5. DEVELOPMENTAL AND REPRODUCTIVE TOXICITY*

5.1. INTRODUCTION

The potential for dioxins and related compounds to cause reproductive and developmental toxicity has been recognized for many years. Recent laboratory studies have broadened our knowledge in this area and demonstrate that altered development is among the most sensitive endpoints of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This chapter reviews much of the literature on dioxin's developmental and reproductive toxicity but is not intended to be exhaustive. Special emphasis is placed on that part of the database that has accumulated since the last major EPA review of this topic (Kimmel, 1988; Peterson et al., 1993). In addition, the database is viewed in light of the Ah receptor model of TCDD action that is being examined for its applicability in the current EPA risk assessment.

To focus the analysis of the database, the chapter is divided into developmental toxicity and male and female reproductive toxicity. The authors recognize the interrelatedness of developmental and reproductive events at all levels of biological complexity. Therefore, the reader should not view the chapter subheadings within each of these divisions as defining discrete endpoints that are exclusive of other endpoints. For example, the effects of TCDD on circulating levels of sex hormones or on responsiveness to sex hormones may be translated into reproductive dysfunction if exposure occurs in adulthood or abnormal development of sexual behavior if exposure occurs perinatally. Likewise, even though organ structure and growth are considered separate manifestations in developmental toxicity that are associated with perinatal exposure to TCDD, the normal development of an organ is dependent on normal growth processes, and inhibiting perinatal growth can significantly disrupt the structural integrity of an organ system.

2,3,7,8-TCDD is one of 75 possible CDD congeners and 135 possible CDF congeners. It is one of the most potent of the CDDs, BDDs, CDFs, BDFs, PCBs, and PBBs, and as such serves as the prototype congener for investigating the toxicity elicited by these classes of chemicals. Developmental and reproductive toxicity is generally believed to be caused by the parent compound; there is no evidence that TCDD metabolites are involved. The toxic potency of TCDD is due to the number and position of chlorine substitutions on the dibenzo-*p*-dioxin molecule. CDD congeners with decreased lateral (2, 3, 7, and 8) or increased nonlateral chlorine and bromine substituents are less potent than TCDD (Safe, 1990); however, most of these

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congeners will produce toxicity, and the pattern of responses within animals of the same species, strain, sex, and age will generally be similar to that of TCDD (McConnell and Moore, 1979; Poland and Knutson, 1982). PCB congeners with zero or one *ortho* chlorines, two *para* chlorines, and at least two *meta* chlorines can assume a coplanar conformation sterically similar to TCDD and also produce a pattern of toxic responses similar to that of TCDD. In contrast, PCB congeners with two or more *ortho* chlorines cannot assume a coplanar conformation and do not resemble TCDD in toxicity (Poland and Knutson, 1982; Safe, 1990).

CDD and CDF congeners chlorinated in the lateral positions, as compared with those lacking chlorines in the 2, 3, 7, and 8 positions, are preferentially bioaccumulated by fish, reptiles, birds, and mammals (Stalling et al., 1983; Cook et al., 1991; U.S. EPA, 1991). Furthermore, coplanar PCBs and/or monoortho-chlorine-substituted analogs of the coplanar PCBs bioaccumulate in fish, wildlife, and humans (Tanabe, 1988; Kannan et al., 1988; Mac et al., 1988; Kubiak et al., 1989; Smith et al., 1990). This is of concern because combined effects of the lateral-substituted CDD, BDD, CDF, BDF, PCB, and PBB congeners acting through an Ah receptor mechanism have the potential of decreasing feral fish and wildlife populations secondary to developmental and reproductive toxicity (Gilbertson, 1989; Walker and Peterson, 1991; Walker et al., 1991; Cook et al., 1991). Humans are not exempt from the developmental and reproductive effects of complex halogenated aromatic hydrocarbon mixtures. Such mixtures that contain both TCDD-like congeners and non-TCDD-like congeners have been implicated in causing developmental and reproductive toxicity in the Yusho and Yu-Cheng poisoning incidents in Japan and Taiwan (Kuratsune, 1989; Hsu et al., 1985; Rogan, 1989). Thus, exposure to TCDD-like congeners is a health concern for humans as well as for domestic animals, fish, and wildlife, although the relative contributions of TCDD-like and non-TCDD-like congeners are not known in some exposure situations.

A mechanism of action that CDD, BDD, CDF, BDF, PCB, and PBB congeners substituted in the lateral positions have in common is that they bind to the Ah receptor, which dimerizes with the Ah receptor nuclear translocator protein (ARNT) in the nucleus. These liganded heterodimeric complexes bind to specific sequences of DNA referred to as dioxin-responsive enhancers (DREs), resulting in alterations in gene transcription. There is evidence that this Ah receptor mechanism may be involved in the antiestrogenic action of TCDD and in its ability to produce the structural malformations of cleft palate and hydronephrosis in mice. Recent interest has also focused on the potential nonnuclear interaction of the Ah receptor within the cell (see Chapter 2).

5.2. DEVELOPMENTAL TOXICITY

The manifestations of developmental toxicity from exposure to TCDD have been divided into three categories for convenience in assessing the database with respect to an Ah receptor-mediated response. These categories include death/growth/clinical signs, structural malformations, and postnatal functional alterations. Exposure-related effects on death/growth/clinical signs are described for fish, birds, laboratory mammals, and humans along with structure-activity results that are consistent with, but do not prove, an Ah receptor-mediated mechanism. Structural malformations, particularly cleft palate formation and hydronephrosis, occur in mice. In other mammalian species, however, postnatal functional alterations, some of which may be irreversible, are the most sensitive adverse developmental effects of TCDD-like congeners. These include effects on the male and female reproductive systems in rats and hamsters, and object learning behavior in monkeys.

5.2.1. Death/Growth/Clinical Signs

5.2.1.1. *Fish*

Early life stages of fish appear to be more sensitive to TCDD-induced mortality than adults. This is suggested by the LD_{50} of TCDD in rainbow trout sac fry (0.4 μg/kg egg weight) being 25 times less than that in juvenile rainbow trout (10 μg/kg body weight) (Walker and Peterson, 1991; Kleeman et al., 1988). The significance of this finding is that early life stage mortality caused by high concentrations of TCDD-like congeners in fish eggs may pose the greatest risk to feral fish populations (Walker and Peterson, 1991; Cook et al., 1991). Cooper (1989) reviewed the developmental toxicity of CDDs and CDFs in fish, and Cook et al. (1991) discussed components of an aquatic ecological risk assessment for TCDD in fish. The reader is referred to this literature for more indepth coverage than is presented here.

TCDD is directly toxic to early life stages of fish. This has been demonstrated for Japanese medaka, pike, rainbow trout, and lake trout exposed as fertilized eggs to graded concentrations of waterborne TCDD. In these species, TCDD causes an overt toxicity syndrome characterized by edema, hemorrhages, and arrested growth and development culminating in death (Helder, 1980, 1981; Wisk and Cooper, 1990a; Spitsbergen et al., 1991; Walker et al., 1991; Walker and Peterson, 1991). Histopathologic evaluation of lake trout embryos and sac fry has shown this syndrome to be essentially identical to that of blue sac disease (Helder, 1981; Spitsbergen et al., 1991). Following egg exposure to TCDD, signs of toxicity are not detected in medaka until after the liver rudiment forms (Wisk and Cooper, 1990a), and in lake trout toxicity is first detected ~1 week prior to hatching but becomes fully manifest during the sac fry stage (Spitsbergen et al., 1991; Walker et al., 1991). Among all fish species investigated thus far, lake trout are the most sensitive to TCDD developmental toxicity. Following exposure of fertilized

lake trout eggs to graded waterborne concentrations of TCDD, the no observable adverse effect level (NOAEL) for sac fry mortality is 34 pg TCDD/g egg, the lowest observed adverse effect level (LOAEL) is 55 pg TCDD/g egg, and the egg TCDD concentration that causes 50% mortality above control at swim up (LD_{50}) is 65 pg TCDD/g egg (Walker et al., 1991). Thus, TCDD is a potent developmental toxicant in fish, and the effect is not secondary to maternal toxicity.

The Ah receptor has not been identified in early life stages of fish; however, it is assumed to be present because PCBs induce hepatic cytochrome P-4501A1 in lake trout and brook trout embryos and fry (Binder and Stegeman, 1983; Binder and Lech, 1984). The Ah receptor has been identified in adult rainbow trout liver (Heilmann et al., 1988) and in a rainbow trout hepatoma cell line (Lorenzen and Okey, 1990). CDD and CDF congeners that are approximate isostereomers of TCDD produce essentially the same pattern of toxic responses as TCDD in early life stages of medaka and rainbow trout, suggesting that they may act through a common mechanism (Wisk and Cooper, 1990b; Walker and Peterson, 1991). Also, in rainbow trout their potencies relative to TCDD (i.e., toxic equivalency factors, TEFs) for causing early life stage mortality (TCDD LD_{50}/congener LD_{50}) are in the same range as those proposed for human health risk assessment based on a diverse spectrum of acute and subchronic toxicity tests in mammalian species (Safe, 1990; Walker and Peterson, 1991). However, for the coplanar PCBs and monoortho-chlorinated analogs of the coplanar PCBs, TEFs based on early life stage mortality in rainbow trout are 1/14 to 1/80 less (Walker and Peterson, 1991) than the TEFs proposed for risk assessment (Safe, 1990).

5.2.1.2. *Birds*

Bird embryos are also more sensitive to TCDD toxicity than adults. The LD_{50} of TCDD in the chicken embryo (0.25 μg/kg egg weight) is 100 to 200 times less than the TCDD dose that causes mortality in adult chickens (25-50 μg/kg body weight) (Greig et al., 1973; Allred and Strange, 1977). The LD_{50} of TCDD injected into fertilized ring-necked pheasant eggs (1.1-1.8 μg/kg egg weight) is 14 to 23 times less than the TCDD dose that causes 75% mortality in ring-necked hen pheasants (25 μg/kg body weight) (Nosek et al., 1993).

Among bird species, most developmental toxicity research has been done on chickens. Injection of TCDD or its approximate isostereomers into fertilized chicken eggs causes a toxicity syndrome in the embryo characterized by pericardial and subcutaneous edema, liver lesions, inhibition of lymphoid development in the thymus and bursa of Fabricius, microphthalmia, beak deformities, cardiovascular malformations, and mortality (Cheung et al., 1981a,b; Brunstrom and Darnerud, 1983; Rifkind et al., 1985; Brunstrom and Lund, 1988; Brunstrom and Andersson, 1988; Nikolaidis et al., 1988a,b). On the other hand, injection of a coplanar PCB into fertilized

turkey eggs at a dose high enough to cause microphthalmia, beak deformities, and embryo mortality did not produce liver lesions, edema, or thymic hypoplasia, all hallmark signs of TCDD toxicity in the chicken embryo (Brunstrom and Lund, 1988). This disparity in signs of TCDD embryotoxicity among bird species is not unique to the turkey and chicken. In fertilized eggs of ring-necked pheasants and eastern bluebirds, injection of TCDD produces embryo mortality, but all of the other signs of toxicity seen in the chicken embryo are absent, including cardiovascular malformations (Thiel et al., 1988; Martin et al., 1989; Nosek et al., 1993). Thus, in bird embryos the signs of toxicity elicited by TCDD and its approximate isostereomers are highly species dependent; the only toxic effect common to all bird species is embryo mortality.

There is evidence in chicken embryos that the Ah receptor may be involved in producing developmental toxicity. The Ah receptor has been detected in chicken embryos (Denison et al., 1986; Brunstrom and Lund, 1988) and the rank order potency of PCB congeners for producing chicken embryo mortality (3,3',4,4',5-PCB > 3,3',4,4'-TCB > 3,3',4,4',5,5'-HCB > 2,3,3',4,4'-PCB > 2,3,4,4',5-PCB, with 2,2',4,5'-TCB, 2,2',4,4',5,5'-HCB, and 2,2',3,3',6,6'-HCB being inactive) is similar to that for a classic Ah receptor-mediated response in the chicken embryo, cytochrome P-4501A1 induction (Rifkind et al., 1985; Brunstrom and Andersson, 1988; Brunstrom, 1989). However, although induction of cytochrome P-4501A1 and toxicity may both be part of a pleiotropic response linked to the Ah receptor, they are not otherwise causally related. This is demonstrated by the nonsteroidal anti-inflammatory drug benoxoprofen that suppresses 3,3',4,4'-TCB-induced toxicity in the chicken embryo without altering its ability to induce microsomal enzyme activity (Rifkind and Muschick, 1983). Also, for 3,3',4,4'-TCB, 3,3',4,4',5,5'-HCB, and TCDD there is a marked dissociation of the dose-response relationship for lethality and enzyme induction in the chicken embryo (Rifkind et al., 1985).

A decreased activity of uroporphyrinogen decarboxylase (URO-D) and an increased accumulation of uroporphyrins are effects that are readily produced by exposure of cultured chicken embryo liver cells to TCDD, 3,3',4,4'-TCB, and other PCBs (Sinclair et al., 1984; Marks, 1985; Lambrecht et al., 1988). Coplanar PCB congeners are more potent inhibitors of URO-D activity in cultured chicken embryo liver cells than are noncoplanar PCB congeners (Sassa et al., 1986), suggesting an Ah receptor-mediated mechanism. Unlike the results in cultured cells, however, a lethal dose of TCDD (6 nmol/egg) does not affect URO-D activity or cause an increased accumulation of uroporphyrins in chicken embryos (Rifkind et al., 1985). Thus, TCDD-induced lethality in chicken embryos is not associated with the effects of TCDD on URO-D activity, even though a decrease in URO-D activity might be expected to occur if a sufficient dose of TCDD could be reached without being lethal.

The chicken embryo heart is a target organ for TCDD and other halogenated aromatic hydrocarbons that act by an Ah receptor mechanism. Expression of the Ah receptor occurs

ubiquitously in cardiac myocytes, while ARNT expression is restricted to myocytes that overlay the atrioventricular canal, outflow tract, and atrial and ventricular septa (Walker et al., 1997). Both Ah receptor and ARNT appear to be absent from the endocardium and endocardial- derived mesenchyme. In addition, cardiac expression of cytochrome P-4501A1 is restricted to myocardium that expresses Ah receptor and ARNT. The classic sign of chick embryo toxicity involving the heart is pericardial edema. However, TCDD has other effects on the chick embryo heart that are less well known. These include its ability to produce cardiovascular malformations and to increase cardiac release of arachidonic acid metabolites. When fertilized chicken eggs are injected with graded doses of TCDD, cardiovascular malformations are produced including ventricular septal defects, aortic arch anomalies, and conotruncal malformations. Approximately 1.6 pmol TCDD/egg (9 ng/kg egg, assuming a 55 g egg weight) causes cardiovascular malformations in 46% of treated embryos versus 29% of control embryos (Cheung et al., 1981a,b). The cardiovascular malformation response may be unique to the chicken embryo because in fertilized ring-necked pheasant and eastern bluebird eggs injected with TCDD the incidence of such malformations is not increased (Thiel et al., 1988; Martin et al., 1989; Nosek et al., 1993).

In the chicken embryo heart, arachidonic acid metabolism is stimulated by TCDD, resulting in increased formation of prostaglandins (Quilley and Rifkind, 1986). Dose-response relationships for the release of immunoreactive PGE_2, PGF_{2a}, and TxB_2 from chick embryonic heart are biphasic, with an apparent maximally effective dose of 100 pmol TCDD/egg. When the egg tetrachloroabenzene (TCDD) dose is further increased, release of these prostaglandins tends to decline towards levels in control hearts. Biphasic dose-response curves for cardiac PGE_2 release also were obtained with 3,3',4,4'-TCB and 3,3',4,4',5,5'-HCB (Quilley and Rifkind, 1986). The thymus and bursa of Fabricius are other TCDD target organs in the chicken embryo. TCDD, 3,3',4,4'-TCB, and 3,3',4,4'-TCAOB cause dose-related decreases in the lymphoid development of both of these immune system organs (Nikolaidis et al., 1988a,b, 1990). Cultured thymus anlage from chick embryos are 100 times more sensitive to TCDD's inhibitory effect on lymphoid development than cultured thymus anlage from turkey and duck embryos (Nikolaidis et al., 1988a). This suggests that the reason thymic atrophy was not seen in turkey embryos at egg doses of 3,3',4,4'-TCB that were overtly toxic (Brunstrom and Lund, 1988) was not because the turkey embryo thymus was incapable of responding to 3,3',4,4'-TCB. Rather, turkey embryos appear to be more sensitive to the lethal than to the immunotoxic effect of this coplanar PCB.

Within the same bird species, the signs of developmental toxicity elicited by TCDD and its approximate isostereomers are similar. In the chicken embryo, TCDD, 3,3',4,4',5-PCB, 3,3',4,4'-TCB, and 3,3',4,4',5,5'-HCB all cause pericardial and subcutaneous edema, liver lesions, microphthalmia, beak deformities, and mortality, and TCDD, 3,3',4,4'-TCB, and 3,3',4,4'-

TCAOB inhibit lymphoid development (Cheung et al., 1981a; Brunstrom and Andersson, 1988; Nikolaidis et al., 1988a,b). In pheasant embryos, an altogether different pattern of responses is seen. Nevertheless, the TCDD-like congeners injected into fertilized pheasant eggs, TCDD and 3,3',4,4'-TCB, produce the same pheasant embryo-specific pattern. This pattern consists of embryo mortality in the absence of edema, liver lesions, thymic hypoplasia, and structural malformations (Brunstrom and Reutergardh, 1986; Nosek et al., 1993).

The lethal potency of TCDD and its approximate isostereomers in embryos of different bird species varies widely. The chicken embryo is an outlier in that it is by far the most sensitive of all bird species to TCDD. Turkey, ring-necked pheasant, mallard duck, domestic duck, domestic goose, golden-eye, herring gull, black-headed gull, and eastern bluebird embryos are considerably less sensitive to the embryo-lethal effect of TCDD and TCDD-like congeners (Brunstrom and Reutergardh, 1986; Brunstrom and Lund, 1988; Thiel et al., 1988; Martin et al., 1989; Elliott et al., 1989; Nosek et al., 1993). TCDD is 4 to 7 times more potent in causing embryo mortality in chicken than pheasant embryos, and 3,3',4,4'-TCB is 20 to 100 times more potent in chicken than turkey embryos (Allred and Strange, 1977; Brunstrom and Lund, 1988; Nosek et al., 1989). In chicken embryos, an egg dose of 3,3',4,4'-TCB of 4 μg/kg increased embryo mortality, whereas an egg dose of 100 μg/kg of the same coplanar PCB had no embryotoxic effect in pheasants and mallard ducks and a dose of 1,000 μg/kg egg had no effect on embryo mortality in domestic ducks, domestic geese, golden eyes, herring gulls, and black-headed gulls (Brunstrom, 1988; Brunstrom and Reutergardh, 1986). In contrast to the above species differences, the potency of 3,3',4,4'-TCB in causing embryo mortality among different strains of chickens is quite similar, with the LD_{50} in six different strains varying less than fourfold (Brunstrom, 1988).

Graded doses of TCDD have been administered to fertilized eastern bluebird and ring-necked pheasant eggs for the purpose of determining a LOAEL and NOAEL for embryotoxicity. Mortality was the most sensitive embryotoxic effect in both species. For eastern bluebirds, the LOAEL was 10,000 pg TCDD/g egg and the NOAEL was 1,000 pg TCDD/g egg (Martin et al., 1989). For ring-necked pheasants, the LOAEL was 1,000 pg TCDD/g egg and the NOAEL was 100 pg TCDD/g egg. The LD_{50} for embryo mortality in the ring-necked pheasant is 1,354 pg TCDD/g egg when the dose is injected into the egg albumin and 2,182 pg TCDD/g egg when the dose is injected into the egg yolk (Nosek et al., 1993). In contrast, for chickens the LD_{50} for embryo mortality is 240 pg TCDD/g egg (Allred and Strange, 1977).

5.2.1.3. *Laboratory Mammals*

5.2.1.3.1. ***Developmental expression of Ah receptor and ARNT***. The Ah receptor and its dimerization partner ARNT are expressed in a specific spatial and temporal pattern in the

developing mammalian embryo and fetus, suggesting that they play a fundamental role in development. Preimplantation mouse embryos express Ah receptor mRNA and protein (Peters et al., 1995), and spatial and temporal patterns of ARNT1, ARNT2, and Ah receptor mRNA expression occur in specific developing tissues and organs of the mouse embryo from gestational day 9.5 to 16 (Abbott et al., 1995; Abbott et al., 1995; Jain et al., 1998). On gestational day 9.5, ARNT1 mRNA is highly expressed in the neuroepithelium of the brain and spinal cord, trigeminal ganglion, branchial arches 1 and 2, heart, hepatic primordia, and primitive gut. ARNT2 message is also expressed in the neuroepithelium and in the remainder of the embryo but at comparatively lower levels. Ah receptor mRNA, in contrast to ARNT1 and ARNT2, is not expressed significantly at gestational day 9.5, but by gestational day 10 Ah receptor mRNA was expressed in the neuroepithelium of the developing brain, in the visceral arches, and in the heart. By day 13.5 or 14 of gestation, Ah receptor mRNA was abundantly expressed in the primitive pituitary, palatal shelf, nasal septal cartilage, dorsal surface of the tongue, developing thymus, lung parenchyma, liver, developing gut mucosa, kidney, urogenital sinus, and tip of the genital tubercle. ARNT1 mRNA was expressed to a high extent in various cell types of endodermal and mesodermal origin such as the lung and tongue muscle and was barely above background in the developing nervous system. The tissue distribution of ARNT2 mRNA was the inverse of ARNT1, being highest in the mantle layer of the spinal cord and brain and lowest in the endodermal and mesodermally derived tissues. The expression patterns observed at gestational day 13.5 or 14 continued to be found at gestational day 15.5 or 16 with the additional finding that ARNT2 was clearly expressed in neural crest derivatives like the dorsal root ganglia, adrenal medulla, and in developing tubules in the renal cortex. Thus, the expression of Ah receptor, ARNT1, and ARNT2 mRNAs was specific for cell type, organ/tissue, and developmental stage. Furthermore, immunohistochemical localization of Ah receptor and ARNT1 protein correlated, in general, with in situ localization of Ah receptor and ARNT1 mRNA expression at each gestational age (Abbott et al., 1995; Abbott and Probst, 1995).

