

**The effects of an in utero exposure to
2,3,7,8-tetrachloro-dibenzo-p-dioxin on male
reproductive function: identification of Ccl5 as a
potential marker.**

Diane Rebourcet, Fanny Odet, Adélie Vérot, Emmanuel Combe, Emmanuelle Meugnier, Sandra Pesenti, Patrick Leduque, Henri Déchaud, Solange Magre, Brigitte Le Magueresse-Battistoni

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1 The effects of an in utero exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin on male
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10 Diane Rebourcet ^{1,2,3,4,5}, Fanny Odet ⁴, Adélie Vérot ⁴, Emmanuel Combe ^{1,2,3,4,5},
11 Emmanuelle Meugnier ^{1,2,3,4,5}, Sandra Pesenti ^{1,2,3,4,5}, Patrick Leduque ⁴, Henri Déchaud ^{4,5,6},
12 Solange Magre ⁷, Brigitte Le Magueresse-Battistoni ^{1,2,3,4,5} ;

13
14 ¹Inserm, U870, Oullins, France; ²INRA, UMR1235, Oullins, France; ³INSA-Lyon, RMND,
15 Villeurbanne, France; ⁴Université Lyon 1, Lyon, France; ⁵Hospices Civils de Lyon, Lyon,
16 France

17 ⁶Inserm U863, Lyon ;

18 ⁷CNRS EAC 7059, Université Paris 7.

19 Address for correspondence: Brigitte Le Magueresse-Battistoni, Inserm U870 INRA 1235,
20 Faculté de médecine Lyon-Sud, 165 Chemin du Grand Revoyet, BP 12, 69921 Oullins cedex,
21 France.

22 email address : brigitte.lemagueresse@inserm.fr

23 Tel 33 4 26 23 59 19; Fax 33 4 26 23 59 16

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27 Summary

28 TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and dioxin-like compounds are widely
29 encountered toxic substances suspected of interfering with the endocrine systems of humans
30 and wildlife and of contributing to the loss of fertility. In this study, we determined the
31 changes in testicular gene expression caused by in utero exposure to TCDD along with the
32 intra-testicular testosterone levels, epididymal sperm reserves, daily sperm production (DSP),
33 and testis histology. To this purpose, female pregnant Sprague-Dawley rats orally received
34 TCDD (10, 100 or 200 ng/kg bodyweight) or vehicle at embryonic day 15, and the offspring
35 was sacrificed killed throughout development. Hepatic Cyp1a1 gene expression was
36 measured in the offspring to confirm the exposure to TCDD. The gross histology of the testes
37 and intra-testicular testosterone levels were normal among the studied groups. Sperm reserves
38 were altered in 67-day-old rats of the TCDD-200 group, but not in 145-day-old animals or in
39 the other TCDD-exposed groups. Nonetheless, fertility was not altered in males of the TCDD-
40 200 group, and the F2 males generated had normal sperm reserves and DSP. Microarray
41 analysis permitted the identification of 8 eight differentially expressed genes in the 4-week-
42 old testes of the TCDD-200 compared with that of the control group (cut-off ± 1.40), including
43 the down-regulated chemokine Ccl5/Rantes. Inhibition of Ccl5/Rantes gene expression was
44 observed throughout development in the TCDD-200 group, and at 67 and 145 days in the
45 TCDD-100 group (animals of younger ages were not examined). Ccl5/Rantes gene expression
46 was mostly confined in Leydig cells. F2 males generated from males of the TCDD-200 group
47 had normal levels of Ccl5/Rantes in testis and Cyp1a1 in liver, which might indicate that
48 Ccl5/Rantes is a marker of TCDD exposure in testis such as Cyp1a1 in liver. In conclusion,
49 we demonstrated a decrease in Ccl5/Rantes RNA levels and a transitory decline in sperm
50 reserves in the testes of rats of TCDD-dosed dams.

51

52 **Introduction**

53 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) and dioxin-like compounds are widely
54 encountered toxic substances suspected of interfering with the endocrine systems of humans
55 and wildlife (Hotchkiss et al., 2008; Diamanti-Kandarakis et al., 2009). They form a large
56 group of structurally-related compounds of which the most toxic is TCDD (Van den Berg et
57 al., 2006). Dioxins are not intentionally produced but are generated as undesired by-products
58 in various industrial processes. They are persistent and, being fat soluble, tend to accumulate
59 in higher animals, including humans. They are found in all environmental compartments
60 although dioxin levels have been decreasing in the recent years in European countries for
61 example, to secure better protection of human health, in particular of children (Lundqvist et
62 al., 2006). Indeed, endocrine disrupters are suspected to be responsible for apparent changes
63 seen over the recent decades, including congenital malformations, cancer and declining sperm
64 counts. Genetic abnormalities are rare and cannot account for the rapid pace of the increase of
65 reproductive disorders. The concept of Testicular Dysgenesis Syndrome (TDS) was therefore
66 proposed (Skakkebaek et al., 2001). It enacts that the adverse changes, i.e., cryptorchidism,
67 hypospadias, impaired spermatogenesis and testicular germ cell cancer, are inter-related and
68 find common origins in fetal life or childhood. Supporting the concept of environmental
69 influence was the demonstration in rats that foetal exposure to high doses of dibutyl phthalate
70 causes a TDS-like phenotype (Fisher et al., 2003; Sharpe & Skakkebaek, 2008).

71 Today, dioxins are found in all humans, and exposure to dioxins in the general
72 population of the European Union for instance, is at a level where subtle health effects might
73 occur and it is, therefore, of utmost importance to improve the assessment of health risk (Gies
74 et al., 2007). Dioxins are not genotoxic but cause a broad spectrum of adverse effects
75 including hepatotoxicity, immune system suppression, developmental toxicity, and skin
76 defects. Meanwhile, there are still controversial data regarding the impact of dioxins on the

77 male reproductive function, specifically in case of maternal exposure. Parameters previously
78 investigated included at least measurement of testosterone levels, testis weight and sperm
79 reserves in the offspring of dosed rat or mouse dams. Declining epididymal sperm reserves
80 were often (Mably *et al.*, 1992; Gray *et al.*, 1995; Theobald & Peterson, 1997; Faqi *et al.*,
81 1998; Simanainen *et al.*, 2004) but not systematically (Wilker *et al.*, 1996; Ohsako *et al.*,
82 2001; 2002; Ikeda *et al.*, 2005; Bell *et al.*, 2007a) reported. Besides, molecular mechanisms
83 have not been clarified.

84 TCDD mediates its toxicity by binding to the aryl hydrocarbon receptor (AhR) and
85 subsequent alteration of the expression of target genes which exhibit dioxin response elements
86 in their promoter moiety including the cytochrome Cyp1a1 (Mimura & Fujii-Kuriyama, 2003;
87 Barouki *et al.*, 2007). Therefore, microarray analyses have been conducted to select the most
88 up- and down-regulated genes in target tissues. The liver has been mostly evaluated because
89 of the strong hepatotoxicity of TCDD, and major genes related to cholesterol biosynthesis,
90 glucose metabolism, and lipogenesis consistent with complementary histopathology have
91 been identified (Fletcher *et al.*, 2005; Sato *et al.*, 2008). Few data are available regarding the
92 testicular transcriptome in rats exposed to TCDD. Two genes of germ cell origin have been
93 identified using a representational difference analysis, and their expression was found to be
94 down-regulated in the testes of adult mice or rats exposed to high doses of TCDD (Kuroda *et al.*
95 *et al.*, 2005; Yamano *et al.*, 2005). In studies investigating the consequences of a maternal
96 exposure to dioxins, foetal pituitary gonadotrophin was defined as an initial target of dioxin
97 indirectly impacting testicular steroidogenesis (Mutoh *et al.*, 2006), although direct testicular
98 effects have also been described (Adamsson *et al.*, 2008).

