1})			
	0	2.4	8	46	0	2.4	8	46
Body weight (g)	299±24	297±25	311±25	286±25	398±35	388±49	376±30	371±30*
Kidneys (g)	1.88±0.2	1.95±0.22	2.03±0.2	1.83±0.18	2.16±0.23	2.2±0.29*	2.18±0.27*	2.08±0.19
ratio	0.63±0.04	0.66±0.07	0.65±0.04	0.64±0.05	0.55±0.04	0.57±0.04	0.58±0.05	0.56±0.03
Spleen (g)	0.66±0.09	0.64±0.08	0.68±0.09	0.71±0.12*	0.68±0.09	0.7±0.12*	0.68±0.1*	0.69±0.09*
ratio	0.22±0.03	0.22±0.03	0.22±0.03	0.25±0.03	0.17±0.02	0.18±0.02	0.18±0.03	0.19±0.03
Liver (g)	11.4±1.3	11.3±0.8	12.3±1.84	10.5±1.6	12±1.3	12.3±2.1	11.7±1.4	11.7±1.49
ratio	3.81±0.3	3.81±0.28	3.9±0.33	3.68±0.43	3.02±0.21	3.16±0.32*	3.12±0.22	3.13±0.23*
Brain (g)	1.81±0.07	1.82±0.08	1.82±0.05	1.79±0.07	1.98±0.08	1.96±0.13	1.93±0.08*	1.94±0.09
ratio	0.61±0.04	0.61±0.04	0.59±0.04	0.63±0.04	0.5±0.04	0.51±0.06	0.52±0.04*	0.53±0.04*
Thymus (g)	0.55±0.13	0.56±0.11	0.62±0.1	0.52±0.1	0.4±0.07	0.39±0.1	0.38±0.08	0.36±0.05
ratio	0.19±0.04	0.19±0.03	0.2±0.04	0.18±0.03	0.1±0.02	0.1±0.02	0.1±0.02	0.1±0.01
Testes (g)	3.4±0.21	3.49±0.21	3.47±0.26	3.0±0.36*	3.63±0.44	3.66±0.27	3.62±0.36	3.63±0.3
ratio	1.16±0.09	1.2±0.12	1.1±0.12	1.06±0.09*	0.92±0.11	0.95±0.11	0.97±0.1	0.99±0.11
Epididymes (g)	1.19±0.19	1.15±0.12	1.15±0.27	1.05±0.15	1.66±0.31	1.63±0.21	1.57±0.23	1.57±0.17
ratio	0.4±0.05	0.39±0.04	0.37±0.09	0.37±0.04	0.42±0.07	0.43±0.07	0.42±0.06	0.43±0.05
Ventral prostate (g)	0.23±0.05	0.25±0.06	0.27±0.06	0.21±0.07	0.46±0.1	0.49±0.1	0.5±0.12*	0.45±0.11
ratio	0.08±0.02	0.08±0.01	0.09±0.02	0.07±0.02	0.11±0.03	0.13±0.02	0.13±0.03	0.12±0.03
Seminal vesicles (g)	0.51±0.13	0.58±0.17	0.52±0.13	0.48±0.17	1.1±0.31	1.04±0.27	1.02±0.25	1.06±0.25
ratio	0.17±0.04	0.19±0.05	0.17±0.04	0.17±0.05	0.27±0.08	0.27±0.07	0.27±0.07	0.29±0.08

The F_1 males from dams treated as described in Table 2, were killed at the indicated time; the number of animals is set out in Table 3. Animals were necropsied and body weight and organ weights analysed using ANCOVA, or one-way ANOVA on absolute organ weights and organ:necropsy body weight ratios, as set out in the Materials and Methods. Results are presented as mean ± Standard Deviation. *= different from control, $P<0.05$.