5.2.1.3.2. ***Transgenic Ah receptor null mice and ARNT null mice.*** Ah receptor null (knockout) mice have been developed to determine which adverse effects of TCDD exposure are Ah receptor mediated, and to determine the effects of absence of the Ah receptor on organ system development and function. Three lines of Ah receptor null mice have been generated using different targeting methods and they are on the following genetic backgrounds: C57BL/6N x Sv/129 (Fernandez-Salguero et al., 1995), substrain of C57BL/6 x Sv/129 (Schmidt et al., 1996) and C57BL/6J x Sv/129 (Mimura et al., 1997). Ah receptor null mice in all the lines are viable, and offspring of both sexes are fertile and capable of reproduction. However, Abbott et al. (1999) reported adverse reproductive outcomes in homozygous AhR null female mice in the line

of Fernandez-Salguero et al. (1995). The range of adverse reproductive effects included deaths of the females during pregnancy and lactation, small litter size at birth, poor survival of pups during the first 2 weeks after birth, and death of Ah receptor null pups after weaning. Because low survival of the weaned homozygous Ah receptor null pups was independent of genotype of the dam, it was probably not caused by maternal factors like lactational insufficiency or aberrant maternal behaviors. However, the increased mortality of fetuses and pups prior to weaning could be due in part to impaired ability of the homozygous Ah receptor null female to support development of the fetuses, to survive pregnancy and lactation herself, and to rear pups until weaning (Abbott et al., 1999c).

The profile of effects observed in the homozygous Ah receptor null offspring were dependent on the Ah receptor null line investigated and consisted of lesions in the immune system, skin, liver, heart, stomach, spleen, and uterus (Fernandez-Salguero et al., 1995, 1996, 1997; Schmidt et al., 1996). These findings suggest that the AhR signaling pathway plays an important physiological role in development and in maintaining homeostasis as offspring age (Fernandez-Salguero et al., 1996, 1997; Schmidt et al., 1996;). Transgenic mice with a null mutation in the ARNT1 gene have also been evaluated, and unlike their AhR counterparts homozygous ARNT1 null embryos are not viable (Kozak et al., 1997; Maltepe et al., 1997). They are affected by neural tube closure defects, forebrain hypoplasia, delayed rotation of the embryo, placental hemorrhaging, visceral arch abnormalities, and death between gestational days 9.5-10.5 (Kozak et al., 1997). The primary cause of embryo mortality may be failure of the embryonic component of the placenta to vascularize and form the labyrinthine spongiotrophoblast, which is consistent with ARNT1's role in hypoxic induction of angiogenesis (Kozak et al., 1997). Thus, the ARNT1 protein appears to play an indispensable role during development that is essential for embryo survival.

5.2.1.3.3. ***Prenatal mortality.*** When exposed to TCDD during adulthood, laboratory mammals display wide differences in the LD_{50} of TCDD. It is interesting to note, however, that when exposure occurs during prenatal development, the potency of TCDD tends to be more similar across species. The LD_{50} of TCDD in adult hamsters, 1,157 to 5,051 μg/kg, makes adult hamsters three orders of magnitude more resistant to TCDD-induced lethality than are adult guinea pigs (Olson et al., 1980; Henck et al., 1981). Yet, a maternal dose of 18 μg TCDD/kg can increase the incidence of prenatal mortality in the hamster embryo/fetus. Because this dose is only twelvefold higher than the dose (1.5 μg TCDD/kg) that increases the incidence of prenatal mortality in the guinea pig, the hamster embryo/fetus approaches other rodent species in its sensitivity to TCDD-induced lethality (Olson and McGarrigle, 1990, 1991). Thus, the magnitude

of the species differences in lethal potency of TCDD is affected by the timing of TCDD exposure during the life history of the animal.

Exposure to TCDD during pregnancy causes prenatal mortality in the monkey, guinea pig, rabbit, rat, hamster, and mouse (Table 5-1). The rank order of susceptibility from the most sensitive to least sensitive species would appear to be monkey = guinea pig > rabbit = rat = hamster > mouse. However, an important caveat must be applied to the information presented in Table 5-1; i.e., that the time period during which exposure of the embryo/fetus to TCDD occurs is just as important a determinant of prenatal mortality as is the dose of TCDD administered. This point will be illustrated in the text that follows when prenatal mortality is described for different strains of mice.

It is important to note that the concept of a critical time period for exposure makes the analysis of lethality data in the embryo/fetus qualitatively different from that which might be applied to similar data in adult animals. For example, a common dosing regimen used in mice, rats, and rabbits (Table 5-1) is to administer 10 daily doses of TCDD to the pregnant dam on days ~6 to 15 of gestation. This dosing regimen is expected to cover the critical period of early development that results in the greatest incidence of prenatal toxicity. In nearly all species of adult laboratory mammals, however, a single lethal dose of TCDD would be expected to produce a similar delayed-onset death regardless of the age of the adult animal. Susceptibility to TCDD-induced prenatal mortality, in contrast, may be greatly dependent on the age of the embryo/fetus. In this case, multiple doses of TCDD that cover this critical period might result in prenatal mortality, whereas a single dose might miss the critical time and not result in prenatal mortality.

The following paragraphs illustrate a type of analysis using an index of cumulative maternal dose similar to the type of analysis that might be applied to lethality data resulting from multiple dosing of adult animals. After presenting the results of applying this type of analysis to prenatal mortality data from different species, the caveat of critical time dependence will be applied to the data obtained by using different strains of mice. This will illustrate the importance of considering dosage regimen when evaluating prenatal mortality data that are available in the literature. In this case, a difference of 1 gestational day might be critically important. It turns out that the form of analysis using cumulative maternal dose may give the greatest possible degree of species variation. As such, different species may actually be more similar with respect to susceptibility to prenatal mortality than would be apparent from results of this type of analysis.

Using the cumulative dose data that are given in Table 5-1, there appears to be a tenfold to twentyfold difference in the fetolethal potency of TCDD when the monkey/guinea pig is compared with the rabbit/rat/hamster. In the CD-1 mouse treated with TCDD on gestational days 7 to 16, it appears that a daily dose of 200 μg TCDD/kg is required to significantly increase prenatal mortality. Given a ~5.5-day half-life of TCDD in the pregnant dam (Weber and

Birnbaum, 1985), the pregnant CD-1 mouse would be exposed to a maximal accumulated dose of ~1,200 μg TCDD/kg by the lowest dosage regimen that significantly increased prenatal mortality. Therefore, by using the index of cumulative dose, the CD-1 mouse would appear to be ~1,200-fold less sensitive than the monkey/guinea pig for TCDD-induced prenatal mortality. However, in NMRI mice administered TCDD only on day 6 of gestation, prenatal mortality begins to increase after a single dose of 45 μg TCDD/kg (Neubert and Dillman, 1972). The NMRI embryo/fetus is less susceptible to TCDD-induced prenatal mortality when the TCDD is administered on later gestational days up to day 15. Thus, there appears to be only an approximate 45-fold difference between the monkey/guinea pig and the NMRI mouse when the NMRI embryo/fetus is exposed specifically on day 6. In C57BL/6 mice, prenatal mortality is significantly increased after a single maternal dose of 24 μg TCDD/kg given on gestational day 6 (Couture et al., 1990a). This mouse strain, therefore, is about twentyfold to thirtyfold less sensitive to TCDD-induced prenatal mortality than is the monkey/guinea pig when exposed specifically on day 6. As with the NMRI mouse, there was little or no increase in prenatal mortality for the C57BL/6 strain when TCDD was administered to the pregnant dam on gestational days 8, 10, 12, or 14.

Peters et al. (1999) administered a single maternal dose of 25 μg/kg of TCDD on gestational day 10 to Ah receptor wild-type or null female mice (Fernandez-Salguero et al., 1995). In the homozygous Ah receptor wild-type dams and their homozygous Ah receptor wild-type fetuses there was no increase in prenatal mortality. However, in the homozygous Ah receptor null dams and their homozygous Ah receptor null fetuses TCDD increased prenatal mortality, as evidenced by an increase in percentage of resorptions. Mimura et al. (1997) also found that TCDD increased resorptions to a greater extent in Ah receptor null dams compared with Ah receptor wild-type dams. These findings suggest that mechanisms that do not require the Ah receptor may mediate, in part, the increase in prenatal mortality caused by TCDD (Peters et al., 1999).

An important finding about predicting TCDD-induced prenatal mortality is that strain differences in lethal potency of TCDD, when animals are exposed in adulthood, does not predict strain differences in lethal potency of TCDD for causing embryo/fetal mortality. Certain rat strains display wide differences in sensitivity to lethality when TCDD is given in adulthood. The Long Evans rat has a wild-type Ah receptor while the Han/Wistar rat contains a point mutation in its Ah receptor gene that results in a splice variant Ah receptor protein that binds TCDD (Pohjanvirta et al., 1998). While Long Evans and Han/Wistar rats are equally sensitive to TCDD-induced hepatic cytochrome P-4501A1 induction, the Han/Wistar strain is far less sensitive to TCDD-induced lethality than the Long Evans strain when both strains are treated with TCDD in adulthood (Pohjanvirta et al., 1993; Unkila et al., 1994). However, when these

same strains are exposed to TCDD during pregnancy, the maternal doses of TCDD administered on gestational days 8 and 12 that cause fetotoxicity and fetal lethality are similar (Huuskonen et al., 1994).

Mammalian pregnancies (including human) are characterized by critical periods or "windows" during which the embryo/fetus exhibits different susceptibilities and responses to chemical exposures. The susceptibility of any particular endpoint depends on the developmental state of that endpoint at the time of exposure. The embryo/fetus is constantly changing at all biological levels (e.g., cellular, tissue, organism), and the mechanisms of action, response, and repair of a particular endpoint at the time of exposure are the determinants of whether a response to a given exposure will result in a developmental alteration or not.

The concept of a critical window for TCDD-induced lethality in the embryo/fetus suggests an explanation for the apparent insensitivity of the CD-1 mouse embryo/fetus exposed to cumulative doses of TCDD. It could very well be that the critical window for prenatal mortality in the mouse occurs on or before gestational day 6. If the embryo/fetus is not exposed to TCDD by gestational day 6, much larger doses of TCDD are required to produce prenatal mortality. Given that exposure of the pregnant CD-1 dams did not begin until gestational day 7, this interpretation is consistent with the ability of a single 24 μg TCDD/kg dose to increase the incidence of prenatal mortality when administered to pregnant C57BL/6 mice on gestational day 6, but not when administered on gestational days 8, 10, 12, or 14 (Couture et al., 1990a). Similarly, Neubert and Dillman (1972) found that the largest increase in prenatal mortality occurred when a single dose of TCDD was given on gestational day 6 compared with prenatal mortality when the TCDD dose was administered on one of gestational days 7 to 15. In addition, this would suggest that the CD-1 embryo/fetus does not have quite the relative insensitivity to the lethal effects of TCDD compared with the embryo/fetus of other species indicated by using cumulative maternal dose as the index of exposure.

It should be noted that the concept of a critical window for prenatal mortality could potentially alter all of the species comparisons made previously that were based on the cumulative maternal doses shown in Table 5-1. If this turned out to be the case, then the true differences between species with respect to their susceptibility to TCDD-induced prenatal mortality could be substantially less than those indicated by using the cumulative maternal dose. This, of course, would involve a comparison between species using only single doses of TCDD given during the critical time period for each species. At the present time, it is not possible to make such a comparison from the information available in the literature.

Similar to fish and birds, the mammalian embryo/fetus is more sensitive to the lethal action of TCDD than the adult. The maternal dose of TCDD that causes 58% fetal mortality in hamsters is 64 to 280 times less than the LD_{50} of TCDD in adult hamsters (Olson et al., 1980;

Henck et al., 1981; Olson et al., 1990). In Sprague-Dawley rats, the cumulative maternal dose of TCDD that causes 41% prenatal mortality is 5 to 10 times less than the approximate LD_{50} of TCDD in adult rats of the same strain (Sparschu et al., 1971; Seefeld et al., 1984). In rhesus monkeys, the cumulative maternal TCDD dose that causes 81% prenatal mortality is 6 and 25 times less, respectively, than the lowest TCDD dose reported to cause mortality in 1-year-old and adult rhesus monkeys (McNulty, 1977, 1985; Seefeld et al., 1979).

Table 5-1 suggests that in many animal species (guinea pig, rabbit, rat, and mouse), TCDD-induced prenatal mortality is most commonly associated with maternal toxicity that is not severe enough to result in maternal lethality. In each of these species, the dose-response relationship for maternal toxicity, indicated by decreased maternal weight gain and/or marked subcutaneous edema of the dam, is essentially the same as that for increased prenatal mortality. Even in the hamster, where maternal toxicity is far less severe, fetuses exhibit increases in neutrophilic metamyelocytes and bands, and increases in leukocyte number and bands are also found in maternal blood (Olson and McGarrigle, 1991). In mice, it has been shown that TCDD exposure causes rupture of the embryo-maternal vascular barrier, which results in hemorrhage of fetal blood into the maternal circulation (Khera, 1992). Also, pregnant CF1 mice treated with 30 μg TCDD/kg on gestational day 12 exhibited 1.9- and 1.5-fold increases in lipid peroxidation in placental and fetal tissues, respectively, on gestational day 14. This was associated with 1.4- to 2.5-fold increases in lipid metabolite levels of malondialdehyde, formaldehyde, acetaldehyde, and acetone in amniotic fluid (Hassoun et al., 1995).

In spite of this general association between maternal and fetal toxicity, prenatal and postnatal lethality can occur in the absence of overt maternal toxicity. Olson and McGarrigle (1991) reported prenatal death but no maternal toxicity in the hamster at 18 μg/kg TCDD, the highest dose in their study. Likewise, studies in the rat demonstrate that both prenatal death (Bjerke and Peterson, 1994) and postnatal death (Gray et al., 1995) can occur in response to exposure during gestation that does not result in overt maternal toxicity (see Section 5.2.3).

In rhesus monkeys, fewer data are available to make the association between prenatal mortality and maternal toxicity. Nevertheless, the results following dietary exposure to 25 ppt TCDD (Bowman et al., 1989a; Schantz and Bowman, 1989) and 50 ppt TCDD (Allen et al., 1977, 1979; Barsotti et al., 1979; Schantz et al., 1979) before and during pregnancy suggest that TCDD-induced prenatal mortality can occur in monkeys in the absence of overt toxic effects on the mother (see Section 5.3.1). In other studies, developmental toxicity in monkeys exposed to a total cumulative maternal dose of 1 μg TCDD/kg administered during the first trimester indicated a high incidence of prenatal mortality (McNulty, 1984, 1985). However, maternal toxicity occurred in some but not all of the mothers exposed. In these monkeys, 13 of 16 pregnancies resulted in prenatal mortality. Within 20 to 147 days after aborting, 8 of the 13 females that had

aborted showed signs of maternal toxicity and 3 of these monkeys died. Thus, the remaining 5 of 13 instances of prenatal mortality apparently occurred in the absence of overt maternal toxicity. The results of these studies indicate that some levels of TCDD exposure can result in prenatal mortality in monkeys even though overt toxicity seems absent in the mother. As will be described (Section 5.3.1.1), however, only limited attention has been given to female reproductive toxicity in general and to the effects of maternal toxicity during pregnancy on fetal development in particular. Therefore, the relationship between maternal toxicity and prenatal mortality in the monkey is not well established. The integrity of the embryo-vascular barrier, for example, has not been evaluated after TCDD exposure.

Gestational exposure to TCDD produces a characteristic pattern of fetotoxic responses in most laboratory mammals consisting of thymic hypoplasia, subcutaneous edema, decreased fetal growth, and prenatal mortality. Added to these common effects on development are other effects of TCDD that are highly species-specific. Examples of the latter are cleft palate formation in the mouse and intestinal hemorrhage in the rat. Table 5-2 shows those maternal and developmental responses that are produced by gestational exposure to TCDD in various species of laboratory mammals. In the mouse, hydronephrosis is the most sensitive effect of prenatal toxicity, followed by cleft palate formation and atrophy of the thymus at higher doses, and by subcutaneous edema and mortality at maternally toxic doses (Couture et al., 1990b; Courtney, 1976; Courtney and Moore, 1971; Neubert and Dillman, 1972). In the rat, TCDD prenatal toxicity is manifested by intestinal hemorrhage, subcutaneous edema, decreased fetal growth, and mortality (Sparschu et al., 1971; Khera and Ruddick, 1973). Structural abnormalities do occur in the rat, but only at relatively large doses (Couture et al., 1990b). In the hamster fetus, hydronephrosis and renal congestion are the most sensitive effects, followed by subcutaneous edema and prenatal mortality (Olson and McGarrigle, 1991). In the rabbit, an increased incidence of extra ribs and prenatal mortality is found (Giavini et al., 1982a), and in the guinea pig and rhesus monkey, prenatal mortality is seen (Olson and McGarrigle, 1991; McNulty, 1984).

5.2.1.4. *Structure-Activity Relationships in Laboratory Mammals*

The structure-activity relationship for developmental toxicity in laboratory mammals is generally similar to that for Ah receptor binding. Gestational treatment of rats with CDD congeners that do not bind the Ah receptor (2-MCDD, 2,7-DCDD, 2,3-DCDD, or 1,2,3,4-TCDD) do not cause TCDD-like effects on development (Khera and Ruddick, 1973). On the other hand, hexachlorodibenzo-*p*-dioxin, which has intrinsic Ah receptor activity, produces fetotoxic responses in rats that are essentially identical to those of TCDD (Schwetz et al., 1973). Similarly, when administered to pregnant rhesus monkeys or CD-1 mice, PCB congeners that act by an Ah receptor-mediated mechanism (3,3',4,4'-TCB and 3,3',4,4',5,5'-HCB) cause the same

type of developmental effects as TCDD. In contrast, 4,4'-DCB, 3,3',5,5'-TCB, 2,2',4,4',5,5'-HCB, 2,2',4,4',6,6'-HCB, and 2,2',3,3',5,5'-HCB, which have essentially no or a very weak affinity for the Ah receptor, do not produce a TCDD-like pattern of prenatal toxicity in mice (Marks and Staples, 1980; Marks et al., 1981, 1989; McNulty, 1985). Thus, most structure-activity results for overt developmental effects of the halogenated aromatic hydrocarbons are consistent with an Ah receptor-mediated mechanism. Nevertheless, one finding that stands out as being inconsistent is that 2,2',3,3',4,4'-HCB, which has a very weak, if any, affinity for binding to the Ah receptor, causes the same pattern of developmental effects in mice as TCDD (Marks and Staples, 1980).

5.2.1.5. *Humans*

In the Yusho and Yu-Cheng poisoning episodes, developmental toxicity was reported in babies born to affected mothers who consumed rice oil contaminated with PCBs, CDFs, and PCQs (Hsu et al., 1985; Yamashita and Hayashi, 1985; Kuratsune, 1989; Rogan, 1989; Masada, 1994; Hsu et al., 1994). In these incidents, it is essentially impossible to determine the contribution of TCDD-like versus non-TCDD-like congeners to the fetal/neonatal toxicity. Nevertheless, high perinatal mortality was observed among hyperpigmented infants born to affected Yu-Cheng women who themselves did not experience increased mortality (Hsu et al., 1985). Thus, in humans the developing embryo/fetus may be more sensitive than the intoxicated mother to mortality caused by halogenated aromatic hydrocarbons.

In most cases, women who had affected children in the Yusho and Yu-Cheng episodes had chloracne themselves (Rogan, 1982). Based on this evidence, Rogan (1982) suggested that "exposure to amounts insufficient to produce some effect on the mother probably lessens the chance of fetopathy considerably." In support of this interpretation, overt signs of halogenated aromatic hydrocarbon toxicity were not observed in infants born to apparently unaffected mothers in the Seveso, Italy, and Times Beach, Missouri, TCDD incidents (Reggiani, 1989; Hoffman and Stehr-Green, 1989).

Effects of chemical exposure on normal development of the human fetus can have four outcomes depending on the dose and time during gestation when exposure occurs: fetal death, growth retardation, structural malformations, and organ system dysfunction. In the Yusho and/or Yu-Cheng incidents, all of these outcomes were found (Yamashita and Hayashi, 1985; Kuratsune, 1989; Rogan, 1989; Masada; 1994; Hsu et al., 1994). Increased prenatal mortality and low birthweight suggesting fetal growth retardation were observed in affected Yusho and Yu-Cheng women (Wong and Hwang, 1981; Law et al., 1981; Yamashita and Hayashi, 1985; Hsu et al., 1985; Miller, 1985; Lan et al., 1989; Rogan et al., 1988). In a follow-up of the Yu-Cheng children at elementary school age, Guo et al. (1994) reported decreased height and muscle

development in children who were the first born to women who were exposed. A structural malformation, rocker bottom heel, was observed in Yusho infants (Yamashita and Hayashi, 1985). Organ dysfunction involving the central nervous system (CNS) that was characterized by delays in attaining developmental milestones and by neurobehavioral abnormalities was reported in Yu-Cheng children exposed transplacentally (Rogan et al., 1988).

Organs and tissues that originate from embryonic ectoderm are well-known targets for toxicity following exposure to TCDD-like halogenated aromatic hydrocarbons. For example, treatment of adult monkeys with TCDD results in effects involving the skin, meibomian glands, and nails (Allen et al., 1977). Similarly, a hallmark sign of fetal/neonatal toxicity in the Yusho and Yu-Cheng episodes is an ectodermal dysplasia syndrome. It is characterized by hyperpigmentation of the skin and mucous membranes, hyperpigmentation and deformation of fingernails and toenails, hypersecretion of the meibomian glands, conjunctivitis, gingival hyperplasia, presence of erupted teeth in newborn infants, altered eruption of permanent teeth, missing permanent teeth, and abnormally shaped tooth roots (Taki et al., 1969; Yamaguchi et al., 1971; Funatsu et al., 1971; Wong and Hwang, 1981; Hsu et al., 1985; Yamashita and Hayashi, 1985; Rogan et al., 1988; Kuratsune, 1989; Rogan, 1989; Lan et al., 1989). Accelerated tooth eruption has been observed in newborn mice exposed to TCDD by lactation (Madhukar et al., 1984), as well as in the human infants mentioned above. In addition, other effects have been reported in Yusho and Yu-Cheng exposed infants that resemble those observed following TCDD exposure in adult monkeys. These include subcutaneous edema of the face and eyelids (Allen et al., 1977; Moore et al., 1979; Law et al., 1981; Yamashita and Hayashi, 1985; Rogan et al., 1988). Also, larger and wider fontanels and abnormal lung auscultation were found in the human infants (Law et al., 1981; Yamashita and Hayashi, 1985; Rogan et al., 1988). The similarities between certain effects reported in human infants exposed during the Yusho and Yu-Cheng incidents, as well as in adult monkeys and neonatal mice exposed to TCDD, enhance the probability that certain effects reported in human infants were caused by the TCDD-like PCB and CDF congeners in the contaminated rice oil ingested by the mothers of these infants.