99 In this study, we determined the changes in testicular gene expression caused by an in
100 utero exposure of female pregnant Sprague-Dawley rats to TCDD along with the

101 measurement of the intra-testicular testosterone levels, epididymal sperm reserves, daily
102 sperm production (DSP), and testis histology in the offspring throughout development.

103

104 **Materials and methods**

105 **Experimental design**

106 Time pregnant Sprague-Dawley females of embryonic day 12 were purchased from
107 Janvier's Breeding (Le Genest, France). They were housed individually in plastic cages with
108 food (Altromin 1310; Genestil, Royaucourt, France) and water provided ad libitum at 23°C
109 and a 12:12 photoperiod. Relative humidity was $50 \pm 10\%$. Animals were randomly assigned
110 to treatment groups. Dams were allowed 3-day acclimatization and were given one oral dose
111 of 2,3,7,8-TCDD (ref ED-901-C) (LGC Promochem, Molsheim, France) in sesame oil on
112 embryonic day 15. Control animals received sesame oil. Maximal volume of gavages was 0.7
113 ml/animal. Aliquots containing various doses of dioxins were assayed by Dioxlab (Dioxlab,
114 Saint-Maurice, France) to ascertain doses given to the animals. A total of 24 animals were
115 used in the reported experiments. Of these animals, nine dams received sesame oil (control
116 group), nine dams received one dose of TCDD 200 ng/kg body weight (bw) (TCDD-200
117 group), three dams received one dose of TCDD 100 ng/kg bw (TCDD-100 group) and three
118 dams received one dose of TCDD 10 ng/kg bw (TCDD-10 group). The outcome of gestation,
119 number of pups, sex-ratio and weight was recorded. Pups were not individually identified in
120 the litters, for bw. Male fertility of the TCDD-200 group (TCDD-F1 males) was assessed
121 using two virgin females per male. It was compared with the fertility of the control-F1 males.
122 Males originating from TCDD-F1 and control-F1 males were considered as TCDD-F2 and
123 control-F2 males, respectively. TCDD-F2 males have not been exposed in utero to TCDD.
124 Rats were sacrificed by cervical dislocation under CO₂ anesthesia at various ages. Testes and

125 epididymes were dissected, weighed, prepared for morphological studies or frozen in liquid
126 nitrogen and stored at -70 °C until processing for RNA analysis. Intratesticular content of
127 testosterone and 4-androstenedione was measured by radioimmunoassay as described
128 elsewhere (Rinaldi *et al.*, 2001). Epididymal sperm reserves and DSP were measured as
129 described (Robb *et al.*, 1978). Livers were dissected for RNA purpose. All experiments were
130 conducted with the approval of the local committee on animal care, and in accordance with
131 the European guidelines (86/609/CEE).

132

133 **Histological analysis and immunofluorescence**

134 For histological and immunohistochemical analyses, tissues were fixed at 4 °C in 0.1 M
135 phosphate buffer, pH 7.4, containing 4% formaldehyde plus 10% picric acid for at least 24 h,
136 dehydrated in a graded series of ethanol, and paraffin-embedded using standard protocols.
137 Sections 5 µm in thickness were stained with the periodic acid-Schiff (PAS)-haematoxylin
138 technique. For immunohistochemical analyses, paraffin-embedded tissues were
139 deparaffinized in xylene, rehydrated in graded ethanol solutions and endogenous peroxidase
140 activity was blocked with 0.3% hydrogen peroxide in methanol for 20 minutes. The sections
141 were sequentially incubated overnight at 4° C with the anti-Ccl5/Rantes primary antibody (sc-
142 1410; diluted 1/100) (Santa-Cruz Biotechnologies Inc., Santa Cruz, CA), and anti-goat Ig-
143 Alexa Fluor 555 secondary antibody (diluted 1/1000) (Invitrogen France, Cergy-Pontoise) for
144 1 h at room temperature. Leydig cell identity was revealed using the anti-3β-hydroxysteroid
145 dehydrogenase (3beta-HSD) antibody (diluted 1/1000; overnight incubation at 4 °C) (kindly
146 provided by Dr I Mason, Reproductive and Developmental Sciences Division, Edinburgh,
147 Scotland, UK) followed by incubations with anti-mouse Ig-Alexa Fluor 555 secondary
148 antibody (diluted 1/1000; 1 h at room temperature) (Invitrogen). Fluorochrome-labeled

149 sections were mounted in vectashield containing DAPI for nuclei visualization (Vector
150 Laboratories Canada, Burlington, ON, Canada). Slides were analyzed with Zeiss Axiovert
151 epifluorescence microscopes (Carl Zeiss, New York, NY, USA), all connected to a digital
152 camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI, USA).

153

154 **Microarray analysis**

155 Total RNA was extracted from testes and livers recovered from the control and TCDD groups
156 of rats using Rneasy mini kit (Qiagen, Courtaboeuf, France). RNA integrity was determined
157 with the Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Massy,
158 France). For microarray analysis, a pool of control testes RNA originating from three
159 different males of three different dams was used as a common reference and compared with
160 three testes originating from three different TCDD-200 F1 males of three different dams. One
161 microgram of total RNA was amplified with the Amino Allyl MessageAmp II aRNA kit
162 (Ambion, Austin, TX, USA) according to the manufacturer's instructions. This mRNA
163 amplification procedure is well validated and it has been demonstrated that it does not distort
164 the relative abundance of individual mRNAs within an RNA population (Wang *et al.*, 2000).
165 Fluorescent probes were synthesized by chemical coupling of 5 µg of aminoallyl aRNA with
166 cyanine (Cy)3 or Cy5 dyes (GE Healthcare Biosciences, Orsay, France). After purification
167 with RNeasy Mini Kit (Qiagen), probes were fragmented with 25X RNA Fragmentation
168 Reagents (Agilent Technologies) and hybridized with 2X Agilent Hybridization Buffer
169 (Agilent Technologies) to *Rattus Norvegicus* opArray (Operon Biotechnologies GmbH,
170 Cologne, Germany) in an Agilent oven at 67 °C for 16 h, following a dye swap experimental
171 procedure to correct for gene-specific dye bias (Churchill, 2002). Microarrays were washed
172 and scanned with a Genepix 4000B scanner (Molecular Devices, Sunnyvale, USA). TIFF

173 images were analysed using Genepix Pro 6.0 software (Molecular Devices). Signal intensities
174 were log-transformed and normalization was performed by the intensity dependent Lowess
175 method. To compare results from different experiments, data from each slide were normalized
176 in log-space to have a mean of zero using Cluster 3.0 software. Only spots with signal to
177 noise ratio above 2 were selected for further analysis. Data were analysed using the
178 significance analysis of microarray procedure with a false discovery rate of 5% (Tuscher *et*
179 *al.*, 2001). Microarray data are available in the GEO database under the number GSE13838.