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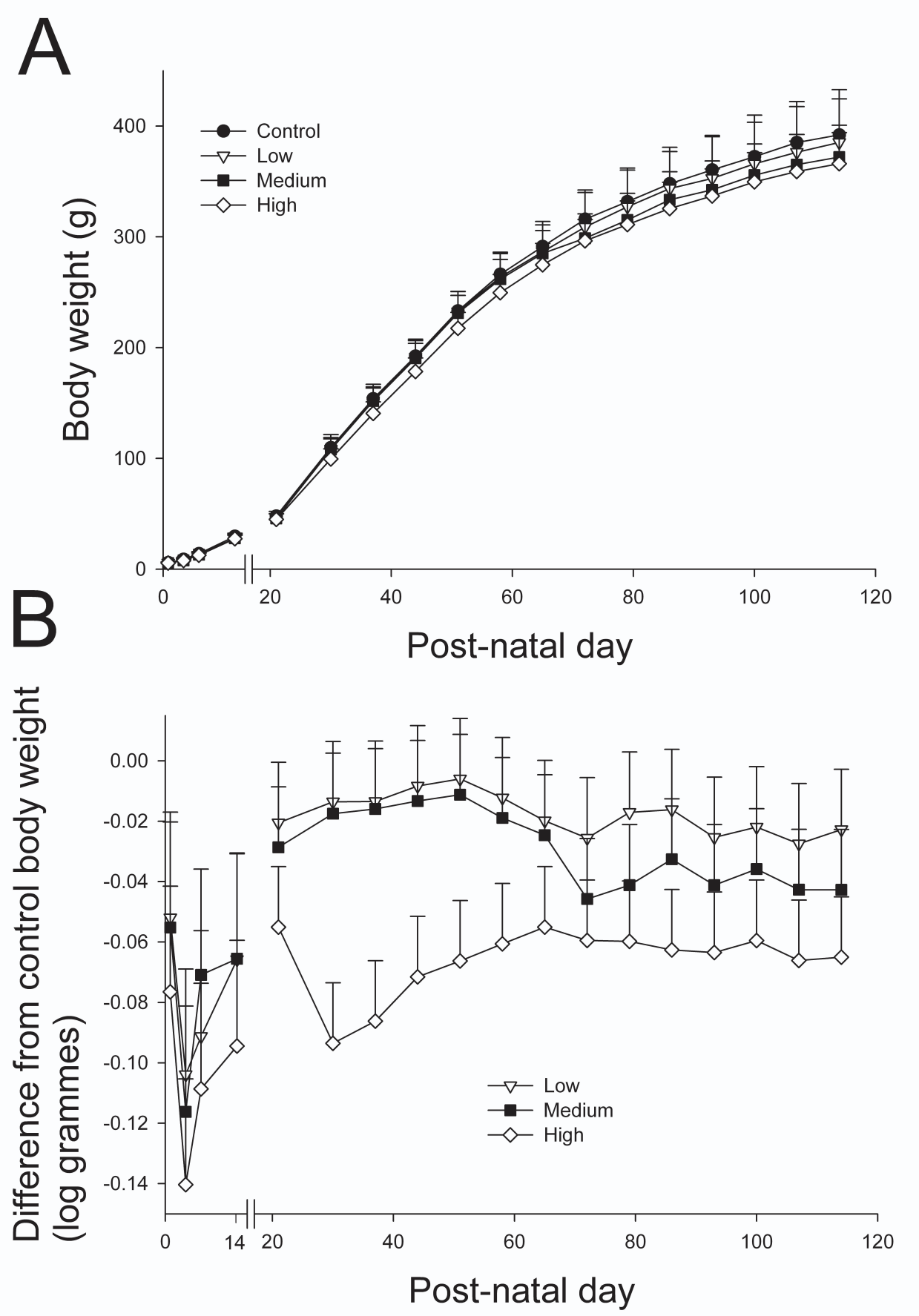
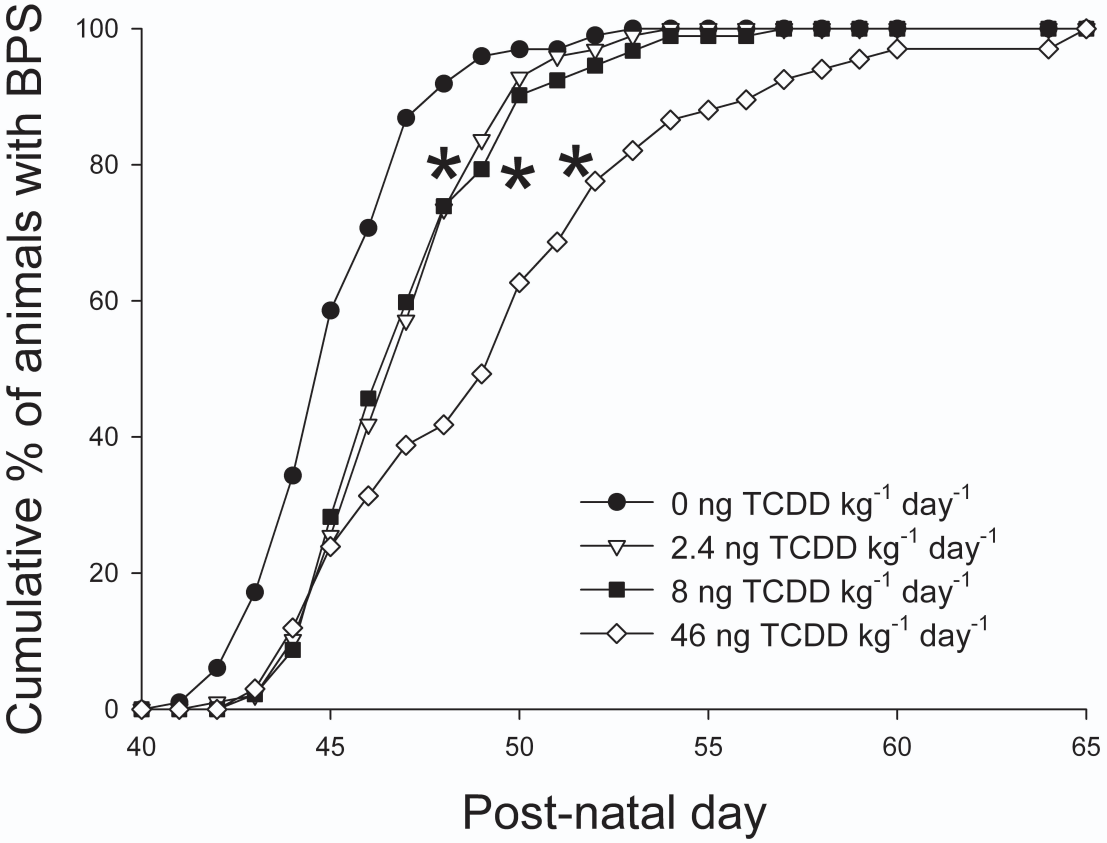
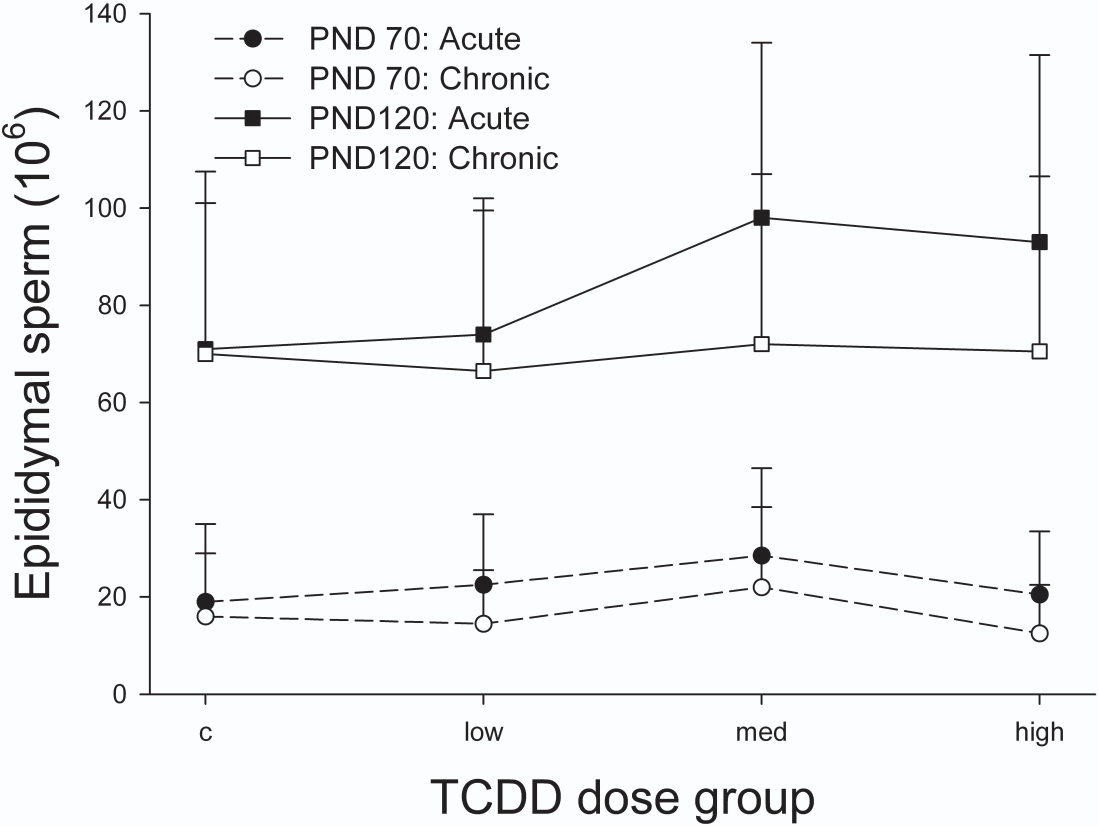

A
Body weight (g)
400
300
200
100
0
Control
Low
Medium
High
0
20
40
60
80
100
120
Post-natal day
B
Difference from control body weight (log grammes)
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
Low
Medium
High
0
14
20
40
60
80
100
120
Post-natal day


Cumulative % of animals with BPS
100
80
60
40
20
0
40
45
50
55
60
65
Post-natal day
*
*
*
0 ng TCDD kg-1 day-1
2.4 ng TCDD kg-1 day-1
8 ng TCDD kg-1 day-1
46 ng TCDD kg-1 day-1


PND 70: Acute
PND 70: Chronic
PND120: Acute
PND120: Chronic
Epididymal sperm (10^6)
140
120
100
80
60
40
20
0
c
low
med
high
TCDD dose group