Although chloracne is the most often cited effect of TCDD exposure involving the skin in adult humans, has an animal correlate in the hairless mouse, and can be studied by using a mouse teratoma cell line in tissue culture (Poland and Knutson, 1982), it has rarely been recognized in the TCDD literature that the nervous system, like the skin, is derived from embryonic ectoderm (Balinsky, 1970). As will be described in Section 5.2.3.2, neurobehavioral effects occur following transplacental and neonatal exposure to TCDD-like congeners in mice, as well as transplacental exposure to TCDD itself in monkeys. In addition, in some of the Yu-Cheng children who were exposed transplacentally to PCBs, PCDFs, and PCQs, there was a clinical impression of developmental delay or psychomotor delay including impairment of intellectual

development (Rogan et al., 1988). As there is a clustering of effects due to TCDD-induced toxicity in organs derived from ectoderm, it is possible to speculate that direct effects of TCDD-like congeners on the CNS are responsible for some of the neurobehavioral effects observed in these children. Effects of TCDD on EGF receptors are associated with certain aspects of the ectodermal dysplasia syndrome such as hyperkeratinization of the skin (Osborne and Greenlee, 1985) and accelerated tooth eruption (Madhukar et al., 1984). Decreased autophosphorylation of the EGF receptor in human placentas is associated with decreased birthweight in infants born to exposed mothers 4 years after the initial Yu-Cheng exposure incident (Sunahara et al., 1987). This last result supports the earlier conclusion that careful study is needed to define the relationship between maternal toxicity, placental toxicity, and developmental toxicity in humans. In addition, further research is needed to characterize and elucidate the mechanisms by which TCDD affects the nervous system.

5.2.2. Structural Malformations

Developmental effects consisting of cleft palate, hydronephrosis, and thymic hypoplasia are produced in mice following in utero exposure to halogenated dibenzo-*p*-dioxin, dibenzofuran, biphenyl, and naphthalene congeners, which bind stereospecifically to the Ah receptor (Weber et al., 1985; Miller and Birnbaum, 1986; Birnbaum et al., 1987a,b, 1991). Of these effects in the mouse, cleft palate is less responsive than hydronephrosis, as the latter is induced in the absence of cleft palate (Couture et al., 1990b). Both responses can be induced at TCDD doses that are not otherwise overtly toxic (Couture et al., 1990a). The oral surface of the palate in the mouse is characterized by 8 or 9 pairs of transverse ridges, rugae. TCDD and 3,3',4,4',5-PCB (PCB 126) produce palatal ruga anomalies in mice (Yasuda et al., 1999). The potency of TCDD for producing teratogenesis in the mouse is clearly evident when one considers that only 0.0003% of a maternally administered dose can be isolated from the fetal palatal shelves or kidneys. More specifically, a maternal TCDD dose of 30 μg/kg administered on gestational day 11 results in a tissue concentration of 0.65 pg TCDD/mg in the palatal shelves 3 days after dosing, and the same tissue concentration of TCDD is present in the kidneys at that time (Abbott et al., 1989).

Susceptibility to the developmental actions of TCDD in mice depends on two factors: genotype of the fetus and stage of development at the time of exposure. One genetically encoded parameter that determines the responsiveness of different mouse strains is the Ah receptor protein. The Ah receptor is thought to mediate the structural malformations caused by TCDD in the mouse, namely cleft palate and hydronephrosis (Poland and Knutson, 1982). After gestational day 12, the Ah receptor and its dimerization partner ARNT are expressed in the embryonic palate and developing urinary tract of the C57BL/6 mouse fetus. Expression of Ah receptor and ARNT mRNA increases significantly during palatal shelf outgrowth from

gestational day 12 to 14. While the increase in Ah receptor expression was not affected by a maternal dose of 24 μg/kg of TCDD administered on gestational day 12, there was a decrease in the expression of ARNT (Abbott et al., 1999b). Similarly, Ah receptor protein levels in the mouse urinary tract increase from gestational days 12 to 14 regardless of exposure to 12 μg/kg of TCDD on gestational day 10, whereas the expression of ARNT protein on gestational day 14 is reduced by TCDD (Bryant et al., 1997). Thus, Ah receptor and ARNT are expressed in the developing palate and urinary tract, and the opportunity exists for the Ah receptor-ARNT complex to regulate gene expression in these developing tissues. It may be important for normal development that an appropriate relative expression of these genes is maintained, and decreasing the availability of ARNT could be a significant factor in the response of the embryonic palate and urinary tract to TCDD.

Mouse strains that produce Ah receptors with relatively high affinity for TCDD respond to lower doses of TCDD than mouse strains that produce relatively low-affinity Ah receptors (Poland and Glover, 1980; Hassoun et al., 1984a). The differences that exist between mouse strains with respect to developmental responsiveness to these chemicals are not absolute, as all strains, including those with Ah receptors of relatively low affinity, respond when exposed to sufficiently large doses during the critical period of organogenesis (Birnbaum, 1991). In the mouse, the peak times of fetal sensitivity vary slightly depending on which developmental effect is used as the endpoint. However, exposure between days 6 and 15 of gestation will produce teratogenesis (Couture et al., 1990a,b).

In inbred strains of mice, the developmental response, characterized by altered cellular proliferation, metaplasia, and modified terminal differentiation of epithelial tissues (Poland and Knutson, 1982), is extremely organ-specific, occurring only in the palate, kidney, and thymus (Birnbaum, 1991). Pharmacokinetic differences are not responsible for this high degree of tissue specificity, and Ah receptors are not found exclusively in the affected organs (Carlstedt-Duke, 1979; Gasiewicz et al., 1983). Therefore, other factors intrinsic to the palate, kidney, and thymus appear to play a role along with the Ah receptors in these tissues in producing the structural malformations. For certain developmental effects, the time at which exposure occurs is important, as there may be a critical period during which the toxicant must be present in order to produce the effect. This critical period can be different for different organs and tissues.

Differences exist between mammalian species with respect to susceptibility to the developmental effects of TCDD. Although genetic differences between species or strains might affect absorption, biotransformation, and/or elimination of TCDD by the maternal system and its absorption across the placenta, such species differences do not account for the lack of cleft palate formation in species other than mice (Birnbaum, 1991). Rather, the species differences in susceptibility to cleft palate formation appear due to differences in the interaction between

TCDD and the developing palatal shelves themselves. This is demonstrated by the occurrence of similar responses when palatal shelves from different species are exposed to TCDD in organ culture (Abbott et al., 1989; Abbott and Birnbaum, 1990a, 1991). The key difference is that in other species much higher concentrations of TCDD are required to elicit essentially the same palatal response that is seen in the mouse (Table 5-3). Thus, since palatal shelves of the mouse are 200 times more sensitive to TCDD than those of the human, it is considered unlikely that human embryos would be exposed to high enough concentrations of TCDD to cause changes in palatal differentiation sufficient to produce cleft palate (Abbott et al., 1999a).

With respect to the occurrence of similar developmental effects in mammalian species other than the mouse, no other species develops cleft palate except at maternal doses that are fetotoxic and maternally toxic (Couture et al., 1990a; Birnbaum, 1991). In mice and hamsters, hydronephrosis can be elicited at TCDD doses that are neither fetotoxic nor maternally toxic (Olson and McGarrigle, 1991), whereas thymic hypoplasia is a fetal response to TCDD observed in virtually all laboratory mammalian species that have been tested (Vos and Moore, 1974). Studies in humans have not clearly identified an association between TCDD exposure and structural malformations (Fara and Del Corno, 1985; Mastroiacovo et al., 1988; Stockbauer et al., 1988; Reggiani, 1989).

5.2.2.1. *Cleft Palate*

5.2.2.1.1. ***Characterization of TCDD effect.*** Palatal shelves in the mouse originate as outgrowths of the maxillary process. Eventually, they come to lie vertically within the oral cavity on both sides of the tongue. In order to form the barrier between the oral and nasal cavities, the shelves in the mouse must reorient themselves from a vertical direction to a horizontal direction. Once they come together horizontally, their medial aspects bring apposing epithelia into close contact (Coleman, 1965; Greene and Pratt, 1976). At this stage, the apposing medial edge epithelia of the separate palatal shelves each consist of an outer layer of periderm that overlays a strata of cuboidal-shaped basal cells. These basal cells, in turn, rest on top of a continuous basal lamina. There is a sloughing of the outer periderm cells followed by the formation of junctions between the newly apposing basal epithelial cells. The midline seam so formed consists of the two layers of basal cells, all of which appear healthy, even though the outer periderm cells are shed before adhesion occurs. As fusion proceeds, the bilayer seam breaks up into small islands of cells. Eventually, the basal lamina disappears and the elongating former basal cells within the small islands extend filopodia into the adjacent connective tissue. During this process, the former basal cells lose epithelial characteristics and gain fibroblast-like features. Essentially, the medial edge epithelium is an ectoderm that retains the ability to transform into mesenchymal cells. Upon completion of this epithelial to mesenchyme transformation, the once separate and

apposing palatal shelves are fused so that a single continuous tissue is formed (Fitchett and Hay, 1989; Shuler et al., 1992).

Cleft palate can result from a failure of the shelves to grow and come together or a failure of the shelves to fuse once they are in close apposition (Pratt et al., 1985). TCDD and other Ah receptor agonists are unusual inducers of cleft palate because the shelves grow and make contact, but the subsequent processes involving loss of periderm, shelf adhesion, and the epithelial to mesenchyme transformation does not occur. Therefore, a cleft is formed as the palatal shelves continue to grow without fusing. When TCDD is administered to pregnant mice on gestational days 6 to 12, the incidence of cleft palate formation increases with time. However, day 12 is a critical window, after which the incidence of cleft palate formation decreases. No cleft palates are formed when TCDD is administered on day 14, since fusion has already occurred (Couture et al., 1990b).

Palatal shelves of the mouse, rat, and human can be removed from the fetus and placed into organ culture. Under these conditions, when the separate shelves are placed in an apposing condition in vitro, sloughing periderm cells are trapped within the seam (Fitchett and Hay, 1989). Thus, due to the presence of these trapped dead cells, the fusion process was previously believed to require programmed cell death to remove epithelial cells at the fusion seam (Coleman, 1965; Greene and Pratt, 1976; Pratt et al., 1984). However, the newer model, which involves transformation of the basal epithelial cells into mesenchyme rather than their death, is believed to be valid under explant conditions in vitro, as well as in vivo (Fitchett and Hay, 1989). When exposed to TCDD as explants in vitro, the palatal shelves of the mouse, rat, and human all respond to TCDD in a similar way by retaining medial epithelial cells that proliferate and differentiate into a stratified epithelium (Abbott et al., 1989; Abbott and Birnbaum, 1989, 1990a, 1991). The epithelial to mesenchyme transformation of the basal epithelial cells does not occur, and instead there is a differentiation into a stratified squamous epithelium such that these cells resemble the squamous keratinizing oral cells within the tissue.

Table 5-3 shows the lowest TCDD concentration that prevents the epithelial to mesenchyme transformation process in isolated palatal shelves (lowest observed effect level, LOEL), TCDD concentration that produces a 100% maximal response (EC_{100}), and lowest concentration of TCDD that produces cytotoxicity. Palatal shelves of rats and humans respond to TCDD in a manner identical to the mouse; however, higher concentrations of TCDD are required to induce the epithelial responses. The relative insensitivity of rat palatal shelves may explain the lack of cleft palate when fetal rats are exposed to nonmaternally toxic doses of TCDD. Sensitivity of human palatal shelves to TCDD in vitro is similar to the rat. This suggests that exposure to maternally toxic and fetotoxic doses of TCDD may be required to cause cleft palate formation in humans.

A disruption in the normal spatial and temporal expression of EGF, thyroid growth factor (TGF)-α, TGF-β1,and TGF-β2 correlates with altered proliferation and differentiation in the medial region of the developing palate, resulting in a palatal cleft. Thus, the abnormal proliferation and differentiation of TCDD-exposed medial cells may be related to reduced expression of EGF and TGF-α. Also, decreased levels of immunohistochemically detectable TGF-β1 could contribute to the continued proliferation and altered differentiation of medial cells (Abbott and Birnbaum, 1990b). It is important to note that EGF and TGF-α both exert their actions by binding to EGF receptors.

Based on these results, biochemical and genetic differences between mouse and human palates have been described that may explain the different sensitivities to cleft palate formation in the mouse and human. Ah receptor concentrations in the mouse palate are 346 times greater than those in the human, and ARNT levels are also greater in the mouse (Abbott et al., 1999a). In addition, gene expression studies have demonstrated that human and mouse palates cultured in vitro are dissimilar with respect to their particular spatial and temporal patterns of EGF, EGF receptor, TGF-α, and TGF-β3 mRNA expression. Because the proteins that are the translation products of these mRNAs are important for palatal development, it has been suggested that species differences in the expression patterns of these genes could contribute to the lower sensitivity of human palates to TCDD when compared with the mouse (Abbott et al., 1998).

The differentiation of basal cells to a stratified squamous epithelium, which resembles the keratinizing oral epithelium within the developing palate that is mentioned above, is similar to certain effects of TCDD that can be studied in cultured human keratinocytes. These effects in cultured human keratinocytes involve altered EGF binding to those cells. In addition, the Ah receptor is implicated in producing this response in cultured cells (Osborne and Greenlee, 1985). Thus, the mechanisms by which TCDD produces a palatal cleft in the mouse may have similarities to the mechanisms by which TCDD produces other effects that are part of the ectodermal dysplasia syndrome. This is consistent with the description given by Fitchett and Hay (1989) that the medial edge epithelium within the developing palate is essentially an ectoderm that retains the ability to transform into mesenchymal cells.

5.2.2.1.2. *Evidence for an Ah receptor mechanism.*

5.2.2.1.2.1. *Genetic.* When wild-type C57BL/6 (Ah^bAh^b) mice are crossed with DBA/2 (Ah^dAh^d) mice that contain a mutation at the Ah locus, all of the heterozygous B6D2F1 progeny (Ah^bAh^d) resemble the wild-type parent in that AHH activity is inducible by TCDD and other halogenated aromatic hydrocarbons (Nebert and Gielen, 1972). Test crosses between the B6D2F1 progeny and each original parent strain, and other B6D2F1 progeny mice, demonstrate that in the C57BL/6 and DBA/2 strains, susceptibility to AHH induction segregates as a simple

dominant trait in the backcross and F_2 progeny. Thus, the trait of AHH induction is expressed in progeny that contain the Ah^bAh^b and Ah^bAh^d genotypes, but is not expressed in the Ah^dAh^d progeny from these crosses. Certain other effects of TCDD, such as its binding affinity for the hepatic Ah receptor (Okey et al., 1979), thymic atrophy (Poland and Glover, 1980), hepatic porphyria (Jones and Sweeney, 1980), and immunosuppressive effects (Vecchi et al., 1983; Nagarkatti et al., 1984) have been shown in similar genetic crosses and test crosses to segregate with the Ah locus that permits AHH induction. Thus, for these effects of TCDD, genetic evidence demonstrates an involvement of the Ah locus (Poland and Knutson, 1982).

Nebert's group was the first to relate developmental toxicity to the Ah locus in mice (Lambert and Nebert, 1977; Shum et al., 1979). Subsequently, Poland and Glover (1980) administered a single 30 μg TCDD/kg dose to pregnant mice on gestational day 10. A 54% incidence of cleft palate was found in homozygous C57BL/6 (Ah^bAh^b) fetuses, a 13% incidence in heterozygous B6D2F1 (C57BL/6 and DBA/2 hybrid, Ah^bAh^d) fetuses, and only a 2% incidence in homozygous DBA/2 (Ah^dAh^d) fetuses. This pattern of inheritance, in which the incidence of developmental toxicity in the heterozygous F1 generation is intermediate between that of the homozygous parental strains, is consistent with the autosomal dominant pattern of inheritance described for AHH inducibility and the Ah locus (Nebert and Gielen, 1972), even if dominance is incomplete in the case of developmental toxicity. However, the pattern of inheritance for developmental toxicity described when Poland and Glover (1980) crossed C57BL/6 and DBA/2 mice is not sufficient proof that the Ah locus is the genetic locus that controls susceptibility to TCDD-induced developmental toxicity in these mouse strains.

To provide such proof, it is necessary to show genetic linkage between the susceptibility for developmental toxicity and the Ah locus. The standard of proof would be that developmental toxicity and a particular allele at the Ah locus must always segregate together in genetic crosses because if the loci are the same there can be no recombination between the loci. This is generally accomplished by demonstrating cosegregation between the two loci, not only in crosses between the two homozygous parental strains, which in and of itself is insufficient proof of genetic linkage, but also in test crosses or backcrosses between the heterozygous F1 hybrids with each homozygous parental strain.

It was stated previously that certain effects of TCDD are well known to segregate with the Ah locus due to the results of appropriate crosses and backcrosses between responsive and nonresponsive mouse strains and their hybrid F1 progeny. With this standard of proof in mind, the evidence that specifically links certain endpoints of developmental toxicity with the Ah locus can be described. It is intended that this information be provided with a considerable degree of detail, so the reader can independently determine whether the standard of proof has been satisfied by the evidence available.

To strengthen their conclusion based on the results of simple crosses between C57BL/6 and DBA/2 mice, Poland and Glover (1980) planned to perform a backcross between the hybrid B6D2F1 and DBA/2. However, the low incidence of cleft palate in B6D2F1 mice would have required characterizing and phenotyping a prohibitively large number of fetuses. Alternatively, the backcross between B6D2F1 and C57BL/6 was considered, in which Ah^bAh^b and Ah^bAh^d progeny would have been distinguished by the amount of high-affinity specific binding for TCDD in fetal liver. In this case, however, overlap between individual mice would have made the results uncertain in some of the progeny. Therefore, it was not possible to obtain satisfactory results from either backcross.

Instead, Poland and Glover (1980) examined the incidence of cleft palate in 10 inbred strains of mice: 5 strains with high-affinity Ah receptors and 5 strains with low-affinity receptors. In the five latter strains, there was only a 0% to 3% incidence of cleft palate formation, whereas four of the five strains with high-affinity Ah receptors developed a ~50% incidence. The one strain with high-affinity Ah receptors that did not follow the pattern, CBA strain, is also resistant to cleft palate formation induced by glucocorticoids. Overall, these results indicate that cleft palate formation probably segregates with the Ah locus.

The incidence of cleft palate formation was studied in fetuses from a cross between C57BL/6 and AKR/NBom mice administered 3,3',4,4'-TCAOB on gestational day 12 (Hassoun et al., 1984b). Although C57BL/6 mice are responsive for AHH induction and cleft palate formation, AKR mice are less responsive, requiring higher doses for both effects. In a manner unlike the result of a cross between C57BL/6 and DBA/2, the incidence of cleft palate formation in the B6AKF1 progeny was <2%, showing that nonresponsiveness segregates as the dominant trait when C57BL/6 mice are crossed with AKR mice. Similarly, cleft palate formation was virtually absent in the progeny of a backcross between AKR/NBom and B6AKF1, demonstrating dominance of the noninducible trait. Although Ah phenotyping of the backcross progeny was not performed in this particular study, Robinson et al. (1974) had previously evaluated segregation of the Ah locus in backcrosses between C57BL/6 and AKR/N mice. They found in these two strains that noninducibility for AHH activity segregates as the dominant trait. Thus, inducibility for cleft palate formation and AHH activity both segregate as dominant traits when C57BL/6 mice are crossed with DBA/2, but noninducibility is dominant for both traits when C57BL/6 mice are crossed with AKR/N. These results are consistent with the interpretation that cleft palate induction probably segregates with the Ah locus.

Like Poland and Glover (1980), Hassoun et al. (1984a) were unable to determine whether cleft palate formation segregates with the Ah locus in C57BL/6 and DBA/2 mice by performing simple backcrosses. Instead, they evaluated cosegregation of the Ah locus and 2,3,7,8-TCDF-induced cleft palate formation using a series of recombinant strains called BXD mice. These

strains are fixed recombinants produced from an original cross between the two parental strains C57BL/6J and DBA/2J. Hybrid B6D2F1 mice were crossed to produce F_2 progeny and these were strictly inbred by sister and brother matings into several parallel strains. The mice used in this study were from the F_{42} and F_{58} generations of inbreeding. It was found that the incidence of TCDF-induced cleft palate formation after matings within eight different BXD strains with high-affinity Ah receptors is >85%. After similar matings with eight different BXD strains with low-affinity Ah receptors, the incidence of TCDF-induced cleft palate formation is <2%. These results of Hassoun et al. (1984a) corroborate those of Poland and Glover (1980) and provide further evidence that cleft palate formation segregates with the Ah locus. Thus, the Ah locus and the Ah receptor are involved in the formation of palatal clefts that are induced by TCDD-like congeners.

Consistent with this interpretation, the Ah receptor null mouse line of Mimura et al. (1997) is completely resistant to TCDD-induced cleft palate formation and the Ah receptor null line of Fernandez-Salguero et al. (1995) is almost entirely resistant to this teratogenic effect of TCDD (Peters et al., 1999). Taken together, these findings support the conclusion that the Ah receptor plays a key role in TCDD-induced cleft palate formation. However, because 9% of the homozygous Ah receptor null fetuses of the Fernandez-Salguero et al. (1995) transgenic line developed cleft palate when exposed to TCDD (compared with 0% of vehicle-exposed wild-type and 0% of vehicle-exposed Ah receptor null fetuses), a TCDD-induced alteration in processes that do not require the Ah receptor might also be involved. Further research is needed to explore this possibility.

As additional evidence for an Ah receptor-mediated mechanism for cleft palate formation by TCDD and related compounds, stereospecific, high-affinity Ah receptors can be isolated from cytosol fractions prepared from embryonic palatal shelves. These receptors are present in palatal shelves of Ah^bAh^b, C57BL/6 fetuses but are not detectable in similar tissue from Ah^dAh^d, AKR/J fetuses (Dencker and Pratt, 1981). However, the significance of this finding may be mitigated to some extent by the following observation. In cytosols prepared from homogenates of whole embryo/fetal tissue (minus head, limbs, tail, and viscera), the concentration of specific binding TCDD receptors is 256 fmol/mg protein in C57BL/6 mice, compared with a concentration of 21 fmol/mg protein in the less responsive DBA/2 strain, 15 fmol/mg protein in the less responsive AKR/J strain, and 19 fmol/mg protein in the less responsive SWR/J strain. However, when embryonic tissue is cultured, the differences between the strains in receptor number are less pronounced, and in the receptors isolated from cultured embryonic cells of different strains, there is only about a twofold difference in the relative binding affinity for ^{3}H-TCDD. The mechanistic reasons for the diminished degree of difference between responsive and less responsive mouse strains during embryonic cell culture are not known (Harper et al., 1991).