180

181 **Quantitative polymerase chain reaction (Q-PCR)**

182 Quantitative polymerase chain reaction was used for validation of the microarray
183 procedure and study of gene expression levels of testicular Ccl5/Rantes and hepatic Cyp1a1
184 in rats from the different experimental groups including the offspring of the dosed-dams and
185 the males of the F2 generation. Each RNA sample used for Q-PCR was prepared from rats
186 originated from different dams. Briefly, first-strand cDNAs were synthesized from 1 µg of
187 total RNA in the presence of 100 U of Superscript II (Invitrogen, Eragny, France) and a
188 mixture of random hexamers and oligo(dT) primers (Promega). Real-time PCR assays were
189 performed in duplicates for each sample with a Rotor-GeneTM 6000 (Corbett Research,
190 Mortlake, NSW, Australia), as described elsewhere (Mazaud Guittot *et al.*, 2008). The list of
191 the primers (Invitrogen, Eragny, France) is available in Table 1. Briefly, PCR was performed
192 with 0.4 µM of each primer and 10 µl Absolute QPCR SYBR Green ROX mix (Thermo
193 Fisher Scientific, Courtaboeuf, France), in a total volume of 20 µl. After the initial
194 denaturation step of 15 min at 95 °C, the reaction conditions were 40 cycles of 95 °C for 15 s,
195 55 or 58 °C (depending on the primers, Table 1) for 10 sec, and 72 °C for 20 sec. The
196 fluorescence intensity of SYBR Green was read on the Rotor-GeneTM at the end of each
197 extension step. Melting curve analyses were performed immediately following the final PCR

198 cycle to verify the specificity of the PCR product by checking its Tm. Rpl19 (ribosomal
199 protein L19) and Gusb (glucuronidase beta) genes were chosen as references for normalizing
200 target genes in the testis (Mazaud Guittot *et al.*, 2008) and liver (unpublished data from the
201 laboratory), respectively. Relative quantification was made by the standard curve method for
202 both target and housekeeping gene (endogenous control) in each sample. A series of dilutions
203 of calibrator sample (external standard) was included in each experiment to generate an
204 external standard curve. Then the concentration of the target in each sample was divided by
205 the concentration of the housekeeping gene in each sample, normalizing the samples. Relative
206 quantification was carried out using the LightCycler® Relative quantification Software
207 (version 1.0). The calculation of data was based on the crossing point (Cp) values obtained by
208 the LightCycler® Software. To correct for sample heterogeneity and variability of detection,
209 results were calculated as the target/reference ratio of the sample divided by the
210 target/reference ratio of the calibrator.

211

212 **Isolation of testicular cells and RT-PCR**

213 Obtention of highly enriched fractions of testicular cells was carried out as described
214 elsewhere (Le Magueresse-Battistoni *et al.*, 1994; 1998; Longin *et al.*, 2001). Briefly, Sertoli
215 and peritubular cells were isolated from 20-day old rats and cultured at 32 °C in a humidified
216 atmosphere of 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM)-Ham's F12 (Life
217 technologies, Grand Island NY) supplemented (peritubular cells) with or without 10% fetal
218 calf serum. Sertoli cells were hypotonically-treated to eliminate the contaminating germ cells.
219 Enrichment of Sertoli and peritubular cells cultured for 6 days was higher than 95%. Leydig
220 cells were isolated from adult rats as previously described elsewhere (Carreau *et al.*, 1988;
221 Mazaud Guittot *et al.*, 2008). Briefly, following a collagenase digestion, interstitial cells were
222 purified on a discontinuous Percoll density gradient. The interface between 40 and 60% was

223 collected and washed to eliminate Percoll. The purity of the fraction ranged from 90 to 95%.
224 It was assessed by the presence of 3beta-HSD activity (Bilinska *et al.*, 1997). Spermatogenic
225 cells were isolated from adult rat testes by trypsinization. The resulting crude germ cell
226 population (containing germ cells from all developmental steps) was submitted to centrifugal
227 elutriation. Two fractions were harvested; the pachytene spermatocyte fraction enriched at 80-
228 85% (contaminated primarily by early spermatids) and the early spermatid fraction (steps 1-8)
229 enriched at 75-80% with primary contamination by both spermatocytes and elongated
230 spermatids.

231 After collection, the different cell populations were processed for RNA extraction
232 using Trizol reagent (Invitrogen, Eragny, France). RT-PCR was conducted as described
233 (Longin *et al.*, 2001) using the primers Ccl5/Rantes (a) and Rpl19 (Table 1) to ensure equal
234 loading in a 2 % agarose gel. A DNA ladder (Promega) was loaded and gels were stained with
235 ethidium bromide. Negative controls contained water instead of cDNA. The PCR product for
236 Ccl5/Rantes (a) was sequenced by GENOME Express (Grenoble, France).

237

238 **Data analysis**

239 Statistical analyses were carried out with the Sigmastat® 3.1 software package (Systat
240 Software, Inc., Point Richmond, CA, USA). All values are expressed as mean \pm SEM unless
241 specified differently. Statistical analysis was performed by ANOVA followed by Dunnett's
242 test for multiple comparisons. Early bw were analysed with a two-way (dose group x day)
243 ANOVA model. Significance was accepted at a confidence level of $p < 0.05$.

244

245 **Results**

246 **Effect of TCDD on F1 males**

247 Preliminary experiments were performed to determine the dose-range of 2,3,7,8-TCDD. We
248 observed that doses of 270 ng/kg bw induced maternal and foetal toxicity, and death of dams
249 was observed at the dose of 1000 ng/kg bw (Appendix S1). Therefore, doses of 10, 100 and
250 200 ng/kg bw were administered to pregnant females at embryonic day 15. No treatment-
251 related differences were noted with regard to the litter size, the sex-ratio or the bw of foetuses
252 in females that were dosed with 10, 100 or 200 ng/kg bw if data were expressed per litter. No
253 significant interaction was evidenced between dose and age (Table 2).

254 However, if male pups at a given age of 5, 7, 10, 12 or 14 days were considered instead
255 of litter as the experimental unit, then male pups were found to be lighter (an average of 8%;
256 $p < 0.05$) in the TCDD-200 group during the lactating period compared with the control group.
257 No effect on weight gain was detected in rats exposed to TCDD from weaning onwards (not
258 shown). The intra-testicular levels of testosterone and 4-androstenedione were in the normal
259 range in 28-, 40-, 67- and 145-day old rats of the TCDD-200 group (not shown). The other
260 groups were not assayed. Testicular and epididymal weights were within the normal range in
261 rats of the differently dosed groups compared with that in the control rats, and the gross
262 histology of the testes was also normal throughout development (not shown).

263

264 **Effect of TCDD on hepatic Cyp1a1 expression levels**

265 Expression of Cyp1a1 was measured by quantitative PCR in the liver of male rats aged 5 and
266 28 days to confirm TCDD exposure. Indeed, the induction of Cyp1a1 is regarded as one of the
267 most sensitive endpoints of AhR activation (Vanden Heuvel *et al.*, 1994). As shown in Fig. 1,
268 expression of Cyp1a1 was dramatically enhanced in the liver of rats of the TCDD-200 group
269 at 5 and 28 days of age (i.e., 1.5 and 5 weeks after the females had been given TCDD),
270 probably as a result of a continued exposure of pups to dioxin through breast milk of the

271 exposed mothers. This is consistent with the finding that the majority of TCDD in offspring of
272 dosed dams has been shown to arise from lactational transfer of TCDD (Li *et al.*, 1995).