The possible influence of maternal toxicity on cleft palate formation was evaluated by performing reciprocal blastocyst transfer experiments using the high-affinity-Ah receptor NMRI and lower affinity-Ah receptor DBA strains of mice (D'Argy et al., 1984). After administration of 30 μg TCDD/kg or 8 mg TCAOB/kg to pregnant dams on gestational day 12, 75% to 100% of all NMRI fetuses developed cleft palates. This is true whether the fetuses remained within the uterus of their natural mother or were transferred into the uterus of a DBA mouse. Under the same conditions, none of the 24 DBA fetuses transferred into an NMRI mother developed a cleft palate, even though 89% of their NMRI litter mates were affected. Thus, these results, along with the presence of Ah receptors in palatal shelves and responsiveness of palatal shelves in organ culture to TCDD, indicate that cleft palate formation in mice is due to a direct effect of TCDD on the palatal shelf itself and is not secondary to maternal toxicity.

5.2.2.1.2.2. ***Structure activity.*** As genetic evidence in mice indicates that the Ah receptor mediates TCDD-induced cleft palate formation and hydronephrosis (see Section 5.3.2.2.2.1), structure-activity requirements based on Ah receptor-binding characteristics should predict the relative potencies of different agonists for producing cleft palate and hydronephrosis. Of the halogenated aromatic hydrocarbons, TCDD has the greatest affinity for binding to the Ah receptor and it is the most potent teratogen in inbred mouse strains. Table 5-4 shows the relative potencies for cleft palate induction and hydronephrosis in C57BL/6 mice for a number of TCDD-like congeners. As TCDD is the most potent, it is assigned a value of 1.000. When examined by probit analysis, the dose-response curve of each congener, compared with all of the others, did not deviate from parallelism. Therefore, the relative potencies of the congeners are valid for any given incidence of cleft palate formation or hydronephrosis. The main finding, however, is that the rank order potency of the various congeners for producing these two developmental effects is generally similar to that for binding to the Ah receptor (see Table 5-4), with the notable exception that the apparent binding affinities for the brominated dibenzofurans have not yet been reported. There are additional ligands for the Ah receptor that cause cleft palate formation in C57BL/6 mice at nonmaternally toxic doses, but they are not listed in the table. These include 3,3',4,4'-TCAOB (Hassoun et al., 1984a), 3,3',4,4'-tetrachlorobiphenyl (Marks et al., 1989), 3,3',4,4',5,5'-hexachlorobiphenyl (Marks et al., 1981), and a mixture that contained 1,2,3,4,6,7- and 2,3,4,5,6,7-hexabromonaphthalenes (Miller and Birnbaum, 1986).

Also consistent with the structure-activity relationships for binding to the Ah receptor is the finding that a number of hexachlorobiphenyls do not induce cleft palate formation. These congeners either lack sufficient lateral substitution or are substituted in such a manner that they cannot achieve a planar conformation. Included in this category are the diortho and tetraortho chlorine-substituted 2,2',3,3',5,5'-; 2,2',3,3',6,6'-; 2,2',4,4'5,5'-; and 2,2',4,4',6,6'-hexachloro-

biphenyls (Marks and Staples, 1980). In addition, it is consistent with the structure-activity relationships that the monoortho chlorine-substituted 2,3,4,5,3',4'-HCB is a weak teratogen. Its potency relative to that of TCDD varies from 3×10^{-5} to 9×10^{-5} for cleft palate formation, AHH induction, and hydronephrosis (see Table 5-4) (Kannan et al., 1988).

A result that would not be expected according to the structure-activity relationships for binding to the Ah receptor is that the diortho chlorine-substituted 2,2',3,3',4,4'-hexachlorobiphenyl causes cleft palate formation and hydronephrosis in mice (Marks and Staples, 1980). However, another diortho chlorine-substituted PCB congener, 2,2',4,4',5,5'-hexachlorobiphenyl, also can cause hydronephrosis and is a very weak inducer of 7-ethoxyresorufin-O-deethylase (EROD) activity (Biegel et al., 1989; Morrissey et al., 1992). It is consistent with the interpretation that 2,2',4,4',5,5'-hexachlorobiphenyl is a partial Ah receptor agonist, that it can competitively displace TCDD from the murine hepatic cytosolic receptor, and that at large enough doses it can inhibit TCDD-induced cleft palate formation and immunotoxicity in C57BL/6 mice (Biegel et al., 1989; Morrissey et al., 1992). These results suggest that PCB congeners do not have to be in a strictly planar configuration to cause teratogenesis.

5.2.2.1.3. ***Species differences.*** Cleft palate is induced in rats only at maternally toxic TCDD doses that are associated with a high incidence of fetal lethality. Schwetz et al. (1973) reported an increased incidence of cleft palate after maternal administration of 100 μg hexachlorodibenzo-*p*-dioxin/kg/day to Sprague-Dawley rats on days 6 to 15 of gestation. Couture et al. (1989) also observed an increased incidence of cleft palate formation after a single dose of 300 μg/kg of 2,3,4,7,8-pentachlorodibenzofuran given to Fischer 344 rats. In Long Evans rats administered 5 μg/kg of TCDD on gestational day 8 there was a 71.4% incidence of cleft palate (Huuskonen et al., 1994). However, in Han/Wistar rats that have a mutated form of the Ah receptor, exposure to 10 μg/kg of TCDD on gestational day 8 failed to cause cleft palate formation (Huuskonen et al., 1994). Thus, there are rat strain differences in susceptibility to cleft palate formation as has been shown for mice. Cleft palate also can be produced in fetal hamsters following maternally toxic and fetotoxic doses of TCDD (Olson et al., 1990).

In monkeys, bifid uvula (Zingeser, 1979) and bony defects in the hard palate (McNulty, 1985) were reported, but there were no corresponding soft tissue defects or clefts of the secondary palate. Cleft palates have not been reported in human fetuses of mothers accidentally exposed to TCDD or mixtures of PCBs and CDFs (Fara and Del Corno, 1985; Mastroiacovo et al., 1988; Stockbauer et al., 1988; Rogan, 1989). Thus, sensitivity of the palate in mice to TCDD is unique. In other species, including humans, other forms of fetal toxicity occur at doses lower than those required for cleft palate formation.

5.2.2.2. *Hydronephrosis*

5.2.2.2.1. ***Characterization of TCDD effect.*** Hydronephrosis is the most sensitive developmental response elicited by TCDD in mice. It is produced by maternal doses of TCDD too low to cause palatal clefting and is characterized as a progressive hydronephrosis preferentially occurring in the right kidney, which can be accompanied by hydroureter and/or abnormal nephron development (Courtney and Moore, 1971; Moore et al., 1973; Birnbaum et al., 1985; Weber et al., 1985; Abbott et al., 1987a,b). Hyperplasia of the ureteric lumenal epithelium results in ureteric obstruction. Therefore, the TCDD-induced kidney malformation in the mouse is a true hydronephrosis in that blockage of urine flow results in back pressure damaging or destroying the renal papilla (Abbott et al., 1987a). In addition, mRNA and protein for both the Ah receptor and ARNT are expressed in the fetal ureters and metanephric tubules of the mouse (Bryant et al., 1997), so it is possible that hydronephrosis is caused by a direct action of TCDD on the developing kidney.

When dissected on gestational day 12 from control embryos, isolated ureters exposed to 1×10^{-10} M TCDD in vitro display evidence of epithelial cell hyperplasia (Abbott and Birnbaum, 1990c). This is significant in that it shows that the hydronephrosis response is due to a direct effect of TCDD on the ureteric epithelium. Embryonic cell proliferation within the ureter may be regulated by the actions of growth factors, including EGF (Abbott and Birnbaum, 1990c). In control ureteric epithelia, the expression of EGF receptors decreases with advancing development, whereas after TCDD exposure the rate of ^{3}H-thymidine incorporation and expression of EGF receptor does not decline. Therefore, in TCDD-treated mice there is a correlation between excessive proliferation of ureteric epithelial cells and inappropriate expression of EGF receptors.

Other effects of TCDD on the developing kidney involve changes in the extracellular matrix components and basal lamina (Abbott et al., 1987b). In TCDD-exposed fetal kidneys, extracellular matrix fibers are of a diameter consistent with Type III collagen similar to such fibers in unexposed fetal kidneys. However, the abundance of these Type III collagen fibers is reduced by TCDD treatment. In the developing kidney, these collagen fibers are associated with undifferentiated mesenchymal cells. Similarly, the expression of fibronectin, which is also associated with undifferentiated mesenchymal cells, is decreased by TCDD exposure. In the glomerular basement membrane, the distribution of laminin and Type IV collagen is altered by TCDD exposure. These changes in the glomerular basement membrane may affect the functional integrity of the filtration barrier and could exacerbate the hydronephrosis and hydroureter. The proteins within the extracellular matrix and basal lamina that are altered by TCDD exposure (laminin, fibronectin, and collagen) are considered markers of a commitment to differentiate into epithelial structures. In the mouse embryo/fetus, TCDD exposure also blocks differentiation

within the epithelium of the developing palate. Although there are effects of TCDD exposure on EGF in the developing ureter as well as the developing palate, the urinary system, unlike parts of the soft palate, is derived from mesoderm. Thus, it is important to note that the ectodermal dysplasia syndrome is intended to denote a clustering of effects that appears to involve ectoderm-derived organs. It is not intended to imply that all TCDD-induced developmental toxicity involves organs derived from ectoderm.

5.2.2.2.2. ***Evidence for an Ah receptor mechanism.***

5.2.2.2.2.1. ***Genetic.*** With respect to involvement of the Ah locus in TCDD-induced hydronephrosis, very few genetic studies have been done. Prior to the discovery of the Ah locus, however, Courtney and Moore (1971) reported a 62% incidence of hydronephrosis in C57BL/6 mice exposed to a maternal TCDD dose of 3 μg/kg/day on days 6 to 15 of gestation, whereas the incidence in similarly exposed DBA/2 mice was only 26%. More recently, Silkworth et al. (1989) reported that when TCDD is administered on gestational days 6 to 15, the incidence of hydronephrosis is dose related. As the maternal dose of TCDD is increased from 0.5 to 4μg/kg/day, the incidence of hydronephrosis in C57BL/6 mice increases from 31% to 92%, whereas in DBA/2 mice the incidence varies from 5% to 37% over the same dose range. In DBA/2 mice the incidence of hydronephrosis increases to 60% when the largest dose of TCDD administered is doubled to 8 μg/kg/day (but does not reach the 92% level seen in C57BL/6 mice at 4μg TCDD/kg). Thus, the incidence of hydronephrosis is higher in the mouse strain that produces high-affinity Ah receptors (C57BL/6) compared with that strain (DBA/2) that produces Ah receptors having lower ligand-binding affinity (Okey et al., 1989). The largest dose of TCDD used in these experiments resulted in hydronephrosis of the fetus without affecting the mean body weight or body weight gain of the dam. In the BXD strains (Hassoun et al., 1984a), the incidence of 2,3,7,8-TCDF-induced hydronephrosis is 34% to 48% in eight strains with high-affinity Ah receptors and 3% to 4% in eight strains with low-affinity Ah receptors. These results obtained in the BXD strains of mice provide the best evidence currently available of an association between the ability of TCDD-like congeners to induce hydronephrosis and the wild-type Ah^b allele. Thus, the Ah locus and the Ah receptor are involved in the hydronephrosis that is induced by TCDD-like congeners.

More recently, transgenic Ah receptor null mutant mice have been used to study the effects of Ah receptor deletion on the ability of TCDD exposure to cause hydronephrosis. Female mice heterozygous for Ah receptor expression were mated to males of the same genotype and exposed during pregnancy to 40 μg TCDD/kg on gestational day 12.5 (Mimura et al., 1997). Nearly all TCDD-exposed wild-type and heterozygous progeny developed hydronephrosis. In sharp contrast, there was no hydronephrosis in offspring from the same litters that were

homozygous for the Ah receptor null mutation. Similarly, Ah receptor null mice generated by a different targeting method (Fernandez-Salguero et al., 1995) were also completely resistant to TCDD-induced hydronephrosis (Peters et al., 1999). These results, coupled with the difference in incidence of TCDD- or TCDF-induced hydronephrosis in C57BL/6 and DBA/2 mouse strains, demonstrate that this teratogenic response to TCDD is Ah receptor mediated. Since haplo-insufficiency was observed for the cleft palate response, but not for hydronephrosis, Mimura and coworkers suggest that the mechanisms by which the Ah receptor mediates these two teratogenic effects of TCDD may be different (Mimura et al., 1997).

5.2.2.2.2.2. ***Structure activity.*** The rank order of potencies for various halogenated aromatic hydrocarbon congeners to cause hydronephrosis in mice is consistent with the structure-activity requirements for binding to the Ah receptor (see Table 5-4). This provides further evidence that the Ah receptor mediates the effects of these TCDD-like congeners on the developing mouse kidney.

5.2.2.2.2.3. ***Species differences.*** Hydronephrosis has been reported after administration of low maternal doses of TCDD to rats and hamsters. Possibly due to the small numbers of fetuses examined, the observed incidences of hydronephrosis in rats after exposure to cumulative maternal doses <2 μg TCDD/kg have not been statistically significant (Courtney and Moore, 1971; Giavini et al., 1983). There are also interstrain differences in rats in susceptibility to hydronephrosis. This is illustrated in the TCDD-resistant Han/Wistar and TCDD-sensitive Long Evans rat strains by 1 and 10 μg/kg of TCDD administered on gestational day 8 causing 3% and 11.9% hydronephrosis in the Han/Wistar strain while a 5 μg/kg dose of TCDD administered on the same day of gestation failing to cause hydronephrosis in the Long Evans strain (Huuskonen et al., 1994). Following a 1.5 μg TCDD/kg dose administered on gestational days 7 and 9, the incidence of hydronephrosis in hamster fetuses was 11% and 4.2%, respectively. This is in contrast to an incidence of <1% in control hamster fetuses. Accordingly, hydronephrosis is one of the most sensitive indicators of prenatal toxicity in hamsters (Olson and McGarrigle, 1991).

5.2.2.3. *Tooth Development*

The interpretation that lactational exposure to CDDs and CDFs may lead to mineralization defects in the first molars of human infants (Alaluusua et al., 1996, 1999) was further investigated in experiments where primordial mandibular molar teeth from mouse embryos were cultured in the presence of 1 μM TCDD (Partanen et al., 1998). In these cultured primordial teeth, TCDD caused toxicity to odonotoblasts and ameloblasts. This led to a failure of dentin to undergo mineralization and a lack of enamel deposition. Cuspal morphology also

was disrupted by TCDD exposure in the cultured teeth. While the concentration of TCDD required to produce these effects was high, 1 μM, the authors suggest that barriers to diffusion inherent in tooth structure may result in TCDD concentrations at the cellular site of action in the primordial teeth being much lower than that in the culture medium (Partanen et al., 1998). Exposure to EGF (10 μg/L) similarly retarded molar tooth development in cultured explants from wild-type embryos, because layers of mineralized dentin and the enamel matrix were thinner than those in vehicle-exposed explants. In cultured primordial molar teeth from EGF receptor null embryos, the effects of EGF were completely ameliorated, and TCDD had only a mild effect. When cultured explants from wild-type mouse embryos were simultaneously exposed to EGF and TCDD, the adverse effects of TCDD on mineralization and enamel deposition were largely, but not completely prevented (Partanen et al., 1998). In utero and lactational exposure of male Holtzman rats to 0.064, 0.16, 0.40, or 1.0 μg/kg of TCDD on gestational day 15 failed to accelerate the age at which incisor eruption occurred. At the highest TCDD dose used there was a tendency for incisor eruption to be accelerated by about 1 day (9.9 days in the control versus 8.9 days in the TCDD group), but the effect was not statistically significant (Mably et al., 1992a). Taken together, these results are consistent with the interpretation that TCDD alters tooth development in organ culture by interfering with EGF receptor signaling. However, TCDD also may affect tooth development by perturbing other signaling pathways, which either act in concert with or interfere with EGF receptor signaling, and probably involve additional mechanisms of cell and/or tissue interactions. Finally, the involvement of EGF receptor signaling in this effect of TCDD is consistent with aberrant tooth development being a part of the TCDD ectodermal dysplasia syndrome.

5.2.3. Postnatal Effects

5.2.3.1. *Eye Opening*

In utero and lactational exposure to TCDD caused external developmental effects in male rodent offspring that are not androgen dependent. The most prominent of these is accelerated eye opening. Mably et al. (1992a) reported that the age of eye opening was accelerated by 1.0 μg/kg of TCDD administered on gestational day 15. Lower doses of TCDD that affected growth and development of several male reproductive tract organs, however, had no effect on eye opening, demonstrating that this endpoint could be clearly dissociated from the more sensitive male reproductive endpoints by the dose of TCDD needed to cause them. Gray et al. (1997) found accelerated eye opening to be one of the most sensitive endpoints in the Long Evans rat, occurring at 0.05 μg/kg of TCDD administered on gestational day 15. This was the lowest dose used in their study, and it also significantly decreased ejaculated sperm numbers, by 25%. In the ICR mouse exposure to 15, 30, or 60 μg/kg of TCDD on gestational day 14 accelerated eye

opening in male pups at all dosage levels (Theobald and Peterson, 1997). However, there was no effect on age to eye opening in female pups from the same TCDD-exposed mouse litters.

5.2.3.2. *Male Reproductive System*

TCDD has been shown to decrease plasma androgen concentrations in the adult male rat (see Section 5.3.2.2). Because TCDD is known to be transferred from mother to young in utero and during lactation (Moore et al., 1976; van den Berg et al., 1987), it can be expected to have an impact on the male reproductive system during early development (Mably et al., 1991). Testosterone and/or its active metabolite 5α-dihydrotestosterone (DHT) are essential prenatally and/or during the early postnatal stage for imprinting and development of accessory sex organs (Chung and Raymond, 1976; Rajfer and Coffey, 1979; Coffey, 1988) and for initiation of spermatogenesis (Steinberger and Steinberger, 1989). For example, exposure of the male rat fetus on gestational days 14 to 16 to a 5α-reductase inhibitor, which inhibits conversion of testosterone to DHT, impairs development of urogenital sinus-derived accessory sex organs such as the prostate (Clark et al., 1993). If perinatal imprinting fails to occur in the Wolffian duct or urogenital sinus-derived accessory sex organs of a neonatal male rat, the result could be that these male sex organs do not develop a normal trophic response to androgenic stimulation and do not grow and develop normally as the animal matures. In addition, aromatization of testosterone to 17ß-estradiol within the CNS is required perinatally for the imprinting of typical adult male patterns of reproductive behavior (Gorski, 1974) and luteinizing hormone (LH) secretion (Barraclough, 1980). Thus, normal development of male reproductive organs and imprinting of typical adult sexual behavior patterns require sufficient testosterone to be secreted by the fetal and neonatal testis at critical times in early development before and shortly after birth (MacLusky and Naftolin, 1981; Wilson et al., 1981).

To determine how the male reproductive system is affected by in utero and lactational TCDD exposure, Mably et al. (1991, 1992a,b,c) treated pregnant rats with a single oral dose of TCDD (0.064, 0.16, 0.4, or 1.0 μg/kg) or vehicle on day 15 of gestation (day 0 = sperm positive). Day 15 was chosen because most organogenesis in the fetus is complete by this time and the hypothalamic/pituitary/testis axis is just beginning to function (Warren et al., 1975, 1984; Aubert et al., 1985). The pups were weaned 21 days after birth. The consequences of this single, maternal TCDD exposure for the male offspring were characterized at various stages of postnatal sexual development. These original studies of male sexual development following in utero and lactational TCDD exposure have been expanded and further defined in subsequent studies using Holtzman, Long Evans, Sprague-Dawley, and Wistar rats, Syrian hamsters, and mice. These studies have been conducted in five different laboratories and in the vast majority of the studies, TCDD was used as the prototype Ah receptor agonist. However, some studies used 3,3'4,4',5-

PCB (PCB 126), 3,3',4,4',5,5'-HCB (PCB 169), and 2,3,4,7,8-PCDF. In general, the collective findings have produced qualitatively similar results that define a significant effect of TCDD and related Ah receptor agonists on the developing male reproductive system. The effects do not appear to result from reduced plasma androgen concentrations during the perinatal period as originally hypothesized by Mably et al. (1992a) and do not overlap completely with developmental effects of known antiandrogens (Roman et al., 1998b; Gray et al., 1999).

5.2.3.2.1. ***Overt toxicity assessment.*** Mably et al. (1992a) found that TCDD treatment had no effect on daily feed intake during pregnancy and the first 10 days after delivery, nor did it have an effect on the body weight of dams on day 20 of gestation or on days 1, 7, 14, or 21 postpartum. Treating dams with graded doses of TCDD on day 15 of gestation had no effect on gestation index, length of gestation, or litter size. Except for an 8% decrease at the highest maternal dose, TCDD had no effect on live birth index. Neither the 4-day nor 21-day survival index was significantly affected by TCDD. In all dosage groups, the number of dead offspring was equally distributed between males and females, and of the females that failed to deliver litters, none were pregnant. Signs of overt toxicity among the offspring were limited to the above-mentioned 8% decrease in live birth index (highest dose only), initial 10% to 15% decreases in body weight (two highest doses), and initial 10% to 20% decreases in feed intake (measured for males only, two highest doses). The latter two effects disappeared by early adulthood, after which the body weights of the maternally exposed and nonexposed rats were similar.

These findings have essentially been confirmed by both Gray's and Peterson's laboratories (Bjerke et al., 1994a; Gray et al., 1995a, 1997; Roman et al., 1995). A single oral exposure of 1 μg/kg TCDD on day 15 of gestation does not result in maternal toxicity, but compromises perinatal viability and growth of the offspring. A difference in the findings was that the reduced viability occurred prenatally in the studies using the Holtzman rat (Bjerke et al., 1994a; Roman et al., 1995) and postnatally in the Long Evans rat (Gray et al., 1995a).

5.2.3.2.2. ***Prenatal plasma androgen levels and testicular androgen production***. Exposure of Holtzman rats to 1.0 μg/kg of TCDD on gestational day 15 was originally reported to decrease plasma testosterone concentrations in male fetuses from gestational days 17 to 21 and in neonates 2 hours after birth (Mably et al., 1992a). However, a subsequent study from the same laboratory was not able to reproduce these findings (Chen et al., 1993). They found, contrary to their original study (Mably et al., 1992a), that exposure of Holtzman rats to 1.0 μg/kg of TCDD on gestational day 15 did not reduce plasma testosterone concentrations in male fetuses on gestational days 18 or 20 or in male neonates 2 hours after birth. Furthermore, perinatal TCDD exposure did not decrease intratesticular testosterone content or interfere with the ability of the

LH analog, human chorionic gonadotropin (hCG), to stimulate testosterone production from bisected testis preparations at these times (Chen et al., 1993). No other studies have examined the effects of in utero and lactational TCDD exposure on plasma testosterone levels or testicular testosterone production in rat fetuses or neonates at these specific times perinatally when plasma testosterone concentrations are elevated. In control rats the neonatal testosterone peak that occurs 2 hours after birth is followed from 6 hours to 5 days after birth by plasma testosterone concentrations that are 70% to 80% lower than the neonatal peak concentration. When evaluated at these times there was no effect of 1.0 μg/kg of TCDD administered on gestational day 15 on plasma testosterone concentrations in male offspring of either the Holtzman or Long Evans strains (Mably et al., 1992a; Gray et al., 1995a). Also, when evaluated 6 hours after birth there was no effect on LH-stimulated testosterone production in neonatal rats of the Long Evans strain exposed perinatally to TCDD (Gray et al., 1995a). Taken together, these results suggest that effects of in utero and lactational TCDD exposure on the male rat reproductive system cannot be explained by decreased testicular testosterone production or plasma testosterone concentrations during perinatal development (Roman and Peterson, 1998b).