273

274 **Sperm counts and DSP in F1 males**

275 Sperm counts were monitored in the caput, corpus and cauda of the epididymis of 67- and
276 145-day old rats and DSP was measured at both ages (Table 3 and not shown). ANOVA and
277 multiple comparison Dunnett's test were run to assess significance. No significant effect was
278 evidenced at 145 days of age for any group or at 67 days of age for the TCDD-10 and TCDD-
279 100 groups regardless of whether the data were reported per litter or per rat (range 6-12). In
280 67 day-old rats, the *p* value was 0.06 for sperm counts in the caput epididymis, 0.056 for
281 sperm counts in the cauda epididymis and 0.052 for DSP when the TCDD-200 group was
282 compared with the control group and data were expressed per litter. The *p*-value was 0.013
283 for sperm counts in the caput epididymis, 0.021 for sperm counts in the cauda epididymis and
284 0.06 for DSP, with data expressed per rat (Table 3).

285

286 **Identification of TCDD sensitive genes**

287 Microarray analysis was developed using 28 day-old rat testes RNA. A restricted number of
288 genes were selected. Specifically, three genes were up- and five genes were down-regulated
289 by ± 1.40 fold in testes of males from TCDD-200 dosed dams compared with that in control
290 testes (SAM procedure with FDR < 5%). Four genes have been reported to exhibit Xenobiotic
291 Response Elements (XRE; <http://drgap.nies.go.jp/pub/page/element>) in the 5' flanking
292 regions of the *Mus musculus* orthologous genes (Table 4). These genes were *Insl3* produced
293 by Leydig cells, the tumour suppressor gene *Glipr1*, and 2 chemokines *Cxcl4* and
294 *Ccl5/Rantes*. They were selected and further studied by Q-PCR (Table 4). *Insl3* and *Cxcl4*
295 gene expression levels which were up-regulated at 28 days of age were found not to be

296 statistically different in TCDD-200 vs. control testes at the other ages studied (5, 67 and 145
297 days for *Ins13* and 67 and 145 days for *Cxcl4*; not shown). *Glpr1* gene expression levels were
298 significantly ($p < 0.05$) enhanced at 28 (Table 4) and 67, but not at 5 days of age (not shown).
299 *Ccl5/Rantes* gene expression levels were down-regulated throughout development in TCDD-
300 200 vs. control testes (Fig. 2). In control testes, gene expression levels of *Ccl5/Rantes*
301 increased as a function of age and a plateau value was reached at 67 days of age. In TCDD-
302 200 testes, *Ccl5/Rantes* gene expression levels also increased as a function of age. However,
303 levels were significantly ($p < 0.05$) decreased in TCDD-200 testes compared with that in the
304 age-matched controls. Specifically, levels in TCDD-200 testes represented 45, 31, 54, 44, and
305 71% of the control levels at 5, 28, 40, 67 and 145 days of age respectively (Fig. 2). We also
306 surveyed *Ccl5/Rantes* levels in TCDD-10 and -100 testes from rats of 67 and 145 days of age.
307 No effect was observed in TCDD-10 testes, and TCDD-100 testes exhibited levels of
308 *Ccl5/Rantes* of the same range as that in TCDD-200 testes (Fig. 3). Data on RNA levels could
309 not be extended to the protein level because the signal for *Ccl5/Rantes* was barely detectable
310 using Western blot analysis (not shown).

311

312 **Identification of the testicular source of *Ccl5/Rantes***

313 Using RT-PCR analysis and specific primers for *Ccl5/Rantes*, a PCR product of the right size
314 and sequence (not shown) was detected in Leydig cells, and to a much lower degree in Sertoli
315 and germ cells (Fig. 3A). To extend these data, we found that the *Ccl5/Rantes*
316 immunoreactivity was confined to interstitial cells positive for 3 β -HSD identifying the
317 Leydig cell population (Fig. 3B). Sections incubated without the primary *Ccl5/Rantes*
318 antibody remained unstained (Fig. 3B).

319

320 **Reproductive performance of F1 males and the F2 generation**

321 F1 males from the control and TCDD-200 groups were mated with two virgin females
322 per male during postnatal week 15. Pregnant females did not receive TCDD during gestation.
323 All females became pregnant and no differences in the outcome of pregnancy, litter size and
324 sex-ratio were recorded between groups (Table 5). No difference in male pup weight was
325 detected between groups, hepatic Cyp1a1 gene expression levels remained at nadir in both
326 groups and Ccl5/Rantes mRNA testicular levels measured in 67- and 145- dayl old rats were
327 in the same range in both groups. Sperm counts and DSP were also in the normal range in
328 male rats of the F2 generation, in both groups (Table 5). Expression per rat instead of per
329 litter did not change the significance of the data in Table 5 (not shown).

330

331 **Discussion**

332 In this study, we demonstrated that exposure of female rats to TCDD 200 ng/kg bw at
333 embryonic day 15 induced decreased sperm reserves in the male offspring at 67 days, but not
334 at 145 days of age. Gene expression profile revealed that Ccl5/Rantes, a chemokine almost
335 exclusively found in Leydig cells, was negatively-regulated in testes of males from exposed
336 dams. No such phenotype was evidenced in males of the subsequent generation.

337 Many studies using rats as experimental models reported the use of maternal doses of
338 up to 1000 ng/kg (Mably *et al.*, 1992; Gray *et al.*, 1995; Sommer *et al.*, 1996; Cooke *et al.*,
339 1998; Haavisto *et al.*, 2006; Adamsson *et al.*, 2008). However, in this study, we observed
340 maternal and foetal toxicity at the dose of 270 ng/kg, and death of dams with the dose of 1000
341 ng/kg. Therefore, to avoid toxic effects that would render the interpretation of our studies
342 difficult, doses of 10, 100 and 200 ng/kg were used in this study. In line with a previous study
343 (Bell *et al.*, 2007a), we observed that neonates from TCDD-200 exposed dams (if considered
344 individually) were about 8% lighter than pups born from control dams during the lactating
345 period. It has been demonstrated that the majority of occurrences of TCDD in offspring of

346 dosed dams arises from lactational transfer of TCDD (Li *et al.*, 1995). Further studies will
347 have to be addressed to determine if breast milk was less nourishing/abundant. It is also
348 possible that TCDD interfered with food intake (Tuomisto *et al.*, 1999). In addition, we found
349 no alteration of the sex-ratio in agreement with recent studies (Rowlands *et al.*; 2006; Bell *et*
350 *al.*, 2007a).