5.2.3.2.3. ***Postnatal plasma androgen levels and testicular androgen production.*** Mably et al. (1992a) failed to find a significant decrease in plasma testosterone or 5α-DHT concentrations in male Holtzman rat offspring that were 32, 49, 63, or 120 days of age and had been exposed on gestational day 15 to either 0.064, 0.16, 0.40, or 1.0 μg/kg of TCDD. Consistent with these negative results, Roman et al. (1995) and Loeffler and Peterson (1999) were also unable to observe any consistent pattern of reduction of plasma testosterone or 5α-androstane-3α,17β-diol concentration in male Holtzman rats that were 21, 32, 49, and 63 days of age and had been exposed to 0.25 or 1.0 μg/kg of TCDD on gestational day 15. Similarly, at the three dosage regimens used for in utero and lactational exposure to TCDD, there was no effect on plasma testosterone concentration in male Wistar rat offspring at postnatal day 70 and only at the highest TCDD dosage regimen was there a reduction in plasma testosterone at postnatal day 170 (Faqi et al., 1998). Gray et al. (1995a) found no effect on serum testosterone concentrations or on basal or LH-stimulated testicular testosterone production from in utero and lactational exposure to 1.0 μg/kg of TCDD administered on gestational day 8 or 15 in male Long Evans rats at 49 or 270 days of age. These investigators also reported that there was no effect of in utero and lactational exposure to 2.0 μg/kg of TCDD administered on gestational day 11 on serum testosterone concentrations of male Syrian hamsters at 140 days of age (Gray et al., 1995a). Theobald and Peterson (1997) failed to find a significant decrease in plasma testosterone concentrations in male ICR mouse offspring that were 44, 65, or 114 days of age and had been exposed on gestational day 14 to either 15, 30, or 60 μg/kg of TCDD. In utero and lactational exposure of rats to other

Ah receptor agonists have produced results similar to those caused by TCDD. 3,3'4,4'5-PCB (PCB 126), 2,3',4,4',5-PCB (PCB 118), or 2,3,4,7,8-PCDF administered on gestational day 1 had no effect on plasma testosterone concentrations in male Wistar rat offspring at 112 days of age (Bouwman et al., 1996). Overall, these findings suggest that the spectrum of effects caused by in utero and lactational exposure to TCDD on the male reproductive system cannot be explained by decreased postnatal testicular androgen production or plasma androgen concentrations (Gray et al., 1995a; Roman and Peterson, 1998b).

5.2.3.2.4. ***External indicators of androgenic status*****.** The androgenic status of the male offspring can be determined from the structure and function of androgen-dependent systems and from the levels of circulating androgens. Anogenital distance, which is dependent on both circulating androgen concentrations and androgenic responsiveness (Neumann et al., 1970), was reduced in 1- and 4-day-old Holtzman male pups by a single maternal TCDD dose as low as 0.16 μg/kg, even when slight decreases in body length were considered (Mably et al., 1992a). However, this effect was not observed in subsequent studies in Holtzman and Long Evans rats exposed perinatally to TCDD when anogenital distance was determined relative to body weight (Gray et al., 1993; 1995a) or crown-rump length (Bjerke et al., 1994a,b; Roman et al, 1995). Also, when Wistar rats were exposed in utero and via lactation to TCDD or 3,3',4,4',5,5'-HCB (PCB 169), anogenital distance was not affected (Faqi et al., 1998a; Smits-van Prooije et al., 1994). This lack of effect on relative anogenital distance was also found when Long Evans rats were exposed to 3,3'4,4'5,5'-HCB (PCB 169) on gestational day 15 (Gray et al., 1999). Thus, these findings suggest that androgenic status of the male rat neonate is not affected by perinatal exposure to TCDD and PCB 169.

Two other external indicators of androgenic status are time to testis descent and time to preputial separation (Rajfer and Walsh, 1977; Korenbrot et al., 1977). These occur in control rats between postnatal days 20-23 and 42-45, respectively. Exposure to 0.16, 0.40, or 1.0 μg/kg of TCDD on gestational day 15 delayed testis descent in the Holtzman rat strain by 1.0 to 1.6 days (Mably et al., 1992a). However, this effect was significant in only two of four rat studies (Mably et al., 1992a; Bjerke et al., 1994a,b; Faqi et al., 1998a) and in the ICR mouse TCDD had no effect on the age at testis descent (Theobald and Peterson, 1997). Puberty, assessed by age at preputial separation, was more reproducibly affected by TCDD across rat strains and species. It was delayed by as much as 3.6 days in Long Evans rats exposed to 1.0 μg/kg TCDD on gestational day 15 (Gray et al., 1995, 1997). The effect was dose-related and significant at a maternal TCDD dose as low as 0.20 μg/kg (Gray et al., 1997). Delays in age at preputial separation were also reported in Holtzman and Wistar rats and in the Syrian hamster following in utero and lactational exposure to TCDD (Bjerke et al., 1994a,b; Roman et al., 1995; Gray et al.,

1995a; Faqi et al., 1998a). The only species studied where TCDD failed to delay the age at preputial separation was the ICR mouse (Theobald and Peterson, 1997). The ability of TCDD to delay puberty in the Long Evans rat was also observed following in utero and lactational exposure to 1.8 mg/kg of 3,3'4,4'5,5'-HCB (PCB 169) administered on gestational day 8 (Gray et al., 1999).

The spectrum of external effects caused by in utero and lactational exposure to TCDD and 3,3',4,4',5,5'-HCB (PCB 169) have been interpreted not to resemble those caused by known antiandrogens such as flutamide (Gray et al., 1999). This is evident in Holtzman and Long Evans rats by perinatal exposure to TCDD or 3,3',4,4',5,5'-HCB (PCB 169) failing to affect external androgen-dependent tissues either by reducing relative anogenital distance or by inducing areolas, retained nipples, or hypospadias (Roman and Peterson, 1998b; Loeffler and Peterson, 1999; Gray et al., 1999). Flank gland development, an androgen-dependent process that occurs in young adulthood in male hamsters, also was not affected by in utero and lactational exposure to TCDD (Gray et al., 1995a). However, other effects of in utero and lactational exposure to TCDD on the androgen-dependent endpoints of preputial separation (weight of the ventral prostate, seminal vesicle, glans penis, testis, and epididymis; daily sperm production; cauda epididymal sperm number; epididymal malformation; demasculinized and feminized sexual behavior; and feminized regulation of LH secretion) resemble effects caused by antiandrogens (Roman and Peterson, 1998b).

5.2.3.2.5. ***Prostate*****.** One of the most sensitive effects of in utero and lactational exposure to TCDD in the male Holtzman rat is a dose-related reduction in ventral prostate weight. The lowest dose of TCDD that caused this effect was 0.064 μg/kg administered on gestational day 15. It reduced ventral prostate weight in male offspring at 32 days of age (Mably et al., 1992a). However, when expressed on a relative body weight basis, 0.16 μg/kg of TCDD was the lowest dose that caused a significant reduction in ventral prostate weight. At a maternal TCDD dose of 1.0 μg/kg, significant decreases in ventral prostate weight have been detected in Holtzman rats as early as postnatal day 14 and as late as postnatal day 120 (Roman and Peterson, 1998; Mably et al., 1992a). In addition to weight of the ventral prostate being reduced by in utero and lactational TCDD exposure in Holtzman rats (Mably et al., 1992a; Bjerke et al., 1994a,b; Roman et al., 1995; Roman and Peterson, 1998), this effect of perinatal TCDD exposure has also been observed in Long Evans and Sprague-Dawley rats (Gray et al., 1997; Wilker et al., 1996) and in ICR and C57BL/6 mice (Theobald and Peterson, 1997; Sommer and Peterson, 1997; Lin et al., 2000). Although a decrease in ventral prostate weight following in utero and lactational exposure to TCDD was not observed in Wistar rats, this may have been caused by the low level of TCDD exposure used in this study (Faqi et al., 1998a). When the same investigators

administered 10 μg/kg of 3,3'4,4'5-PCB (PCB 126) on gestational day 15 to Wistar rats, ventral prostate weights in 70- and 170-day-old offspring were reduced (Faqi et al., 1998b). Administration of 1.8 mg/kg of 3,3',4,4',5',5-HCB (PCB 169) on gestational day 8 reduced ventral prostate weight in Long Evans rats at 65, 260, and 600 days of age (Gray et al., 1999). However, exposure to 100 μg/kg of 3,3'4,4'-TCB (PCB 77) on gestational day 15 had no effect on ventral prostate weight of Wistar rats at 70 and 170 days of age (Faqi et al., 1998b). Taken together, these results demonstrate that in utero and lactational exposure to certain Ah receptor agonists, namely, TCDD, PCB 126, and PCB 169, are capable of reducing ventral prostate weight in various strains of rats and mice. PCB 77 does not appear to share this effect with the other Ah receptor agonists, and this may be due to the more rapid rate of metabolism and elimination of PCB 77 in the rat than TCDD and the other coplanar PCB congeners tested.

The ability of in utero and lactational exposure to TCDD to decrease ventral prostate weight in the rat is greatest from the earliest age at which the organ can be accurately weighed until just after puberty (50 days of age). Thereafter, the magnitude of the weight reduction is progressively attenuated with advancing age, either completely or partially, depending on the dose of TCDD administered during pregnancy (Mably et al., 1992a). At minimally effective doses the reduction in ventral prostate weight is transient and not seen in adulthood. However, at maximally effective doses ventral prostate weight of adult males is reduced significantly. This has been demonstrated for TCDD in Holtzman rats, PCB 169 in Long Evans rats, PCB 126 in Wistar rats, and TCDD in ICR mice (Mably et al., 1992a; Gray et al., 1999; Faqi et al., 1998b; Theobald and Peterson, 1997). In utero and lactational exposure to TCDD also decreases weight of the dorsolateral prostate and anterior prostate (coagulating gland) in the Holtzman rat, ICR mouse, and C57BL/6 mouse (Roman et al., 1995; Theobald and Peterson, 1997; Sommer and Peterson, 1997; Roman and Peterson, 1998; Loeffler and Peterson, 1999; Lin et al., 2000). Thus, TCDD exposure is capable of interfering with ventral, dorsolateral, and/or anterior prostate growth and morphogenensis early in development. Depending on the dose administered during pregnancy, timing of the exposure, species or strain of animal, and lobe of the prostate, TCDD is capable of causing a prostate lesion that cannot be compensated for later in life. Besides size of the ventral prostate being smaller in adulthood, its responsiveness to testosterone stimulation in adulthoood is also impaired by perinatal exposure to TCDD (Bjerke et al., 1994b).

The mechanism by which in utero and lactational exposure to TCDD impairs prostate growth and development is unknown. It cannot be explained in Holtzman rats by TCDD decreasing plasma androgen concentrations (Chen et al., 1993; Roman et al., 1995; Gray et al., 1995a) or inhibiting the conversion of circulating androgens to DHT in the prostate (Roman et al., 1995; Theobald et al., 2000). TCDD probably acts directly on the urogenital sinus from which the prostate develops and on the developing lobes of the prostate as they undergo

differentiation. The Ah receptor and ARNT are expressed in both the rat urogenital sinus and the developing ventral and dorsolateral prostate (Roman et al., 1998a; Sommer et al., 1998) and the infantile rat ventral prostate is responsive to in utero and lactational TCDD exposure in terms of CYP1A induction (Roman and Peterson, 1998). Also, various androgen-regulated mRNAs that code for secretory proteins that are produced by prostate luminal epithelial cells and are markers for functional differentiation of these cells show transient decreases in response to perinatal TCDD exposure in the Holtzman rat (Roman and Peterson, 1998a).

In utero and lactational exposure to TCDD begins to impair rat prostate development during fetal life (Roman et al., 1998a). Therefore, it is important to determine the concentration of TCDD that is present in the fetal urogenital tract after gestational day 15 because this is when fetal prostate development is initiated in the rat. Administration of 1.15 μg/kg of TCDD on gestational day 8 to Long Evans rats results in concentrations of TCDD in the urogenital tract of the fetus of 0.04 and 1.1 pg/g on gestational days 16 and 21, respectively (Hurst et al., 1998). This is significant because a slightly lower dose of TCDD, 1.0 μg/kg, administered to Long Evans rats on either gestational day 8 or 15, causes a decrease in ventral prostate weight of the offspring peripubertally that disappears in adulthood (Gray et al., 1993, 1995a, 1997, 1999). Furthermore, 1.0 μg/kg of TCDD administered on gestational day 15 to Holtzman rats impairs prostate growth and development postnatally (Roman and Peterson, 1998b) and reduces the number of prostatic buds that emerge from the fetal urogenital sinus on gestational day 20 to form the various lobes of the prostate (Roman et al., 1998a). In addition, this same dose of TCDD decreased cell proliferation in the ventral prostate of Holtzman rat neonates that were 1 day of age (Roman et al., 1998a). Subsequent analysis of the effects of TCDD on early postnatal development of the ventral prostate revealed that differentiation of both smooth muscle cells and luminal epithelial cells was delayed and striking alterations in histology of the ventral prostate were apparent in male offspring at 32 days of age (Roman et al., 1998a). These alterations consisted of epithelial hyperplasia, decreased abundance of fully differentiated luminal epithelial cells, increased density of basal epithelial cells, altered spatial distribution of the androgen receptor, and increased thickness of the periductal smooth muscle sheath. Thus, the effects of in utero and lactational TCDD exposure on prostate growth and development are contributed by impaired growth of the developing organ prenatally and neonatally and by delayed and/or impaired differentiation postnatally that, if the dose of TCDD is high enough, could be permanent.

Essentially nothing is known about the long-term consequences of in utero and lactational exposure to Ah receptor agonists on the prostate of laboratory rodent species during old age. The only study available found that the incidence of acute prostatitis in the dorsolateral prostate of 600-day-old Long Evans rats was increased significantly by exposure to a single dose of 1.8

mg/kg of 3,3',4,4',5,5'-HCB on gestational day 8 (Gray et al., 1999). Also, 1 of 9 males displayed diffuse epithelial hypertrophy of the ventral prostate compared with 0 of 15 control males (Gray et al., 1999).

To understand the mechanism by which TCDD impairs the initial step in rat prostate formation, it is important to note that both the Ah receptor and ARNT proteins are expressed in high concentrations in the fetal Holtzman rat urogenital sinus (Sommer et al., 1999). The fetal rat prostate develops from the urogenital sinus. Solid cords of basal epithelial cells (prostatic buds) emerge from the urogenital sinus and invade the surrounding mesenchyme on gestational day 18.5. By gestational day 20.5 this budding process, which TCDD partially blocks (Roman et al., 1998a), is complete. Ah receptor and ARNT proteins were expressed at high levels in the rat urogenital sinus on gestational days 16, 18, and 20, with mean concentrations of 600 fmoles Ah receptor and 140 fmoles ARNT per mg total tissue lysate (Sommer et al., 1999). From a mechanism point of view, it is significant that Ah receptor protein levels were approximately four times greater than ARNT. Since ARNT dimerizes with several members of the bHLH PAS family of transcription factors, it raises the possibility that TCDD activation of the Ah receptor in the urogenital sinus might sequester ARNT from participating in endogenous protein-protein interactions that may be essential for prostate development. Whatever the mechanism, it is clear that the Ah receptor plays a role. The recent finding that impairment of ventral prostate growth and development in Ah receptor wild-type mice by in utero and lactational exposure to 5 μg/kg of TCDD on gestational day 14 is blocked in Ah receptor knockout mice administered the same dose of TCDD supports this view (Lin et al., 2000).

Ah receptor and ARNT proteins are also expressed in human fetal, benign hyperplastic, and malignant prostate (Kashani et al., 1998). Also, TCDD exposure in a human prostate cancer cell line, LNCaP, dose-dependently inhibits androgen-dependent transcriptional activity and prostate-specific antigen expression (Jana et al., 1999). Thus, the human prostate, like the rat and mouse prostate, is capable of responding to TCDD.

5.2.3.2.6. ***Seminal vesicle.*** Weight of the seminal vesicle is decreased in Holtzman, Sprague-Dawley, and Long Evans rats and Syrian hamsters by in utero and lactational exposure to TCDD (Mably et al., 1992a; Bjerke and Peterson, 1994; Gray et al., 1995a; Wilker et al., 1996; Gray et al., 1997). The same effect has been observed in Long Evans rats with 3,3',4,4',5,5'-HCB (PCB 169) and in Wistar rats with 3,3'4,4'-TCB (TCB-77) (Gray and Kelce, 1996; Gray et al., 1999; Faqi et al., 1998b). The lowest maternal dose of TCDD to decrease seminal vesicle weight was 0.16 μg/kg administered on gestational day 15. It significantly decreased seminal vesicle weight in Holtzman rat offspring at 32 and 63 days of age (Mably et al., 1992a).

In Wistar rats a multiple dosing regimen with TCDD that caused a significant decrease in cauda epididymal sperm numbers, daily sperm production, and sperm morphology failed to decrease seminal vesicle weight (Faqi et al., 1998a). This implies that the TCDD-induced decrease in seminal vesicle weight is not the most sensitive effect of TCDD on the developing male reproductive system. 3,3',4,4',5-PCB (PCB 126) administered as a single dose of 10 μg/kg on gestational day 15 also had no effect on seminal vesicle weight in the Wistar rat even though it did significantly decrease ventral prostate weight (Faqi et al., 1998b). Thus, the TCDD-induced reduction in seminal vesicle weight is not as sensitive as some of the other developmental reproductive system endpoints in the Wistar rat (Faqi et al., 1998a). Similarly, in Holtzman rats and ICR mice sensitivity of the seminal vesicle to TCDD is not as great as that of the ventral prostate (Mably et al., 1992a; Roman et al., 1995; Theobald and Peterson, 1997). In Long Evans rats the two accessory sex organs seem equivalent in their sensitivity to TCDD administered on day 15 of gestation (Gray et al., 1997).

The time course of the response of the prostate and seminal vesicle to in utero and lactational TCDD exposure in the Holtzman rat is very different (Roman et al., 1995). The ventral and dorsolateral prostate respond with the greatest relative weight reduction early in development and the magnitude of the response lessens with increasing age. In the case of the seminal vesicle, the opposite is true. Significant TCDD-induced decreases in weight are generally not detected until the peripubertal stage when androgen concentrations are rapidly increasing (Roman et al., 1995). Also, the magnitude of the decrease in seminal vesicle relative weight is not as great as it is for the prostate. The difference in time course of weight reduction between the seminal vesicle and prostate suggest that small TCDD-induced reductions in plasma androgen concentrations peripubertally might account for the small decreases in weight of the seminal vesicles in Holtzman rats at this age (Roman et al., 1995; Roman and Peterson, 1998a). A deficiency in number of androgen receptors in the seminal vesicle is not involved, because the decrease in seminal vesicle weight in 330-day-old Long Evans rat exposed to 1.0 μg/kg of TCDD on gestational day 15 was not associated with a decrease in the concentration of androgen receptors in the seminal vesicle (Gray et al., 1995a).

Another difference between the two accessory sex organs is the effect of in utero and lactational exposure to TCDD on their responsiveness to androgenic stimulation in adulthood. The adult ventral prostate is clearly affected by such exposure and is relatively refractory in its responsiveness to androgens. On the other hand, responsiveness of the adult seminal vesicle to testosterone stimulation is not affected by in utero and lactational exposure to TCDD (Bjerke et al., 1994b).

Consistent with the findings in Holtzman rats (Roman et al., 1995), Long Evans rats exposed on gestational day 15 to 1.0 μg/kg of TCDD and assessed on postnatal days 15, 25, 32,

49, 63, and 120 did not exhibit a decrease in weight of the paired seminal vesicles and attached coagulating glands until postnatal day 32 (Hamm et al., 2000). Furthermore, in utero and lactational exposure to TCDD reduced the amount of secretory fluid contained in the seminal vesicles at this age, which contributed to their decreased weight (Hamm et al., 2000). As with the TCDD-exposed ventral prostate at 32 days of age (Roman et al., 1998a), there were striking alterations in histology of the seminal vesicle at 32 days of age (Hamm et al., 2000). Compared with control animals where seminal vesicle epithelium displayed extensive branching, TCDD-exposed rats had seminal vesicles with fewer and shorter epithelial branches. The epithelium of control rats was characterized by tall columnar cells, whereas that of TCDD-exposed rats contained smaller cells with a lower cytoplasmic-volume to nuclear-volume ratio (Hamm et al., 1999, 2000). Immunostaining for proliferating cell nuclear antigen (PCNA) in control seminal vesicles at 32 days of age was localized to undifferentiated basal cells and no immunoreactivity was observed in terminally differentiated luminal epithelial cells. In contrast, the undifferentiated seminal vesicles of TCDD-exposed rats at the same age exhibited PCNA immunoreactivity at both the basal and luminal surfaces of poorly branched glands (Hamm et al., 2000). Thus, the collective database demonstrates that in utero and lactational exposure to TCDD interferes with epithelial proliferation and differentiation in the seminal vesicle as has been reported for the rat prostate (Hamm et al., 2000; Roman et al., 1998a).

5.2.3.2.7. ***Glans penis.*** Like the prostate and seminal vesicle, the glans penis is an androgen-dependent tissue. In utero and lactational exposure to 1.0 μg/kg of TCDD on gestational day 15 decreased glans penis diameter and absolute weight in Holtzman rats at 63 days of age, but had no effect on glans penis length or relative weight (Bjerke and Peterson, 1994). Weight of the glans penis was reduced in 450-day-old Long Evans rats exposed on gestational day 15 to either 0.20 or 0.80 μg/kg of TCDD (Gray et al., 1997).

5.2.3.2.8. ***Testis weight.*** In utero and lactational exposure to a single dose of TCDD administered on gestational day 15 decreases testis weight in Holtzman, Long Evans, and Sprague-Dawley rats (Mably et al., 1992c; Gray et al., 1995a; Wilker et al., 1996). The effect is transient, being manifested to the greatest extent peripubertally and then decreasing with age. It is not as sensitive an endpoint as the decrease in ventral prostate weight or reduction in cauda epididymal sperm numbers in Holtzman rats or the decrease in ejaculated sperm numbers in Long Evans rats (Mably et al., 1992a,c; Gray et al., 1995a, 1997). Also, in Wistar rats exposed in utero and via lactation to TCDD in a multiple dosing regimen, testis weight is not affected at levels of TCDD exposure that decrease daily sperm production (Faqi et al., 1998a). In utero and lactational exposure to TCDD had no effect on testis weight in the ICR mouse, but did decrease

it in the Syrian hamster (Theobald and Peterson, 1997; Gray et al., 1995a). Thus, among laboratory rodent species there are both strain and species differences in susceptibility to TCDD-induced decreases in testis weight.

There is also variability in the extent to which in utero and lactational exposure to individual non-ortho-substituted PCB congeners are capable of producing this effect. Exposure to 3,3',4,4',5,5'-HCB (PCB 169) on gestational day 8, or to 3,3',4,4'-TCB on gestational day 15, reduced testis weight in Wistar rat offspring (Gray and Kelce, 1996; Gray et al., 1999; Faqi et al., 1998b). However, exposure to 3,3',4,4',5-PCB (PCB 126) on gestational day 15 had no effect on testis weight in the Wistar rat strain (Faqi et al., 1998b).

5.2.3.2.9. ***Epididymis weight and malformation.*** In utero and lactational exposure to TCDD has been shown to reduce epididymal weight in Holtzman, Long Evans, and Sprague-Dawley rat strains (Mably et al., 1992c; Bjerke and Peterson, 1994; Gray et al., 1995a, 1997, 1999; Wilker et al., 1996). In these studies TCDD was administered on gestational day 15, except for the studies by Gray et al. (1995a), where it was also administered on gestational day 8. In the Wistar rat strain a multiple dosing regimen was used for in utero and lactational TCDD exposure and it was not associated with a reduction in the epididymal weight of the progeny (Faqi et al., 1998a). Weight of the epididymis was also not reduced by in utero and lactational TCDD exposure in the ICR mouse (Theobald and Peterson, 1997), but cauda epididymal weight was reduced by TCDD exposure on gestational day 11 in the Sryrian hamster (Gray et al., 1995a). In rat strains that responded to perinatal TCDD exposure by reducing epididymal weight in progeny, certain non-ortho-substituted PCB congeners had the same effect. Administration of 3,3'4,4'5,5'-HCB (PCB 169) on gestational day 8 decreased whole epididymal weight in Long Evans rats, whereas weight of the epididymides in Wistar rats was either not affected or slightly reduced by 3,3',4,4'-TCB (PCB 77) or 3,3',4,4',5-PCB (PCB 126) administered on gestational day 15 (Gray and Kelce, 1996; Gray et al., 1999; Faqi et al., 1998b) .

Compared with decreased testis weight, decreased epididymis and cauda epididymis weights in the Holtzman rat were more sensitive and persistent effects of in utero and lactational TCDD exposure. This is demonstrated by dose-related decreases in weight of the cauda epididymis occurring in Holtzman rats at 120 days of age and cauda epididymal weight being reduced significantly at this age by the lowest dose of TCDD used in the study, 0.064 μg/kg (Mably et al., 1992c). The lowest dose of TCDD reported to decrease epididymal weight in other studies was 0.20 μg/kg in the Long Evans rat (Gray et al., 1997) and the lowest dose tested in the Sprague-Dawley rat, 0.5 μg/kg (Wilker et al., 1996).

The reduction in epididymal weight following in utero and lactational exposure to 1.0 μg/kg of TCDD on gestational day 15 could be permanent in certain rat strains. Significant

decreases in whole epididymal or cauda epididymal weights have been observed in 120-day-old Holtzman rats and 240- to 330-day-old Long Evans rats (Mably et al., 1992c; Gray et al., 1995a). Since epididymal growth is androgen dependent, a TCDD-induced androgenic deficiency and/or decrease in androgen responsiveness of the epididymis could account for decreased size of the organ (Setty and Jehan, 1977; Dhar and Setty, 1990). However, if an antiandrogenic mechanism is involved, it does not appear to be associated with decreases in plasma androgen levels or epididymal androgen receptor levels. This is because in utero and lactational exposure to TCDD has been shown not to reduce circulating androgen concentrations in Holtzman or Long Evans rats at various stages of postnatal development (Roman et al., 1995; Gray et al., 1995a). Also TCDD does not reduce androgen receptor concentrations in either the caput or corpus epididymis when measured in 240- to 330-day-old Long Evans rat progeny exposed perinatally to TCDD (Gray et al., 1995a).

The highest dose of TCDD to be investigated on epididymal development was 2.0 μg/kg administered on gestational day 15 to Sprague-Dawley rats (Wilker et al., 1996). The effects of this high dose on morphological development of the rat epididymis are useful in providing insight into possible mechanisms of action of TCDD on the epididymis. More specifically, this dose of TCDD induced a high incidence of malformations in the epididymis (27%) that were characterized by the segmental absence of regions of the epididymis (Wilker et al., 1996). This high incidence is similar to that reported for rats and mice exposed in utero to the antiandrogen flutamide (van der Schoot, 1992; Cain et al., 1994a,b) and suggests that TCDD may alter testosterone-dependent differentiation of the Wolffian duct into the epididymis (Wilker et al., 1996).

5.2.3.2.10. ***Testicular and epididymal sperm numbers.*** Among the most robust, sensitive, persistent, and reproducible effects of in utero and lactational TCDD exposure in rats, hamsters, and mice are reductions in sperm numbers (Roman and Peterson, 1998b). Generally when TCDD is administered as a single dose during pregnancy, daily sperm production is affected the least. Caput epididymal sperm numbers are reduced to a greater extent than daily sperm production (Gray et al., 1997). Cauda epididymal sperm numbers are reduced more than caput epididymal sperm numbers by in utero and lactational exposure to TCDD. In fact, statistically significant, dose-related reductions in cauda epididymal sperm numbers have been reported for four strains of rats, mice, and hamsters following in utero and lactational exposure to TCDD (Mably et al., 1992c; Gray et al., 1995a, 1997; Wilker et al., 1996; Theobald and Peterson, 1997; Faqi et al., 1998a). The greatest magnitude of reduction in sperm numbers at any given dose of TCDD, however, has been reported for ejaculated sperm numbers in two strains of rats and the hamster (Gray et al., 1995a, 1997; Sommer et al., 1996). Thus, the overall effect of exposure to a

single dose of TCDD administered on gestational day 15 in rats and hamsters is that it causes a progressively greater percentage decrease in sperm numbers in going from the testis (daily sperm production), to caput epididymal sperm numbers, to cauda epididymal sperm numbers, to ejaculated sperm numbers. Taken together, these results imply that in utero and lactational TCDD exposure alters epididymal function such that epididymal sperm storage is permanently reduced (Gray et al., 1995a).

An entirely different profile of inhibitory effects of in utero and lactational exposure to TCDD on sperm numbers, however, was observed in Wistar rats that were exposed to multiple doses of TCDD during mating, pregnancy, and lactation (Faqi et al., 1998b). With this multiple-dosage TCDD exposure paradigm in Wistar rats, the percentage decrease in daily sperm production was greater than the percentage decrease in cauda epididymal sperm numbers (Faqi et al., 1998a)—just the opposite of what was observed in Long Evans and Holtzman rats where TCDD was administered as a single dose (Gray et al., 1995a, 1997; Sommer et al., 1996). It is possible that the difference in rat strain or the TCDD exposure paradigm between these studies accounts for the testis being more sensitive than the cauda epididymis to TCDD-induced decreases in sperm numbers in the study by Faqi and coworkers (1998a).

The lowest single dose of TCDD to decrease cauda epididymal sperm numbers in 120-day-old Holtzman rats was 0.064 μg/kg administered on gestational day 15 (Mably et al., 1992c). The lowest single dose to decrease ejaculated sperm numbers in 450 day old Long Evans rats was 0.050 μg/kg of TCDD administered on gestational day 15 (Gray et al., 1997). Faqi and coworkers (1998a), using a multiple dosing regimen for in utero and lactational exposure of Wistar rat progeny to TCDD, found that the lowest dosing regimen to significantly decrease daily sperm production and cauda epididymal sperm numbers in 170-day-old Wistar rats was a 0.025 μg TCDD/kg loading dose followed by a 0.005 μg TCDD/kg weekly maintenance dose (Faqi et al., 1998a). When the male offspring exposed in utero and via lactation to TCDD in this manner were weaned at 22 days of age, the mean concentration of TCDD in the testis and liver was 0.25 ng/g and 0.24 ng/g, respectively (Faqi et al., 1998a).

5.2.3.2.10.1. ***Daily sperm production.*** In utero and lactational exposure to TCDD in Holtzman rats caused a dose-related decrease in daily sperm production in progeny at 49, 63, and 120 days of age (Mably et al., 1992c). The lowest dose of TCDD administered on gestational day 15 to reduce daily sperm production in 120-day-old Holtzman rats was 0.064 μg/kg. Other studies have also reported that in utero and lactational exposure to TCDD is capable of significantly decreasing daily sperm production in Holtzman, Long Evans, and Wistar rats (Bjerke and Peterson, 1994; Sommer et al., 1996; Gray et al., 1997; Faqi et al., 1998b), but this effect was not observed in Sprague-Dawley rats (Wilker et al., 1996), ICR mice (Theobald and Peterson, 1997),

or Syrian hamsters (Gray et al., 1995a). In the three rat strains where decreased daily sperm production is observed, the response lessens in severity as the progeny age and in some cases returns to control levels (Mably et al., 1992c; Gray et al., 1995a). The Long Evans rat is an example of a rat strain where the decrease in daily sperm production caused by perinatal TCDD exposure is transient (Gray et al., 1995a, 1997). Among the most persistent effects of TCDD on daily sperm production was found in 170-day-old Wistar rats exposed to TCDD dosing during mating, pregnancy, and lactation (Faqi et al., 1998b). It has also been observed in Long Evans rats that administration of TCDD on gestational day 15 is more effective than administering it on gestational day 8 (Gray et al., 1995a; Gray and Kelce, 1996).

Effects on daily sperm production in Long Evans and Wistar rat strains by certain non-ortho-substituted PCBs was variable. In utero and lactational exposure to 3,3',4,4',5',5-HCB (PCB 169) on gestational day 8 reduced daily sperm production in Long Evans rats (Gray et al., 1995b, 1999; Gray and Kelce, 1996). However, exposure of Wistar rats on gestational day 15 to 3,3',4,4',5-PCB (PCB 126) had no effect (Faqi et al., 1998b). Furthermore, in utero and lactational exposure to 3,3',4,4'-TCB (PCB 77) increased both testis size and daily sperm production in 65- and 140-day-old Wistar rats. It was hypothesized that this latter paradoxical effect for an Ah receptor agonist of increasing daily sperm production may be secondary to a possible non-Ah receptor-mediated effect of PCB 77, such as neonatal hypothyroidism (Faqi et al., 1998b).

While severe undernutrition of rat pups and weanlings can adversely affect the male reproductive system and decrease spermatogenesis (Ghafoorunissa, 1980; Jean-Faucher et al., 1982a,b; Glass et al., 1986), it is unlikely that this was involved in reducing daily sperm production caused by in utero and lactational exposure of Holtzman rats to TCDD (Mably et al., 1992c). At the two highest maternal TCDD doses, 0.40 and 1.0 μg/kg, feed consumption and body weight of male offspring were decreased up to 21%, but there was essentially no effect on feed intake or body weight at the two lowest doses, 0.160 and 0.064 μg/kg. However, reduction in daily sperm production, cauda epididymal sperm numbers, and certain sex organ weights, occurred at the two lowest doses. Thus, undernutrition cannot account for these effects (Mably et al., 1992a,c).

Because follicle-stimulating hormone (FSH) and testosterone are essential for quantitatively normal spermatogenesis (Steinberger and Steinberger, 1989), an alternative explanation for the decreases in daily sperm production is a decrease in FSH and/or testosterone levels. In rats, the duration of spermatogenesis is 58 days (Blazak et al., 1985; Amann, 1986; Working and Hurtt, 1987), so the decreases in plasma FSH concentrations in 32-day-old male offspring could contribute to the reductions in daily sperm production when the progeny were 49 and 63 days of age (Mably et al., 1992c). However, the modest depressant effect of perinatal

TCDD exposure on plasma FSH concentrations was transitory, with no effect on plasma FSH levels being found when the offspring were 49, 63, and 120 days old (Mably et al., 1992c). Therefore, it was concluded that reduced daily sperm production in 120-day-old rats perinatally exposed to TCDD is not due to decreases in plasma FSH levels when the animals were 49 to 120 days of age (Mably et al., 1992c). Also, administration of 1.0 μg/kg of TCDD on gestational day 15 to Holtzman rats did not affect testicular testosterone production of their progeny at either prenatal or postnatal stages of development (Chen et al., 1993; Roman et al., 1995). Therefore, a reduction in intratesticular testosterone levels following such TCDD exposure would not be sufficient to reduce spermatogenesis (Zirkin et al., 1989; Mably et al., 1992c; Gray et al., 1993).

In normal rats, daily sperm production does not reach a maximum until 100 to 125 days of age (Robb et al., 1978), but in rats perinatally exposed to TCDD it takes longer for sperm production to reach the adult level. Furthermore, the length of the delay for daily sperm production to attain control levels appears to be directly related to TCDD dose (Mably et al., 1992c). If the dose is high enough, the reduction in daily sperm production may be permanent. This is suggested by in utero and lactational exposure to TCDD decreasing daily sperm production in 170-day-old Wistar rats (Faqi et al., 1998b) and 300-day-old Holtzman rats (Moore et al., 1992). However, in Long Evans rat the TCDD-induced reduction in daily sperm production is transient and does not last beyond the peripubertal stage of development (Gray et al., 1997).

5.2.3.2.10.2. *Testis histology*. A key observation for postulating mechanisms by which perinatal TCDD exposure reduces spermatogenesis in the Holtzman rat strain in adulthood is the finding that the ratio of leptotene spermatocytes per Sertoli cell in the testes of 49-, 63-, and 120-day-old Holtzman rats is not affected by in utero and lactational TCDD exposure even though daily sperm production is reduced (Mably et al., 1992c). Because Sertoli cells provide spermatogenic cells with functional and structural support (Bardin et al., 1988) and the upper limit of daily sperm production in adult rats is directly dependent on the number of Sertoli cells per testis (Russell and Peterson, 1984), three possible mechanisms for the decrease in daily sperm production in Holtzman rats may be involved. TCDD could increase the degeneration of cells intermediate in development between leptotene spermatocytes and terminal-stage spermatids (the cell type used to calculate daily sperm production); decrease postleptotene spermatocyte cell division (meiosis); and/or decrease the number of Sertoli cells per testis (Orth et al., 1988). In a histological study of the testis in Holtzman rats exposed to TCDD in utero and via lactation, it was found that spermatogenesis was qualitatively normal; there was no indication of a gross histological lesion nor any evidence of germ cell degeneration (Shinomiya et al., 1994).

In Long Evans rats exposed in utero and via lactation to TCDD, testicular histopathology was not typically affected (Gray et al., 1995a). That is, there was generally no histological evidence for degeneration of the seminiferous tubules, Sertoli cell abnormalities, or retained spermatids in progeny exposed in utero and via lactation to TCDD (Gray et al., 1997). On occasion, however, both Long Evans rat progeny and Syrian hamster progeny of dams exposed to TCDD exhibited severe atrophy of the seminiferous tubules that was associated with a marked loss of spermatogenic activity (Gray et al., 1997).

In Wistar rats that were exposed to TCDD using a multiple dosing regimen during mating, pregnancy, and lactation, the decrease in daily sperm production observed at the two lowest TCDD exposure levels was not associated with any testicular pathology (Faqi et al., 1998a). However, at the highest level of in utero and lactational TCDD exposure some seminiferous tubuli showed pyknotic nuclei and cell debris in the lumen (Faqi et al., 1998a). In utero and lactational exposure to 1.0 μg/kg of TCDD on gestational day 15 did not cause abnormal testicular histology in 62-day-old Sprague-Dawley rats (Wilker et al., 1996). The number of Sertoli cells per testis and number of Sertoli cells per gram testis was not affected by TCDD. However, the ratio of the number of elongated spermatids in testicular homogenates to the number of Sertoli cells per testis was significantly lower in TCDD-exposed progeny (Wilker et al., 1996).

5.2.3.2.10.3. ***Epididymal sperm numbers.*** The epididymis has two functions: in the caput and corpus epididymis, proximal regions of the organ, spermatozoa mature gaining the capacity for motility and fertility, whereas in the cauda epididymis, the distal region, mature sperm are stored before ejaculation (Robaire and Hermo, 1989). Mably et al. (1991, 1992c) found that motility and morphology of sperm taken from the cauda epididymis on postnatal days 63 and 120 were unaffected by perinatal TCDD exposure. Exposure of Wistar rats to TCDD in a multiple-dosing regimen during mating, pregnancy, and lactation caused small but significant increases in the percentage of abnormal sperm in 170-day-old male offspring (Faqi et al., 1998a). However, in Wistar rats exposed in utero and via lactation to a single dose of 3,3',4,4'-TCB (PCB 77) or 3,3',4,4',5-PCB (PCB 126) on gestational day 15, no effect on the percentage of abnormal sperm was found in 65- or 140-day-old male progeny (Faqi et al., 1998b).

It bears repeating at this time that the most sensitive, robust, persistent, and reproducible effect of in utero and lactational exposure to TCDD on the male reproductive system of laboratory rodents is a decrease in cauda epididymal sperm numbers. This effect has been demonstrated for Holtzman, Long Evans, Sprague-Dawley, and Wistar rats as well as ICR mice and Syrian hamsters (Mably et al., 1992c; Gray et al., 1995a, 1997; Wilker et al., 1996; Theobald

and Peterson, 1997; Faqi et al., 1998a). The lowest dose of TCDD to produce this effect is 0.064 μg/kg administered on gestational day 15 to Holtzman rats (Mably et al., 1992c).

Following in utero and lactational exposure to a single dose of TCDD there is a graded decline in sperm numbers as they travel from the testis through the caput, corpus, and cauda epididymis and are ejaculated (Gray et al., 1995a, 1997; Sommer et al., 1996). While these results suggest that sperm transit rate through the epididymis should be increased by in utero and lactational TCDD exposure, three studies that have determined epididymal sperm transit rates have reached different conclusions. The most rigorous examination of this endpoint was the study by Sommer et al. (1996). They found that in Holtzman rats administered TCDD on gestational day 15 there was no effect on epididymal sperm transit rate. This rules out the possibilities of sperm loss via spontaneous ejaculation or abnormal introduction of sperm into urine (Sommer et al., 1996). In contrast, in utero and lactational exposure of Sprague-Dawley rats to TCDD on gestational day 15 was reported to increase in epididymal sperm transit rate (Wilker et al., 1996), and using a multiple-dosing regimen for in utero and lactational TCDD exposure in Wistar rats, a decrease in epididymal sperm transit rate was found (Faqi et al., 1998a).

In association with the reduction in cauda epididymal sperm numbers, there is a distinct tendency for an increased incidence of a chronic inflammatory reaction in the epididymis of Long Evans rats exposed in utero and via lactation to 3,3',4,4',5,5'-HCB (PCB 169) (Gray et al., 1999) and in Holtzman rats exposed to TCDD (Sommer and Peterson, unpublished results). Furthermore, the decrease in cauda epididymal sperm numbers in adult hamsters following in utero and lactational exposure to TCDD is associated with an increased incidence of sperm granulomas in epididymides and/or testes. This lesion was characterized by a nodular accumulation of fibrous connective tissue and mixed inflammatory cells surrounding degenerating sperm in the interstitium of the epididymides and testes. Taken together, these findings suggest that the reduction in cauda epididymal sperm numbers caused by in utero and lactational exposure to TCDD in the hamster are due in part to sperm resorption from the epididymis. Furthermore, since resorption of sperm in the epididymis is often associated with the accumulation of inflammatory cells in the organ, sperm resorption from the epididymis might also be occurring in postpubertal Holtzman rats exposed to TCDD in utero and via lactation (Sommer and Peterson, unpublished results).

5.2.3.2.10.4. ***Ejaculated sperm numbers.*** Although it has been assessed in only two rat strains, Long Evans and Holtzman, and in the Syrian hamster, the effect on the male reproductive system that is detected at the lowest dose of TCDD administered during pregnancy is that which causes a decrease in ejaculated sperm numbers. The lowest single dose of TCDD administered during

pregnancy that causes this effect is 0.050 μg/kg administered on gestational day 15 in the Long Evans rat with ejaculated sperm numbers assessed in adulthood (Gray et al., 1997). In addition to the reduction in total number of sperm ejaculated during the mating period there was also a reduction in the number of sperm in copulatory plugs. The small reduction in sperm produced by the testis of Long Evans rats, exposed perinatally to TCDD, was not sufficient to account for the larger reductions in cauda epididymal sperm numbers and ejaculated sperm numbers. Finally, there was no reduction in the number of copulatory plugs produced by TCDD-exposed males, indicating no interference with copulation (Gray et al., 1995a).

5.2.3.2.11. ***Reproductive capability.*** To assess reproductive capability, male Holtzman rats born to dams given TCDD (0.064, 0.16, 0.40, or 1.0 μg/kg) or vehicle on day 15 of gestation were mated with control virgin females when the males were 70 and 120 days of age (Mably et al., 1991, 1992c). The fertility index of the males is defined as number of males impregnating females divided by number of males mated. The two highest maternal TCDD doses decreased the fertility index of the male offspring by 11% and 22%, respectively. However, these decreases were not statistically significant, and at lower doses the fertility index was not reduced. The gestation index, defined as the percentage of control dams mated with TCDD-exposed males that delivered at least one live offspring, was also not affected by in utero and lactational TCDD exposure.

With respect to progeny of these matings, the results of the above study (Mably et al., 1992c) and more recent studies are somewhat inconsistent possibly due to differences in the rat strain used. Gray et al. (1995a) reported in the Long Evans that there were fewer implants in females mated to gestational day 15 TCDD-treated male offspring. On the other hand, all male Wistar rat offspring exposed during mating, pregnancy, and lactation to TCDD were able to impregnate unexposed female rats and to produce viable fetuses (Faqi et al., 1998a). For these TCDD-exposed male rat progeny, their mating, pregnancy, and fertility indices were similar to control. Also, the number of implantations, resorption rate, number of viable and dead fetuses, and sex ratio of the progeny were similar among control and TCDD treatment groups (Faqi et al., 1998a). Similar to the results in Wistar rats, Mably et al. (1992c) reported that there was no effect on litter size, live birth index, or 21-day survival index for male Holtzman rat offspring that were mated to unexposed females.