351 Maternal exposure to TCDD lead to controversial data regarding sperm reserves in the
352 male offspring, as stated in the Introduction. One explanation for this debated question may
353 reside in the use of different strains as it is known that rats may be differentially resistant to
354 TCDD exposure (Simanainen *et al.*, 2004). In this study, we showed a significant decrease in
355 caput and cauda sperm reserves of the TCDD-200 group at 67 days of age if data were
356 expressed per rat. The origin of the reduced production of sperm cells in the young adult rats
357 is presently unknown. Indeed, the decline was transitory, i.e. these parameters were in the
358 normal range in 145-day old rats from TCDD-200 exposed dams. Gross histology of the testis
359 and number of apoptotic germ cells, as well as intratesticular testosterone levels, and
360 epididymal and testes weights, were all in the normal range compared with that of the
361 controls. In addition, dams were dosed at embryonic day 15, at the onset of testicular
362 testosterone production. Considering that TCDD half-life is close to 3 weeks in rat (Bell *et al.*,
363 2007b), it implies that foetuses were maximally exposed in the first few days following
364 dosing, i.e. during the early programming critical window for masculinization of the
365 reproductive tracts in rat (Welsh *et al.*, 2008). Collectively, these observations would suggest
366 that the androgenic signalling pathway was not targeted by a prenatal TCDD exposure in
367 postnatal rats, which is consistent with previous studies (Gray *et al.*, 1995; Haavisto *et al.*,
368 2006).

369 Gene expression profiles were used to select differentially expressed genes which
370 could be regarded as markers of a dioxin exposure. We used 28 day-old rat testes because at

371 that age, spermatids massively populate the tubules and first elongated spermatids are
372 differentiated at 28 days of age. Germ cells are not over-represented, thus allowing the
373 identification of a potential defect in somatic cells. In addition, hepatic Cyp1a1 levels were
374 highly induced at 28 days of age, indicating that at this age TCDD was still exerting a direct
375 genomic action. Using RNAs extracted from testes of the TCDD-200 group, we selected a
376 restricted number of genes with fold-changes ± 1.40 . Three genes were up-regulated and five
377 genes were down-regulated. Four genes were further selected based on their identity and the
378 presence of XRE in their flanking regions, indicating that they might represent direct target
379 genes. The upregulated *Glpr1* gene has been found to exert tumour suppressor functions and
380 is a p53 target gene (Li et al., 2008). In addition, it has previously been identified as a dioxin-
381 sensitive gene in human subjects (McHale *et al.*, 2007). Further studies will be required to
382 identify if its up-regulation in 28 and 67-day old rat testes from the TCDD-200 group could
383 contribute to the decline in DSP and sperm reserves observed at 67 days of age.

384 *Insl3* is involved in testicular descent during embryo development (Nef & Parada,
385 1999), and has been suggested to act as a pro-survival factor in germ cells of adult testes
386 (Kawamura *et al.*, 2004). Therefore, it might be speculated that the up-regulation of *Insl3*
387 would counterbalance deleterious effects induced by TCDD. However, expression levels were
388 in the normal range in adult rats of exposed dams. In addition, expression levels at 5 days of
389 age were very low, resulting in high variations in the Q-PCR dosage, and lack of significance.

390 The two other genes validated by quantitative PCR were the chemokines *Cxcl4* and
391 *Ccl5/Rantes*. Chemokines have previously been identified as dioxin-sensitive genes in a
392 microarray analysis of adipocytes treated with TCDD in vitro (Hanlon *et al.*, 2005). *Cxcl4*
393 which was up-regulated at 28 days of age but not at the older ages investigated was not
394 further studied. *Ccl5/Rantes* was expressed as a function of age and down-regulated in the
395 testes of males from TCDD-200 dosed dams throughout development. It has previously been

396 reported in the testis (Le Goffic *et al.*, 2002), and we identified Leydig cells as the major site
397 of Ccl5/Rantes expression. Leydig cells are also a target of the chemokine because specific
398 receptors were detected through RT-PCR (DR, EC and BLMB, unpublished observations).

399 Rantes (for 'regulated upon activation normal T cell expressed and secreted') now
400 given the immunologic designation Ccl5 was originally identified as a typical chemokine as it
401 was able to recruit leukocytes to sites of inflammation. From its discovery, it was found that
402 in addition to T-Cells, Ccl5/Rantes is produced by many other types of cells, including
403 fibroblasts, endothelial and epithelial cells. Moreover, its activity is not merely restricted to
404 chemotaxis. Beneficial activities have been described, including antimicrobial and antiviral as
405 well as detrimental effects. For example, Ccl5/Rantes enhances inflammatory processes and
406 has been associated with the induction or promotion of cancer (Appay & Rowland-Jones,
407 2001; Levy, 2009). Interestingly, Ccl5/Rantes is present in both the male and female genital
408 tract fluids and spermatozoa exhibit specific receptors for the chemokine. However,
409 consistent with high levels found in diseases related to infertility including genital tract
410 infections, Ccl5/Rantes has been shown to impact sperm fertilizing ability negatively
411 (Barbonetti *et al.*, 2008). Therefore, the decrease in Ccl5/Rantes gene expression levels
412 observed in this study in testes of rats born from exposed dams could be part of a protecting
413 mechanism against TCDD impact. It remained to determine if (and to which extent) decreased
414 Ccl5/Rantes gene expression levels contributed to the transitory sperm count decline. For
415 example, sperm counts were normal in 145-day old rats, whereas Ccl5/Rantes gene
416 expression levels were significantly lower than that of controls at that age. In addition, we
417 may have bypassed important genes impacted as well by a TCDD exposure, and contributing
418 with or independently of Ccl5/Rantes in decreasing sperm counts. A transcriptomic study
419 using testes of 67-day old rats may be useful to answer this point.

420 Inhibition of *Ccl5/Rantes* gene expression levels exerted by dioxin was observed 20
421 weeks after dams were given TCDD either 100 or 200 ng/kg bw, and 16 weeks after weaning
422 if considering that the majority of TCDD in offspring of dosed dams has been shown to arise
423 from lactational transfer of TCDD (Li *et al.*, 1995). As TCDD half-life is close to 3 weeks in
424 rats (Bell *et al.*, 2007b), it is possible that increased transcriptional activity coupled to mRNA
425 stability could account for the persisting decreased effects observed at 67 and 145 days of age.
426 Further studies using primary cultures of Leydig cells are warranted to fuel this hypothesis.

427 Several studies have provided evidence that endocrine disrupters might cause
428 epigenetic alterations which could be transmitted to subsequent generations through the male
429 germ line (Anway *et al.*, 2005) or the maternal lineage (Newbold *et al.*, 2006). We thus
430 examined the reproductive performance of the F1 males from TCDD-200 dosed dams, and F3
431 males were also generated using F2 males (not shown). Interestingly, we observed that sperm
432 reserves, DSP, and testicular *Ccl5/Rantes* gene expression levels were in the normal range in
433 the F2 and F3 (not shown) generations. However, because exposure was acute and performed
434 at embryonic day 15 thus at the onset of a common programming window in which androgen
435 action is essential for normal reproductive tract masculinization (Welsh *et al.*, 2008), but a
436 few days after reprogramming of the germ line has occurred (Sasaki & Matsui, 2008; Trasler,
437 2009), chronic dosing studies covering the whole developmental period should be carried out
438 before one could conclude the lack of transgenerational effect of dioxin.