Effects of in utero and lactational exposure to non-ortho-substituted PCB congeners has also been investigated on the reproductive capability of male rat progeny when they reach sexual maturity. Exposure to 1.8 mg/kg of 3,3',4,4',5,5'-HCB (PCB 169) on gestational day 1 reduced the fertility of the PCB 169-exposed male Wistar rat progeny when they were mated with unexposed control females (Smits-van Prooije et al., 1993). In contrast, treatment with either

3,3',4,4'-TCB (PCB 77) or 3,3',4,4',5-PCB (PCB 126) on gestational day 15 had no effect on the outcomes of matings between PCB 77- or PCB 126-exposed male Wistar rat progeny mated with unexposed females for the following endpoints: implantations per litter, viable fetuses per litter, or percentage resorptions (Faqi et al., 1998b).

Since rats produce and ejaculate 10 times more sperm than is necessary for normal fertility and litter size (Aafjes et al., 1980; Amann, 1982), the absence of a reduction in fertility of male rats exposed perinatally to TCDD is not inconsistent with the substantial reductions in testicular spermatogenesis and epididymal sperm reserves. In contrast, reproductive efficiency in human males is very low, the number of sperm per ejaculate being close to that required for fertility (Working, 1988). Thus, measures of fertility using rats are not appropriate for low-dose extrapolation in humans (Meistrich, 1992). A percentage reduction in daily sperm production in humans, similar in magnitude to that observed in rats (Mably et al., 1991, 1992c), could be associated with reduced fertility in men.

5.2.3.2.12. Possible mechanisms for effects on male reproductive tract growth and development. The mechanisms by which TCDD and related compounds impair male reproductive tract development are not known. Several possibilities exist and since these have been reviewed recently (Roman and Peterson, 1998b), only an overview will be presented here. It is generally assumed that most effects of in utero and lactational exposure to TCDD on development of the male reproductive system are Ah receptor mediated. While this is probable, it has not yet been proven conclusively for these endpoints. A comparison of male reproductive endpoints in wild-type and Ah receptor knockout mice, following in utero and lactational exposure to TCDD, is needed to provide this insight. Such studies in Ah receptor wild-type and Ah receptor knockout mice have shown that the decrease in prostate and seminal vesicle weight caused by perinatal exposure to TCDD is dependent on the Ah receptor (Lin et al., 2000). However, this kind of information is not available for any of the other male reproductive endpoints. Three possible Ah receptor-dependent mechanisms by which in utero and lactational exposure to TCDD could impair male reproductive development are as follows. First, the liganded Ah receptor could dimerize with ARNT and this complex could then bind to dioxin-responsive elements in the 5' regulatory regions of genes and alter their transcription in male reproductive tissues during the endocrine phase of fetal and neonatal sexual differentiation. Second, the liganded Ah receptor could compete with other PAS proteins for dimerization with ARNT at critical periods of male repoductive tract development and downregulate genes dependent on ARNT and an alternative dimerization partner for transcription. Third, the ligand-activated Ah receptor could be rapidly depleted from cells, thereby decreasing the pool of Ah receptor available for binding of an endogenous ligand or activating the transcription of genes

important in normal male reproductive development at the cellular level. Treatment of adult rats with TCDD is known to downregulate Ah receptor expression in several male rat reproductive tract tissues (Roman et al., 1998c). However, it was recently found that this does not occur in the developing rat prostate when 1.0 μg/kg of TCDD is administered on gestational day 15, making this latter Ah receptor mechanism seem less likely (Sommer et al., 1999).

In utero and lactational exposure to TCDD produces a novel constellation of growth and developmental alterations in the male rat reproductive system. These consist of decreases in accessory sex organ weights, delays in preputial separation, decreases in daily sperm production by the testis and sperm storage in the cauda epididymis, decreases in ejaculated sperm numbers, and partial demasculization of sexual behavior and partial feminization of sexual behavior and the regulation of LH secretion. Taken together, these effects are consistent with decreased testicular androgen production and/or circulating androgen concentrations. But these parameters have not been shown to be affected perinatally or at later times by perinatal exposure to TCDD (Mably et al., 1992a; Chen et al., 1993; Roman et al., 1995; Gray et al., 1995a). Nevertheless, the possibility remains that the androgenic deficiency-like syndrome caused by developmental exposure to TCDD could be the result of interference with androgen action at the level of the androgen receptor. While no effect of in utero and lactational exposure to TCDD on androgen receptor concentrations in the caput epididymis, cauda epididymis, ventral prostate, or seminal vesicle was found in 336- to 339-day-old Long Evans rats (Gray et al., 1995a), alterations in the spatial distribution of the androgen receptor were found in the ventral prostate of infantile and weanling Holtzman rats exposed perinatally to TCDD (Roman et al., 1998a). This latter effect of TCDD may explain why the ventral prostate exhibits decreased growth and abnormal differentiation in the presence of normal circulating levels of androgens (Roman et al., 1998a). Thus, it is possible that TCDD acts at or beyond the androgen receptor to interfere with prostate development.

Just because development of androgen-dependent tissues such as the prostate, seminal vesicle, epididymis, and testis are affected by in utero and lactational TCDD exposure does not necessarily mean that an antiandrogenic action of TCDD is the only mechanism by which TCDD could disrupt their development (Roman and Peterson, 1998b; Gray et al., 1999). Impaired growth and development of these organs could arise by TCDD acting on multiple components of the endocrine axis to alter concentrations of other hormones, growth factors and/or their receptors. Epidermal growth factor, prolactin, thyroid hormones, and growth hormones can influence development of certain of these organs, and their signaling pathways may be modulated by perinatal exposure to TCDD (Gray et al., 1999). Also, an important finding is that the spectrum of TCDD's effects on male reproductive system development and function does not quite match that which is produced by perinatal exposure to known antiandrogens, 5α-reductase

inhibitors, or antiestrogens (Roman and Peterson, 1998b; Gray et al., 1999). Therefore, it is possible that TCDD modulates cell proliferation and differentiation in these tissues by interfering with nonhormonal aspects of these processes. For example, prostatic budding and ductal morphogenesis are of course androgen-dependent but they also involve important mesenchymal-epithelial interactions occurring downstream of androgen receptor action that might be modulated by TCDD (Roman and Peterson, 1998b).

5.2.3.2.13. ***Sexual differentiation of the CNS*****.** Sexual differentiation of the CNS is dependent on the presence of androgens during early development. In rats, the critical period of sexual differentiation extends from late fetal life through the first week of postnatal life (MacLusky and Naftolin, 1981). In the absence of adequate circulating levels of testicular androgen during this time, adult rats display high levels of feminine sexual behavior (e.g., lordosis), low levels of masculine sexual behavior, and a cyclic (i.e., feminine) pattern of LH secretion (Gorski, 1974; Barraclough, 1980). In contrast, perinatal androgen exposure of rats will result in the masculinization of sexually dimorphic neural parameters, including reproductive behaviors, regulation of LH secretion, and several morphological indices (Raisman and Field, 1973; Gorski et al., 1978). The mechanism by which androgens cause sexual differentiation of the CNS is not completely understood. In the rat, it appears that 17ß-estradiol, formed by the aromatization of testosterone within the CNS, is one of the principal active steroids responsible for mediating sexual differentiation (McEwen, 1978); however, androgens also are involved.

5.2.3.2.13.1. ***Demasculinization of sexual behavior*****.** Mably et al. (1991, 1992b) assessed sexually dimorphic functions in male rats born to dams given graded doses of TCDD or vehicle on day 15 of gestation. Masculine sexual behavior was assessed in male offspring at 60, 75, and 115 days of age by placing a male rat in a cage with a receptive control female and observing the first ejaculatory series and subsequent postejaculatory interval. The number of mounts and intromissions (mounts with vaginal penetration) before ejaculation was increased by a maternal TCDD dose of 1.0 μg/kg. The same males exhibited twelvefold and elevenfold increases in mount and intromission latencies, respectively, and a twofold increase in ejaculation latency. All latency effects were dose related and significant at a maternal TCDD dose as low as 0.064 μg/kg (intromission latency) and 0.16 μg/kg (mount and ejaculation latencies). Copulatory rates (number of mounts + intromissions/time from first mount to ejaculation) were decreased to less than 43% of the control rate (Mably et al., 1992b). This effect on copulatory rates was dose related, and a statistically significant effect was observed at maternal TCDD doses as low as 0.16 μg/kg. Postejaculatory intervals were increased 35% above the control interval, and a statistically

significant effect was observed at maternal doses of TCDD as low as 0.40 μg/kg. Collectively, these results demonstrate that perinatal TCDD exposure demasculinizes sexual behavior.

Because perinatal exposure to a maternal TCDD dose of 1.0 μg/kg has no effect on the open field locomotor activity of adult male rats (Schantz et al., 1991), the increased mount, intromission, and ejaculation latencies in Holtzman rats (Mably et al., 1992b) appear to be specific for these masculine sexual behaviors, not secondary to a depressant effect of TCDD on motor activity. The reported postpubertal plasma testosterone and DHT concentrations in litter mates of the rats evaluated for masculine sexual behavior were as low as 56% and 62%, respectively, of controls (Mably et al., 1991, 1992a). However, plasma testosterone concentrations that were only 33% of controls are still sufficient to masculinize sexual behavior of adult male rats (Demassa et al., 1977). Therefore, the modest reductions in adult plasma androgen concentrations following perinatal TCDD exposure were not of sufficient magnitude to demasculinize sexual behavior.

Reductions in perinatal androgenic stimulation can inhibit penile development and subsequent sensitivity to sexual stimulation in adulthood (Nadler, 1969; Södersten and Hansen, 1978). Therefore, the demasculinization of sexual behavior could, to some extent, be secondary to decreased androgen-dependent penile development. However, perinatal TCDD exposure had no effect on gross appearance of the rat penis. In addition, TCDD-exposed males exhibited deficits in such masculine sexual behaviors as mount latency and postejaculatory interval, which do not depend on stimulation of the penis for expression (Sachs and Barfeld, 1976). Thus, although some effects of TCDD, such as decreased copulatory rate and prolonged latency until ejaculation, could be due to reduced sensitivity of the penis to sexual stimulation, the twelvefold increase in mount latency and increase in postejaculatory interval cannot be explained by this mechanism.

The effect of in utero and lactational exposure to 0.7 μg/kg of TCDD on gestational day 15 on the expression of masculine sexual behavior was assessed in male Holtzman rats at 61-65 and 75-79 days of age (Bjerke et al., 1994). A partial demasculinization of sexual behavior was evidenced by increased intromission latencies and a greater number of intromissions prior to ejaculation. Overall, the effects were more similar to those observed in Holtzman rats exposed on gestational day 15 to 0.4 μg/kg of TCDD (Mably et al., 1992b).

The effect of in utero and lactational exposure to 1.0 μg/kg of TCDD administered on gestational day 8 or 15 on masculine sexual behavior was assessed in Long Evans rats (Gray et al., 1998a). The expression of masculine sexual behaviors was altered to a greater extent in rats exposed to TCDD on gestational day 15 than gestational day 8. In males exposed to TCDD on gestational day 15, partial demasculinization of sexual behavior was evidenced by increases in total number of mounts prior to ejaculation, number of mounts with intromissions prior to

ejaculation, number of mounts without intromissions prior to ejaculation, and latency prior to ejaculation (Gray et al., 1995a). While the same profile of results was obtained in males exposed to TCDD on gestational day 8, the effects were not as great and were not statistically significant (Gray et al., 1995a).

Masculine sexual behavior was also assessed in male Wistar rats exposed in utero and via lactation to TCDD administered to dams during mating, pregnancy, and lactation (Faqi et al., 1998a). Mount latency and intromission latency were increased at two of three TCDD exposure levels. However, ejaculation latency, number of mounts with intromissions prior to ejaculation, and intromission frequency were not affected. Of the five endpoints of masculine sexual behavior assessed in male Wistar rat progeny that were exposed to 10 μg/kg of 3,3',4,4',5-PCB (PCB 126) on gestational day 15, only one endpoint was affected, the number of mounts with intromissions prior to ejaculation, and it was increased (Faqi et al., 1998b).

Taken together, these results demonstrate in three different rat strains, Holtzman, Long Evans, and Wistar, that in utero and lactational exposure to TCDD affects some, but not all, endpoints of masculine sexual behavior. Therefore, TCDD only partially demasculinizes sexual behavior. The response is not as robust as other endpoints, and the degree to which TCDD affects the expression of masculine sexual behavior depends on the rat strain, Ah receptor agonist, and dose administered. It is notable that male hamster progeny do not exhibit demasculinized sexual behavior following perinatal exposure to TCDD, making it difficult to extrapolate this response with certainty to other species.

5.2.3.2.13.2. ***Feminization of sexual behavior*****.** Mably et al. (1991, 1992b) determined if the potential of adult male rats to display feminine sexual behavior was altered by perinatal TCDD exposure. Male offspring of dams treated on day 15 of gestation with various doses of TCDD up to 1 μg/kg or vehicle were castrated at ~120 days of age, and beginning at ~160 days of age were injected weekly for 3 weeks with 17ß-estradiol benzoate, followed 42 hours later by progesterone. Four to six hours after the progesterone injection at weeks 2 and 3, the male was placed in a cage with a sexually excited control stud male. The frequency of lordosis in response to being mounted by the stud male was increased from 18% (control) to 54% by the highest maternal TCDD dose, 1.0 μg/kg. Lordosis intensity, scored after Hardy and DeBold (1972) as 1 for light lordosis, 2 for moderate lordosis, and 3 for a full spinal dorsoflexion, was increased in male rats by perinatal TCDD exposure. Both effects on lordosis behavior in males were dose related and significant at maternal TCDD doses as low as 0.16 μg/kg (increased lordotic frequency) and 0.40 μg/kg (increased lordotic intensity). Together, they indicate a feminization of sexual behavior in these animals. Although severe undernutrition from 5 to 45 days after birth potentiates the display of lordosis behavior in adult male rats (Forsberg et al., 1985), the

increased frequency of lordotic behavior was seen at a maternal TCDD dose of 0.16 μg/kg, which had no effect on feed intake or body weight. It was concluded that perinatal TCDD exposure feminizes sexual behavior in adult male rats independent of undernutrition.

Defeminization of sexual behavior in male rats occurs during the first week or so after birth (Goy and McEwen, 1980; Perakis and Stylianopoulou, 1985). Therefore it was hypothesized that if TCDD interferes with defeminization of sexual behavior that lactational exposure to TCDD would be more important than in utero exposure. A subsequent study by Bjerke and Peterson (1994) is consistent with this hypothesis. In their cross-fostering study on the effects of in utero versus lactational TCDD exposure in the Holtzman rat, it was found, as predicted, that feminization of male sexual behavior required lactational exposure. When exposure was restricted to the in utero period, the male offspring did not display a significant increase in lordotic behavior, whereas such behavior was increased following exposure during the lactational period, either alone or in combination with in utero exposure. Also, in a separate study it was shown following in utero and lactational exposure to 1.0 μg/kg of TCDD administered on gestational day 15 that feminine sexual behavior of male rats was partially feminized as indicated by an increase in lordosis quotient (Bjerke et al., 1994b).

In contrast to the above three studies that show in utero and lactational TCDD exposure increases the expression of feminine sexual behavior in male Holtzman rats (Mably et al., 1992b; Bjerke and Peterson, 1994; Bjerke et al., 1994b), this effect was not observed in male Long Evans rats (Gray et al., 1995a). They showed no significant increase in lordotic behavior as adults following in utero and lactational exposure to 1.0 μg/kg of TCDD on gestational day 8 or 15 (Gray et al., 1995a). This may be due to a rat strain difference in susceptibility to this endpoint.

5.2.3.2.13.3. ***Feminization of LH secretion regulation.*** The effect of perinatal TCDD exposure on regulation of LH secretion by ovarian steroids was determined in male offspring at ~270 days of age. There is normally a distinct sexual dimorphism to this response. In rats castrated as adults, estrogen-primed females greatly increase their plasma LH concentrations when injected with progesterone, whereas similarly treated males fail to respond (Taleisnik et al., 1969). Progesterone had little effect on plasma LH concentrations in estrogen-primed control males, but significant increases were seen in males exposed to maternal TCDD doses as low as 0.40 μg/kg. Thus, perinatal TCDD exposure increases pituitary and/or hypothalamic responsiveness of male rats to ovarian steroids in adulthood, indicating that regulation of LH secretion is permanently feminized.

5.2.3.2.13.4. ***Estrogen receptor concentrations in the brain and volumes of sexually dimorphic brain nuclei.*** In the Holtzman rat, in utero and lactational exposure to TCDD partially demasculinizes and feminizes sexual behavior in adult male rats, possibly by causing incomplete sexual differentiation of the CNS. To determine if TCDD exposure affects other aspects of sexual differentiation of the CNS in this rat strain, the effects of perinatal exposure to 0.7 μg/kg of TCDD administered on gestational day 15 on estrogen receptor binding in specific brain nuclei was examined along with effects on the volumes of brain nuclei that are dependent on hormone stimulation during the period of CNS sexual differentiation (Bjerke et al., 1994b). It was found that estrogen receptor concentrations in three brain nuclei—the medial preoptic nucleus (MPO), the ventrolateral aspect of the ventromedial nucleus, and the periventricular preoptic area—were higher in control females than males, but in utero and lactational exposure to TCDD had no effect on estrogen receptor concentrations in these sexually dimorphic brain nuclei (Bjerke and Peterson, 1994). It also had no effect on estrogen receptor concentrations in other brain nuclei where there was not a sex difference in estrogen receptor concentrations.

The volumes of sexually dimorphic brain nuclei were also not affected by in utero and lactational exposure to TCDD in Holtzman rats. In control rats the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) is greater in males, whereas the MPO is greater in females. Perinatal TCDD exposure had no effect on the volume of either nucleus in male and female Holtzman rat offspring in adulthood (Bjerke and Peterson, 1994). Thus, in utero and lactational TCDD exposure is capable of partially demasculinizing and partially feminizing sexual behavior of Holtzman rat progeny (Mably et al., 1992b; Bjerke and Peterson, 1994; Bjerke et al., 1994b), but it is not associated with an effect on sexual differentiation of the estrogen receptor system in the brain or the volume of sexually dimorphic brain nuclei (Bjerke et al., 1994b; MacLusky et al., 1998).

5.2.3.2.13.5. ***Comparison to other Ah receptor-mediated responses.*** The induction of hepatic cytochrome P-4501A1 and its associated EROD activity are extremely sensitive Ah receptor-mediated responses to TCDD exposure. Yet in 120-day-old male Holtzman rats that had been exposed to TCDD perinatally, alterations in sexual behavior, LH secretion, sex organ weights, and sperm numbers were observed when induction of hepatic EROD activity could no longer be detected (Mably et al., 1991, 1992a,b,c). These results suggest that TCDD affects sexual behavior, gonadotrophic function, and sperm counts when virtually no TCDD remains in the body. Therefore, the partial demasculinization and feminization of sexual behavior, partial feminization of LH secretion, and reduced cauda epididymal sperm numbers caused by in utero and lactational exposure to TCDD have the potential to be irreversible effects of transient

exposure to TCDD during the endocrine phase of fetal and neonatal sexual differentiation (Mably et al., 1992b,c).

5.2.3.2.13.6. ***Possible mechanisms for effects on sexual behavior*.** The most plausible explanation for the demasculinization of sexual behavior and feminization of sexual behavior and LH secretion is that perinatal exposure to TCDD impairs sexual differentiation of the CNS. Neither undernutrition, altered locomotor activity, reduced sensitivity of the penis to sexual stimulation, nor modest reductions in adult plasma androgen concentrations of the male offspring can account for this effect (Mably et al., 1992b). On the other hand, exposure of the developing brain to testosterone, conversion of testosterone into 17ß-estradiol within the brain, and events initiated by the binding of 17ß-estradiol to its receptor are all critical for sexual differentiation of the CNS and have the potential to be modulated by TCDD. If TCDD interferes with any of these processes during late gestation and/or early neonatal life, it could irreversibly demasculinize and feminize sexual behavior (Hart, 1972; McEwen et al., 1977; Whalen and Olson, 1981) and feminize the regulation of LH secretion (Gogan et al., 1980, 1981) in male rats in adulthood. However, results that argue against this hypothesis are that in utero and lactational exposure to TCDD does not alter either estrogen receptor concentrations in various brain nuclei or volumes of sexually differentiated brain nuclei of male and female Holtzman rat progeny at doses that affect the expression of masculine and feminine sexual behavior (Bjerke et al., 1994). Also, while in utero and/or lactational exposure to TCDD may cause similar effects on sexual behavior in other animal species, including nonhuman primates (Pomerantz et al., 1986; Thornton and Goy, 1986; Goy et al., 1988), in which sexual differentiation is under androgenic control; this was not able to be demonstrated for male hamster progeny exposed in utero and via lactation to TCDD (Gray et al., 1995a). In humans, there is evidence that social factors account for much of the variation in sexually dimorphic behavior; there is also evidence that prenatal androgenization influences both the sexual differentiation of such behavior and brain hypothalamic structure (Erhardt and Meyer-Bahlburg, 1981; Hines, 1982; LeVay, 1991).

5.2.3.2.14. ***Cross-species comparisons*.** Gray et al. (1995a) demonstrated that many of the results observed on male rat reproductive system development following in utero and lactational TCDD exposure are also observed in male hamster offspring exposed perinatally to TCDD. Pregnant hamsters were exposed to 2 μg/kg of TCDD on gestational day 11. This exposure level caused no maternal toxicity or decrease in viability of the offspring. The number of litters in the study was small (three dams/treatment group). Nevertheless, growth retardation, reduced adrenal and brain weight at postnatal day 136 to 140, delayed eye opening, and reduced sperm in the epididymis and ejaculate were observed in the male hamster offspring exposed to TCDD.

Anogenital distance and testicular sperm number were not affected. As regards androgen-dependent organ development, age at preputial separation was delayed and seminal vesicle weight was reduced, but flank gland development was not affected. In contrast to the rat, none of the masculine sexual behavior parameters measured in male hamster offspring appeared to be altered. At sacrifice (postnatal day 136 to 140), there was an increase in sperm granulomas and in the severity of kidney lesions in the TCDD-exposed male hamster progeny. While the findings of this study show some species specificity in the hamster's response to in utero and lactational TCDD exposure, they generally support the above findings in male rat offspring.