439 In conclusion, our data demonstrated that maternal exposure to TCDD at doses of 100
440 and 200 ng/kg bw impacted the reproductive function of the F1 males. We propose that the
441 chemokine *Ccl5/Rantes* might be regarded as a marker of TCDD exposure. Future studies will
442 help to determine to which extent *Ccl5/Rantes* in Leydig cells may regulate spermatogenesis.
443 In addition, given that TCDD has been shown to exhibit various endocrine disrupting effects
444 both interacting with the estrogenic and androgenic pathways (Ohtake *et al.*, 2003), it would

445 be of interest to determine whether Ccl5/Rantes is modulated as well by chemicals with
446 estrogenic and/or antiandrogenic activities.

447

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455

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665 (SRF-2) gene expression affected by TCDD treatment. *Endocrine Journal* 52, 75-81.
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669 **FIGURE LEGENDS**

670 **Figure 1:** Relative expression of *Cyp1a1* assessed by Q-PCR analysis in the livers of TCDD-
671 200 male rats of the F1 generation at 5 and 28 days of age. The *Gusb* levels-normalized
672 values are the mean \pm S.E.M. of n=3-4 different animals from 3-4 different litters. a, $p<0.05$
673 as compared to age-matched controls.

674
675 **Figure 2:** Relative expression of *Ccl5/Rantes* assessed by Q-PCR analysis in the testes of
676 TCDD-200 and control rats of 5 to 145 days of age. Testes of TCDD-10 and TCDD-100 rats
677 were studied at 67 and 145 days of age. The *Rpl19* levels-normalized values are the mean \pm
678 S.E.M. of n= of 3 to 5 animals from 3 to 5 different litters. a, $p<0.05$ as compared to controls.

679
680 **Figure 3:** Identification of the testicular source of *Ccl5/Rantes*. (A) Total RNA was extracted
681 from 6-day old cultured peritubular cells (Pc) or Sertoli cells (Sc) recovered from 20-day old
682 rat testes; from a fraction enriched in Leydig cells (Lc), a crude fraction of germ cells (TGc)
683 elutriated fractions enriched in spermatids (Stids) or in pachytene spermatocytes (Scytes) (all
684 from adult rat testes). RT-PCR analysis was conducted on 4 independent series of samples,
685 and one representative series is shown. *Rpl19* was used to roughly estimate differences of
686 expression between samples. (B) Immunohistochemical localization of *Ccl5/Rantes* (red
687 labeling) on testis paraffin sections. Staining for 3β -HSD (red labeling) was used to identify
688 Leydig cells in the interstitium. Leydig cells were strongly labeled for *Ccl5/Rantes* by contrast
689 to seminiferous tubules which were weakly labeled. Nuclei are blue-labeled (DAPI staining).
690 Arrowheads point to Leydig cells. Control (-) for *Ccl5/Rantes* indicates that the primary
691 antibody against *Ccl5/Rantes* was omitted. Bar: 100 μ m.

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Table 1: List and sequence of primers used for PCR analysis

Gene Symbol	Accession #	Forward 5' to 3'	Reverse 5' to 3'	Size (bp)	Topt
Ccl5/Rantes (a)	NM_031116	CTTGCAAGTCGTCCTTTGTCAC	GACTAGAGCAAGCAATGACAG	158	58
Ccl5/Rantes (b)	NM_031116	ACCTTGCAAGTCGTCCTTTGTC	ATCTATGCCCTCCCAGGAATG	224	55
Cyp1a1	NM_012540	CAAGAGCTGCTCAGCATAGTC	GCTCAATGAGGCTGTCTGTG	229	58
Glipr1	NM_001011987	TCTCTGCACTAACCCACAACG	GGAGAAGTACTTAGCGATG	124	58
Gusb	NM_017015	CTTCATGACGAACCAGTCAC	GCAATCCTCCAGTATCTCTC	117	58
Insl3	NM_053680	CTGTCTCACTGGCTGCACC	GGGTGTTTCATTGGCACAG	119	58
Pf4	NM_001007729	TTCTTCTGGGTCTGCTGTTG	ATTCTTCAGCGTGGCTATG	197	55
Rpl19	NM_031103	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC	195	58
Sycp1	NM_012810	TTGGGAGAGGTTGAGAAAGC	CCTTTGCTGAAGACTGTTCC	205	58

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The size of the expected PCR fragment in base pairs (bp) and the optimal temperature (Topt) for annealing are reported. Two reference genes were used, Gusb (Glucuronidase Beta) and Rpl19 (Ribosomal protein L19). Two couples were used for Ccl5/Rantes, Ccl5/Rantes (a) for RT-PCR and Ccl5/Rantes (b) for RT-Q-PCR.

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Table 2: F1 pups and weight of the offspring

2.3.7.8 TCDD (ng/kg)	0	10	100	200	
Mean number of pups/dam	12.5±2.7 (9)	11.7±1.5 (3)	13.7±2.5 (3)	10.91±2.9 (9)	
Mean % of males/litter	47.6±19 (9)	62.4±19.2 (3)	59.2±10.1 (3)	48.4±13 (9)	
Male pup weight (g) from 5 to 14 postnatal days					
	5	14.3±2.4 (5)	13.7±1.3 (3)	13.3 ± 1.3 (3)	12.9±1.6 (5)
		13.7±1.7 [32]	13.7±1.3 [17]	13.3± 1.3 [21]	12.6± 1.7 [26] ^a
	7	20.4±3.2 (5)	18.8±1.8 (3)	18.0±1.4 (3)	17.7±2.9 (5)
		19.5±2.2 [28]	18.7±1.8 [19]	17.9±1.4 [22]	17.2±3.0 [22] ^a
	10	28.1±4.0 (5)	26.5±1.6 (3)	26.3±0.8 (3)	25.4±4.1 (5)
		26.9±3.0 [28]	26.4±2.0 [18]	26.3±1.1 [22]	24.7± 4.3 [22] ^a
	12	34.9±5.5 (5)	33.7±1.3 (3)	32.7±1.0 (3)	31.9±4.5 (5)
		33.4±4.1 [28]	33.7±1.7 [18]	32.6± 1.4 [22]	31.0±4.6 [22] ^a
	14	42.0±5.6 (5)	40.6±2.4 (3)	39.9±1.1 (3)	38.5±5.4 (5)
		40.3±4.5 [28]	40.2±2.6 [18]	39.8±1.5 [22]	37.5± 5.6 [22] ^a

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The number of dams studied is indicated in parentheses. The total number of pups weighed is indicated in brackets. Male pups were regularly weighed during the first two weeks. Values are mean ± SEM of (n) litter. For pup weight values are mean ± SD of (n) litter or [n] pups. Statistical analysis was performed using ANOVA followed by the multiple comparisons Dunett's test. No significance was observed if data were expressed per litter; a, p<0.05 as compared to time-matched controls if considering data expressed per male pup.

747 **Table 3: Epididymal sperm reserves and daily sperm production (DSP) in 67-day old rats of the**
 748 **TCDD-10, TCDD-100, TCDD-200 and control groups.**

2,3,7,8 TCDD (ng/kg bw)		0	10	100	200
Epididymal Sperm Reserves (10⁶)					
Caput	(litter)	48.8±0.65 (9)	46.9±2.3 (3)	42.4±2.6 (3)	39.9±4.3 (9) P=0.06
	(rat)	48.5±2 (12)	47.2±1.6 (7)	42.1±1.5 (8)	39.5±3.6 (10) P=0.013
Corpus	(litter)	7.6±0.52 (9)	8.8±1.7 (3)	6.8±1.1 (3)	8.6±1.9 (9)
	(rat)	7.4±0.72 (12)	8.4±1.3 (7)	6.9±0.7 (8)	8.3±1.6 (10)
Cauda	(litter)	59.9±3.2 (9)	67.7±1.3 (3)	59.4±1.1 (3)	46.5±3.4 (9) P=0.056
	(rat)	62.4±3.3 (12)	70.5±7.1(7)	59.9±6.4 (8)	45.9±3.1(10) P=0.021
Daily Sperm Production (10⁶)					
	(litter)	25.9±0.9 (9)	25.2±3 (3)	25.7±1.8 (3)	22.5±1 (9) P=0.052
	(rat)	26.3±1.04 (12)	25.3±1.9 (7)	25.5±6.4 (8)	22.4±0.9 (10) P=0.06

774 Values are mean ± SEM and are expressed per litter (3 to 9) and per rat (7 to 12). Statistical analysis
 775 was performed using ANOVA followed by the multiple comparisons Dunett's test, and the *p* value is
 776 reported for the TCDD-200 group as compared to the control group.

781 **Table 4: List of genes up and down regulated in 28 day-old rat testes from the TCDD-200 group**

Gene symbol and known function or localization	Accession number	Micro Array Fold Change	Q PCR Fold Change (n=3)	Number of 5' flanking XRE in mouse orthologous genes
Insl3 Leydig cells	NM_053680	1.49	1.41 ± 0.10	6
Glipr1 Tumor suppressor	NM_001011987	1.45	1.65 ± 0.10	1
Cxcl4 Chemokine	NM_001007729	1.39	1.66 ± 0.24	2
Q4V7D5	XM_576624	-1.48	nd	nd
RGD1561017	XM_577094	-1.53	nd	nd
RT1-CE5 variant 1, variant 2	NM_001008843, NM_001033986	-1.53	nd	nd
LOC679900	XM_574899	-1.83	nd	nd
Ccl5/Rantes Chemokine	NM_031116	-1.86	-2.70 ± 0.05	3

782 Data for Q PCR are mean ± SEM of 3 testis samples from 3 different litters. nd, not defined.

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Table 5: Generation of F2 males raw data of end-points surveyed

End-points surveyed		Control	TCDD 200 ng/kg bw
Number of males		8	7
Number of dams		16	14
Mean number of pups/dam		13.19±3 (16)	12.64±3 (16)
Mean % of males/litter		51.6±12.2 (16)	47.2±13 (16)
Male pup weight (g)	5 days	12.7±1.1 (8)	13.9±2.2 (7)
		12.6± 1.4 [51]	13.2± 1.9 [30]
	8 days	19.9±1.7 (8)	20.2±3.9 (7)
		19.7± 2.4 [51]	19.2±3.7 [30]
Q PCR data (relative expression)			
Hepatic cyp1a1 levels	20 days	2.7±3.2 (3)	1.6±1.9 (3)
Testicular Ccl5/Rantes levels	67 days	0.84±0.09 (4)	1.06±0.45 (3)
	145 days	1.05±0.13 (4)	1.31±0.23 (3)
Daily Sperm Production (10⁶)	67 days	22.7±1.02 (4)	25.3±4.1 (3)
	145 days	31.3±3.4 (4)	31.4±1.3 (3)
Epididymal Sperm Reserves (10⁶)	67 days	73.5±9.7 (4)	95.5±9.8 (3)
	145 days	244.9±29.2 (4)	261.5±12.9 (3)

The number of dams studied is indicated in parentheses. The total number of pups weighed is indicated in brackets. Values for pup weight are the mean ± SD of (n) litters or [n] pups. Data were not significant. Q PCR data, DSP and sperm reserves were expressed as mean ± SEM of (n) samples, each sample originated from a different litter.

825 Supplemental Data: Description of the 2 preliminary experiments settled up to determine the
826 dose-range of TCDD

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828 Experiment 1: Four groups of five dams each were orally treated with 0, 27, 140 or
829 270 ng/kg bw. Timing of dosing was Embryonic Day 11. Animals were killed at different
830 developmental ages. Intra-testicular content of testosterone and 4-androstenedione were
831 performed in samples collected at different periods of development. We also performed
832 histological analysis and fertility tests. Sperm reserves were counted in animals aged of 159
833 days. No treatment-related differences were noted with regard to the outcome of gestation, the
834 litter size, the sex-ratio or the body weights of fetuses from females that were dosed with the
835 two lower doses of TCDD. However, one dam had aborted and a second one gave birth to 10
836 dead fetuses in the group administered the highest dose. We did not collect samples from the
837 dead fetuses, and the origin of their death has not been investigated. The gross histology of
838 the in utero exposed rat testes of the different TCDD dosed groups including the highest
839 dosed-group was comparable to the control group. Intra-testicular hormone levels in the
840 treated animals were also in the normal range. Animals could reproduce and sperm reserves
841 assessed at 159 days of age were in the normal range.

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843 Experiment 2: A unique dose of TCDD of 1000 ng/kg bw was administered at
844 Embryonic Day 15 to mimic the design of previous experiments leading to a spectrum of
845 effects in the reproductive system of the male offspring (Ref. in Introduction). Two groups of
846 5 dams each were handled. The five control dams had normal gestation, normal litter size and
847 sex-ratio. In the TCDD-dosed dams group, one dam died before giving birth, 2 dams had
848 eaten their fetuses soon after delivery, one dam gave birth to one male, and one dam gave
849 birth to one male and two females. The four pups died a few days after birth. The three
850 surviving dams were killed showing apathy and stuck and bristly hair.

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852 Aliquots containing various doses of dioxins were assayed by Dioxlab (Dioxlab,
853 94417 Saint-Maurice) to ascertain doses given to the animals. Therefore, maternal and foetal
854 toxicity were observed in our hands at the dose of 270 ng/kg bw, and death of dams occurred
855 at the dose of 1000 ng/kg bw.

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Figure 1:

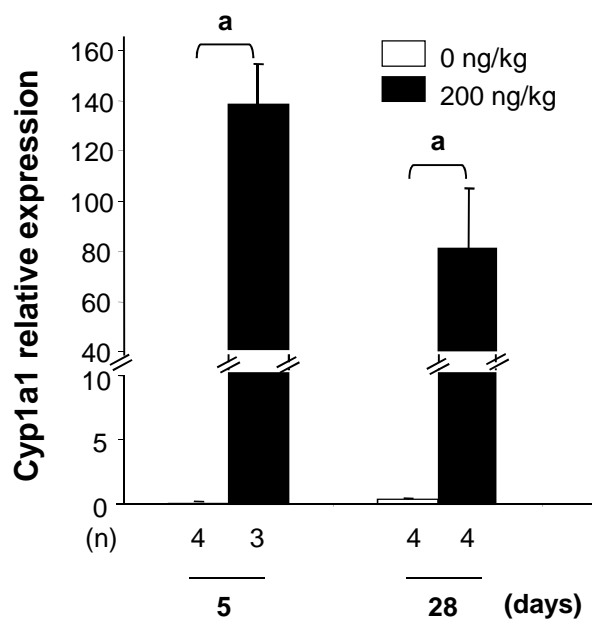


Figure 2:

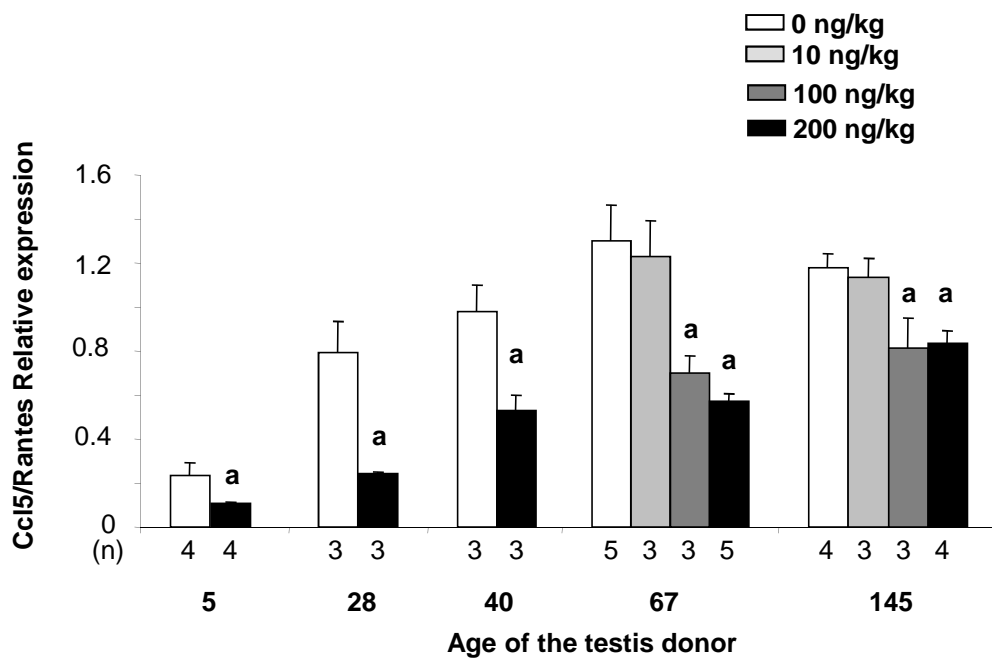


Figure 2:

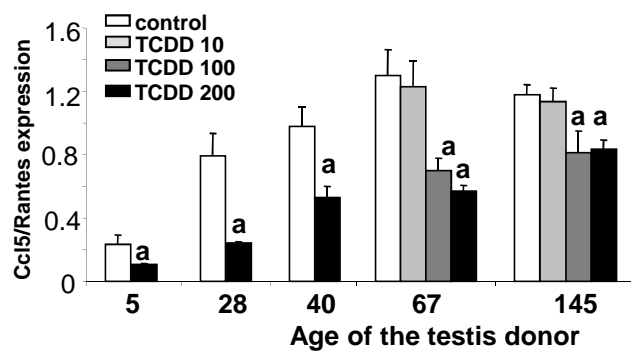
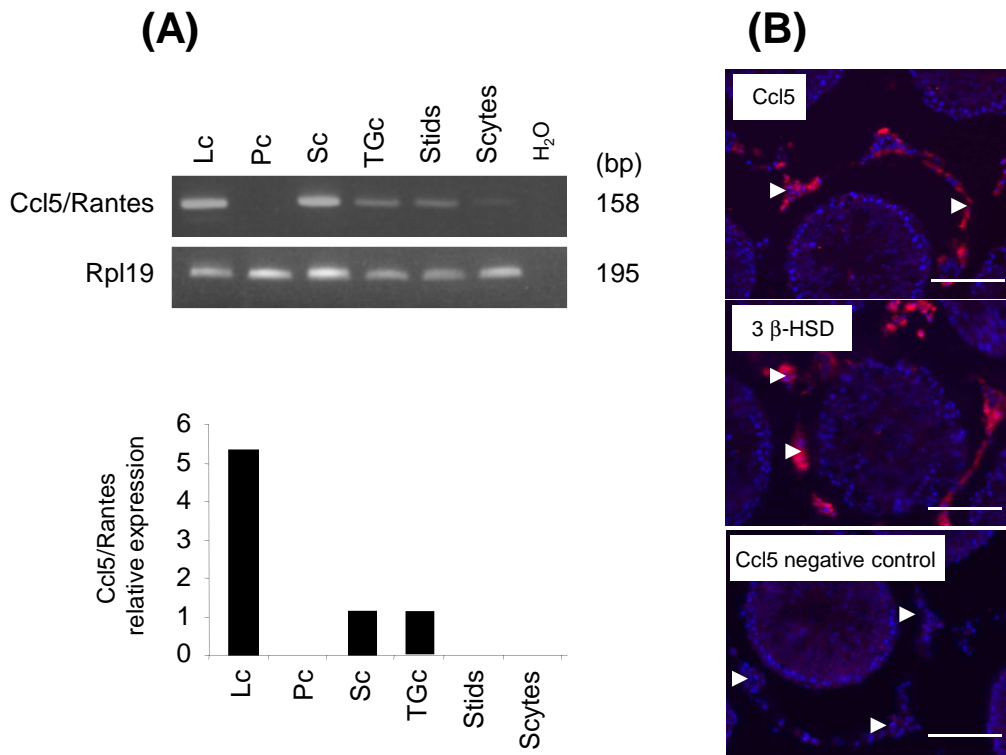


Figure 3:



2,3,7,8 TCDD (ng/kg bw)		0	10	100	200
Epididymal Sperm Reserves (10⁶)					
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	(rat)	62.4±3.3 (12)	70.5±7.1(7)	59.9±6.4 (8)	45.9±3.1(10) P=0.021
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Male pup weight (g) from 5 to 14 postnatal days				
	5			
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	13.7±1.7 [32]	13.7±1.3 [17]	13.3± 1.3 [21]	12.6± 1.7 [26] ^a
	7			
	20.4±3.2 (5)	18.8±1.8 (3)	18.0±1.4 (3)	17.7±2.9 (5)
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	10			
	28.1±4.0 (5)	26.5±1.6 (3)	26.3±0.8 (3)	25.4±4.1 (5)
	26.9±3.0 [28]	26.4±2.0 [18]	26.3±1.1 [22]	24.7± 4.3 [22] ^a
	12			
	34.9±5.5 (5)	33.7±1.3 (3)	32.7±1.0 (3)	31.9±4.5 (5)
	33.4±4.1 [28]	33.7±1.7 [18]	32.6± 1.4 [22]	31.0±4.6 [22] ^a
	14			
	42.0±5.6 (5)	40.6±2.4 (3)	39.9±1.1 (3)	38.5±5.4 (5)
	40.3±4.5 [28]	40.2±2.6 [18]	39.8±1.5 [22]	37.5± 5.6 [22] ^a

Male pups were regularly weighed during the first two weeks. Values are the mean ± SD of (n) litter. The number of total pups weighed is reported in [] and statistical analysis was performed using ANOVA followed by the multiple comparison Dunnett's test. ^a p<0.05 as compared to time matched controls.

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	145 days	244.9±29.2 (4)	261.5±12.9 (3)

Data are mean ±SD of (n) litter for pups weight and ±SEM for QPCR, DSP and sperm reserves. The number of rats for male pup weight is indicated in [].