Male ICR mice, CD-1 derived, exposed in utero and via lactation to TCDD are also affected by developmental toxicity to the reproductive system (Theobald et al., 1997). The doses of TCDD used in this study did not alter maternal or offspring body weights and were not associated with prenatal or postnatal mortality. However, ventral prostate weight assessed on postnatal days 44, 65, and 114 was significantly decreased in male offspring after exposure to 15 μg TCDD/kg administered on gestational day 14. Coagulating gland weight was also reduced on the same postnatal days, but this lobe of the prostate was not as sensitive as the ventral prostate because larger doses of TCDD were required to produce the effect. The dorsal prostate in the ICR mouse was not affected even at the largest maternal TCDD dose (60 μg/kg). There was no statistically significant decrease in daily sperm production after TCDD exposure, but whole epididymal sperm numbers were decreased at maternal doses of 30 and 60 μg TCDD/kg. Thus, ventral prostate weight in this strain of mouse was a more sensitive endpoint of TCDD exposure than the reduction in epididymal sperm number. The C57BL/6 mouse is more sensitive to the developmental effects of TCDD on accessory sex organ growth (Lin et al., 2000). Ventral prostate, dorsolateral prostate, and coagulating gland weight in mouse progeny exposed to 5 μg/kg of TCDD on gestational day 13 were all significantly reduced when assessed on postnatal days 21 to 90. Histological examination revealed an impairment of ductal development in the ventral prostate on postnatal day 21, and only 50% of the luminal epithelial cells in ventral prostate from TCDD-exposed mice expressed androgen receptors, compared with 100% of the epithelial cells in tissue from vehicle-exposed mice. Urogenital sinus epithelial complexes from male offspring exposed to a single maternal dose of 5 μg/kg of TCDD administered on gestational day 13 were examined by scanning electron microscopy, and complete agenesis of ventral prostate buds on gestation day 18 was found. In Ah receptor knockout mice obtained from Bradfield (Schmidt et al., 1996) and backcrossed into C57BL/6, there was no reduction in seminal vesicle or prostate weight due to in utero and lactational TCDD exposure, and prostatic bud formation occurred normally by gestational day 18. Therefore, these effects on the prostate and seminal vesicle of perinatal TCDD exposure in the mouse appear to be Ah receptor mediated.

Taken together, these results indicate that there is species specificity in sensitivity to certain effects of in utero and lactational TCDD exposure on male reproductive system development. In general, however, the results reported in the hamster and mouse are consistent with those reported in male rat offspring. There is evidence that at least some of these effects are Ah receptor mediated, namely those that occur in the prostate and seminal vesicle. Because the fetal human prostate expresses the Ah receptor (Kashani et al., 1998), it is plausible that the human prostate could be affected by sufficient exposure to TCDD and TCDD-like Ah receptor agonists during development.

5.2.3.3. *Female Reproductive System*

Effects of in utero and lactational exposure to TCDD on female reproductive system development has not been investigated for as long as the male reproductive system. However, the results from these studies clearly show that the effects of gestational exposure to TCDD is not limited to the male offspring.

5.2.3.3.1. ***Vaginal thread malformation.*** One of the most sensitive effects of in utero and lactational exposure to TCDD on the female reproductive system is the occurrence of a vaginal thread malformation. It has been detected in two strains of rats, Long Evans and Holtzman, but not in ICR mice or Syrian hamsters (Gray et al., 1995; Flaws et al., 1997; Theobald and Peterson, 1997; Gray et al., 1997b; Wolf et al., 1999; Dienhart et al. 2000). The lowest dose of TCDD to significantly increase the incidence of vaginal threads in female progeny is 0.20 μg/kg administered on gestational day 15 to Long Evans rat dams (Gray et al., 1997b). This dose was effective in increasing the incidence of the malformation when expressed either as percentage of females with a temporary or permanent vaginal thread or as percentage of females with a permanent vaginal thread (Gray et al., 1997b). In utero and lactational exposure to other Ah receptor agonists are also capable of producing this same type of malformation in Long Evans rats. 3,3',4,4',5,5'-HCB (PCB 169) administered on gestational day 8 at a dose of 1.8 mg/kg caused a significant increase in the percentage of female progeny with vaginal threads (Gray et al., 1999).

The original study to report an increase in vaginal thread malformation following in utero and lactational exposure to TCDD was that of Gray and Ostby (1995). They reported on the effects in Long Evans rats of a single maternal exposure to 1 μg/kg of TCDD on either gestational day 8 or 15 on postnatal vaginal development in female offspring. Both exposures were associated with incomplete (vaginal thread) or absent vaginal opening, and a smaller vaginal orifice. Similar malformations involving the vaginal canal have been reported for Holtzman rats (Gray and Ostby, 1995; Flaws et al., 1997). The incidence of vaginal threads was

greater in the Long Evans rat when TCDD was administered on gestational day 15 compared with gestational day 8 (Gray and Ostby, 1995). On the other hand, in the Holtzman rat the incidence of vaginal threads was essentially the same when TCDD was administered on gestational days 11, 15, or 18 (Flaws et al., 1997).

The vaginal thread is manifested in pubertal rats as a persistent thread of mesenchymal tissue surrounded by keratinized epithelium that partially occludes the vaginal opening (Flaws et al., 1997). However, it was not known how early in development this abnormality could be detected. Vaginal threads in TCDD-exposed Holtzman rat offspring were identified in histological sections of the developing vagina in 2-day-old pups, demonstrating that this malformation was actually present at birth (Flaws et al., 1997). This suggested that prenatal exposure to TCDD should be sufficient to cause the vaginal thread malformation. This was confirmed in a cross-fostering study in Long Evans rats (Gray et al., 1997b) where female progeny that received prenatal TCDD exposure developed vaginal threads but those that received only postnatal exposure to TCDD did not. The earliest time during fetal development that morphologic signs of this malformation were present was gestational day 19 in Holtzman rats (Dienhart et al., 2000) and gestational day 18 in Long Evans rats (Hurst et al., 1999). At this time there was an increased thickness of mesenchymal tissue between the caudal Mullerian ducts. The presence of this mesenchymal tissue caused the Mullerian ducts to fail to fuse, a process that is normally completed prior to birth. TCDD was also found to block regression of the Wolffian ducts, which contributed to the changes in morphology of the vagina (Dienhart et al., 2000). Thus, prenatal TCDD exposure leads to altered development of the rat vagina as early as gestational day 18 or 19 depending on the strain, 3 or 4 days after treatment of the dams. This effect is produced by TCDD interfering with two critical morphogenetic events involved in the formation of the female reproductive tract, namely, regression of the Wolffian ducts and fusion of the Mullerian ducts (Hurst et al., 1999; Dienhart et al., 2000).

The mechanisms by which TCDD produces these effects at the molecular level are unknown. TCDD modulates cellular responses to both hormones and growth factors including androgens, estrogens, EGF, and TGF (Abbott, 1997; Birnbaum, 1998; Roman and Peterson, 1998b). Developmental processes such as the timing of morphogenetic signals and events like cell proliferation, cell movement, receptor expression, apoptosis, and terminal differentiation are tightly regulated by these and other hormones and growth factors. Thus, TCDD modulation or interference with the activity of hormones and/or growth factors in the female rat reproductive tract may play a role in causing the vaginal thread malformation (Dienhart et al., 2000).

5.2.3.3.2. ***Cleft phallus and mild hypospadias*****.** Other morphological effects of in utero and lactational exposure to TCDD on the female reproductive tract are cleft phallus and mild

hypospadias. The hypospadias are considered mild because the urethral opening was always separate from the vaginal opening. These two types of malformations have been observed in rats and hamsters, but not in mice (Gray and Ostby, 1995; Flaws et al., 1997; Theobald and Peterson, 1997; Gray et al., 1997b; Wolf et al., 1999; Dienhart et al., 2000). The lowest dose of TCDD to significantly increase the incidence of cleft phallus and mild hypospadias is 0.80 and 0.20 μg/kg of TCDD, respectively, administered on gestational day 15 in Long Evans rats (Gray et al., 1997b). The morphometric indices of mild hypospadias that were significantly affected by exposure to 0.2 μg/kg of TCDD on gestational day 15 were length of the urethral slit (increased by TCDD), distance from the tip of the phallus to the urethral opening (increased by TCDD), and distance from the urethral to vaginal opening (decreased by TCDD) (Gray et al., 1997b). Other Ah receptor agonists are also capable of producing cleft phallus and mild hypospadias in female Long Evans rats. 3,3',4,4',5,5'-HCB (PCB 169) administered on gestational day 8 at a dose of 1.8 mg/kg caused a significant increase in the percentage of female progeny with cleft phallus (Gray et al., 1999). It also caused the female offspring to have a significantly longer urethral slit and a shorter distance between the urethral and vaginal openings (Gray et al., 1999). The incidence of cleft phallus was greater in female Long Evans rat progeny administered TCDD on gestational day 15 compared to gestational day 8 (Gray and Ostby, 1995). In Holtzman rats the incidence was greater when TCDD was administered on gestational day 11 compared with gestational day 15 or 18 (Flaws et al., 1997).

These TCDD-induced malformations of the external genitalia in female rats and hamsters (cleft phallus and mild hypospadias) closely resemble the mild form of hypospadias caused by in utero exposure to diethylstilbestrol (DES) and other potent estrogens. In hamsters estradiol causes cleft phallus (Whitsett et al., 1978) and in rats DES and the synthetic estrogen RU2858 are capable of producing a mild form of hypospadias (Voherr et al., 1979; Vannier et al., 1980). This raises the possibility that TCDD, which is often characterized as being an antiestrogen, might cause these effects through an estrogen-like developmental action (Gray et al., 1997b). In this context, it is important to stress that the other type of malformation produced by in utero and lactational exposure to TCDD in the female rodent, vaginal thread formation, is unique to TCDD and TCDD-like Ah receptor agonists. Vaginal thread formation, which can be detected as early as gestational day 19 in the rat, is not known to be produced by any other class of chemical, including potent estrogens like DES.

Gray and Ostby reported that in utero and lactational exposure to TCDD caused a significant reduction in ovarian and brain weights when necropsied as adults (Gray and Ostby, 1995). Hamster offspring, like rats, display clefting of the phallus, mild hypospadias, and reduced ovarian weight, but not formation of the vaginal thread (Gray and Ostby, 1995; Gray et al., 1997b; Wolf et al., 1999). Female ICR mouse offspring were not susceptible to either cleft

phallus or vaginal thread malformations nor were their ovarian or brain weights reduced by perinatal TCDD exposure (Theobald and Peterson, 1997).

5.2.3.3.3. ***Ovary.*** In utero and lactational exposure to TCDD decreased ovarian weight in the rat and hamster but not in the mouse (Gray and Ostby, 1995; Theobald and Peterson, 1997; Wolf et al., 1999). Shiverick and Muther (1983) reported that there was no change in circulating levels of estradiol in the rat after exposure to 1 μg/kg/day on gestational days 4 to 15. Similarly, Gray et al. have found no effect on serum estradiol levels after perinatal exposure to a single maternal dose of 1 μg TCDD/kg administered on gestational day 15 in the Long Evans rat, evaluated on postnatal days 21 and 28 (Gray et al., 1997b). In addition, these authors found no effect on ovarian estradiol production when ovaries from vehicle- and TCDD-exposed rats removed on postnatal days 21 and 28 were placed in organ culture for 3 hours. However, Chaffin et al. found that serum estradiol and ovarian secretion of estradiol in vitro were decreased by a similar exposure regimen in the Holtzman rat (Chaffin et al., 1996, 1997). Histologic examination of ovaries from 21- to 22-day-old rats that had been exposed to a single maternal dose of 1 μg TCDD/kg in utero and via lactation revealed decreases, compared with vehicle-exposed rats, in the number of ovarian follicles without alterations in ovarian size, or apoptosis in the affected follicular regions (Heimler et al., 1998). Similarly, the administration of a single maternal dose of 0.6 mg/kg of 3,3',4,4',5,5'-HCB (PCB 169) on gestational day 1, combined with daily doses of 1 mg/kg of 3,3',4,4'-TCB (PCB 77) on gestational days 2-18 resulted in a statistically significant increase in the incidence of cystic dilated ovarian follicles (Smits-van Prooije et al., 1994).

5.2.3.3.4. ***Estrous cyclicity and reproductive performance.***

5.2.3.3.4.1. ***Rats.*** With regard to effects on estrous cyclicity and reproductive performance, Long Evans rats exposed on gestational day 8 to 1.0 μg/kg of TCDD had a significantly increased number of the female offspring displaying constant estrus by 1 year of age (Gray and Ostby, 1995). This was accompanied by a significant reduction in fertility during a continuous breeding trial. The gestational day 15 exposure did not have the same effect on cyclicity, and the occurrence of constant estrus was not significantly different from control rats. There was also no effect on female sexual behavior. Nevertheless, the number of mounts of control males and the latency to ejaculation were increased in matings with females exposed to TCDD on gestational day 15. This was possibly due to the vaginal abnormalities interfering with normal copulation.

Gray and Ostby (1995) also compared the gestational day 15 exposure to 1.0 μg/kg of TCDD in the Long Evans female offspring with that in the Holtzman. There was a greater reduction in neonatal viability in Holtzman than Long Evans female offspring (50% vs. 11%, respectively) following TCDD exposure. In the surviving Holtzman offspring, the

morphological effects were similar to those in the Long Evans offspring, including genital clefting and vaginal threads. Reproductive behavior was not assessed in the Holtzman strain.

As Gray and Ostby (1995) have noted, their observations are consistent with previous reports of infertility in female offspring after in utero exposure to TCDD (Khera and Ruddick, 1973) and are likely due to the alterations in estrous cyclicity and ovarian function. In utero and lactational exposure of Wistar rats to 0.5 μg/kg/day of TCDD administered on gestational days 6 to 15 caused infertility in both sexes (Khera and Ruddick, 1973). Also, in utero and lactational exposure to 1.8 mg/kg of 3,3',4,4',5,5'-HCB (PCB 169) on gestational day 1 decreased mating success and female fecundity in female Wistar rat progeny (Smits-van Prooije et al., 1994). Taken together, in utero and lactational exposure to TCDD and TCDD-like Ah receptor agonists in the rat causes morphological and functional reproductive alterations in female offspring at relatively low doses that do not induce overt maternal toxicity.

5.2.3.3.4.2. ***Hamsters.*** TCDD produced adverse effects in female hamsters that persisted through two generations (F1 and F2). This occurred despite the F1 generation being the only generation that was exposed in utero and via lactation to 2.0 μg/kg of TCDD administered on gestational day 11.5 (Wolf et al., 1999). In the female progeny of the F1 generation, vaginal opening was delayed and vaginal estrous cycles were altered. However, most TCDD-exposed females had regular 4-day behavioral estrous cycles. This suggests in utero and lactational TCDD exposure did not cause a marked disruption in the hypothalamic-pituitary-gonadal hormonal cyclicity. While the F1 TCDD-exposed females mated successfully with a control male, 20% of them did not become pregnant and 38% of those that did become pregnant died near term. Both the number of implants in pregnant TCDD-exposed hamsters and the number of pups they produced that were born alive were reduced significantly.

An important finding was that survival of F2 generation offspring through weaning was virtually eliminated by treating with TCDD the dams that produced the F1 generation (Wolf et al., 1999). The cause of death of the F2 generation offspring has not been reported.

5.2.3.3.5. ***Histopathology of the aging female reproductive tract.*** In utero and lactational exposure to TCDD affects the histopathology of the female rat reproductive tract (Gray et al., 1997b). In the ovary of TCDD-exposed female offspring, cystic follicles with luteinization and sertoliform hyperplasia were observed. Diffuse squamous hyperplasia of the cervix and hyperkeratosis of the vagina were seen in the TCDD-exposed progeny, but not in the controls.

5.2.3.3.6. ***Ovarian and mammary gland tumors.*** Ovarian tumors were found in female rats following in utero and lactational exposure to TCDD, but not in control rats (Gray et al., 1997b).

In addition, in utero and lactational exposure of rats to 1.0 μg/kg of TCDD on gestational day 15 rendered mammary glands of the female offspring more susceptible to tumor formation induced by 7,12-dimethylbenz[a]anthracene (DMBA) (Brown et al., 1998).

5.2.3.4. *Neurobehavior*

Because differentiated tissues derived from ectoderm, namely, skin, conjunctiva, nails, and teeth, are sites of action of halogenated aromatic hydrocarbons in transplacentally exposed human infants, another highly differentiated tissue derived from ectoderm, the CNS, should be considered a potential site of TCDD action. In support of this possibility, sexual differentiation of the CNS in adult male rats is irreversibly altered in a dose-related fashion by perinatal exposure to TCDD (Mably et al., 1991, 1992b). As will be shown below, the central nervous systems of mice transplacentally exposed to 3,3′,4,4′-TCB, monkeys perinatally exposed to TCDD, and children transplacentally exposed to a mixture of PCBs, CDFs, and PCQs in the Yu-Cheng incident are also affected. Thus, functional CNS alterations, which may or may not be irreversible, are observed following perinatal exposure to halogenated aromatic hydrocarbons.

5.2.3.4.1. ***Ah receptor and ARNT in the central nervous system.*** Ah receptors have been identified in rat brain (Carlstedt-Duke, 1979). However, while an early study suggested the Ah receptor may be associated with glial cells rather than neurons (Silbergeld, 1992), a more recent study of the adult male rat brain that used in situ hybridization to localize mRNAs for the Ah receptor and ARNT proteins found mRNAs for both proteins in the same neuronal populations in the olfactory bulb, hippocampus, cerebral, and cerebellar cortices (Kainu et al., 1995). Unexpectedly, detectable levels of Ah receptor mRNA were not detected in the hypothalamus. The significance of these findings is that they suggest that TCDD and related Ah receptor agonists may act in discrete neuronal populations in the brain.

Following administration of ^{14}C-TCDD in the rat, the highest concentrations of TCDD-derived ^{14}C are found in the hypothalamus and pituitary. Much lower concentrations are found in the cerebral cortex and cerebellum (Pohjanvirta et al., 1990). In another study, the Ah receptor was not detected in whole rat or mouse brain but was detected in the cerebrum of the hamster and cerebrum and cerebellum of the guinea pig (Gasiewicz, 1983).

5.2.3.4.2. ***Neurobehavior in mice.*** CD-1 mice exposed transplacentally to 3,3',4,4'-TCB at a maternal oral dose of 32 mg/kg administered on days 10 to 16 of gestation exhibited neurobehavioral, neuropathological, and neurochemical alterations in adulthood (Tilson et al., 1979; Chou et al., 1979; Agrawal et al., 1981). The neurobehavioral effects consisted of circling, head bobbing, hyperactivity, impaired forelimb grip strength, impaired ability to traverse a wire

rod, impaired visual placement responding, and impaired learning of a one-way avoidance task (Tilson et al., 1979). The brain pathology in adult mice exhibiting this syndrome consisted, in part, of alterations in synapses of the nucleus accumbens (Chou et al., 1979). This suggested that in utero exposure to 3,3',4,4'-TCB may interfere with synaptogenesis of dopaminergic systems. In support of this possibility, Agrawal et al. (1981) found that adult mice transplacentally exposed to 3,3',4,4'-TCB had decreased dopamine levels and decreased dopamine receptor binding in the corpus striatum, both of which were associated with elevated levels of motor activity. It was concluded that transplacental exposure to 3,3',4,4'-TCB in mice may permanently alter development of striatal synapses in the brain.

Eriksson (1988) examined the neurobehavioral effects of 3,3',4,4'-TCB in NMRI mice exposed to a single oral dose of 0.41 or 41 mg/kg on postnatal day 10. Following sacrifice of the mice on day 17, muscarinic receptor concentrations in the brain were significantly decreased at both dose levels. This effect was shown to occur in the hippocampus but not in the cortex. More recently (Eriksson et al., 1991), NMRI mice were exposed to the same two doses of 3,3',4,4'-TCB similarly administered on postnatal day 10. At 4 months of age, the effects of the PCB on locomotor activity were assessed. At both dose levels, abnormal activity patterns were exhibited in that the treated mice were significantly less active than controls at the onset of testing, but were more active than controls at the end of the test period. This pattern of effects can be interpreted as a failure to habituate to the test apparatus. In contrast to the previous results with CD-1 mice, circling or head bobbing activities were not observed in these animals. Upon sacrifice after the activity testing was complete, a small but statistically significant increase (as opposed to the decrease found after sacrifice on postnatal day 17) in the muscarinic receptor concentration of the hippocampus was found in animals from the high-dose group. These results suggest that the neurochemical effects of 3,3',4,4'-TCB are complex. Cholinergeric as well as dopaminergic systems in the brain are involved.

Of all the developmental and reproductive endpoints reported in this chapter for laboratory animals, the only ones that have not yet been demonstrated to occur following perinatal exposure to TCDD are the above neurotoxic effects in mice. These have only been studied following perinatal exposure to 3,3'4,4'-TCB. In addition, there is as yet no evidence to show (1) that among inbred mouse strains having low- and high-affinity Ah receptors, susceptibility to 3,3',4,4'-TCB-induced neurotoxicity segregates with the Ah locus or (2) that the rank order binding affinity of congeners for the Ah receptor correlates with their rank order potency for causing these neurotoxic effects in mice. The rapid metabolism of 3,3',4,4'-TCB compared with the relatively slow metabolism of TCDD in mice causes some uncertainty about the potential involvement of the Ah receptor in 3,3',4,4'-TCB-induced neurotoxicity. Contributing to this uncertainty is the hypothesis that 3,3',4,4'-TCB might produce CNS effects

by being converted to a hydroxylated metabolite that is neurotoxic. Although there is no evidence for or against this hypothesis, there is also no evidence for or against the Ah receptor mechanism hypothesis of 3,3',4,4'-TCB neurotoxicity. Further research is needed to test these hypotheses. In so doing, it should become apparent whether 3,3',4,4'-TCB-induced neurotoxicity effects are relevant to TCDD-induced developmental toxicity.

5.2.3.4.3. ***Neurobehavior in rats.*** A considerable number of neurobehavioral endpoints have been evaluated following perinatal exposure to either TCDD and coplanar PCBs that are Ah receptor agonists, or to ortho-substituted PCBs that do not interact with the Ah receptor. Interest in the latter, for the purposes of this section, arises from the fact that mixtures to which children have been exposed in utero and via lactation typically contain Ah receptor agonists and non-Ah receptor agonists. Therefore, it is important from a mechanistic point of view to determine whether the effects of Ah receptor agonists can be distinguished from the effects of structurally similar non-Ah receptor agonists. It is plausible that some effects of TCDD on neurobehavior in rodents could be Ah receptor mediated because this protein has been detected in the developing neuroepithelium of the mouse fetus (Abbott et al., 1995) and in neuronal tissue of the adult rat brain (Kainu et al., 1995).

Two hypotheses have been advanced: (1) TCDD and TCDD-like PCB congeners do not produce behavioral impairment at biologically relevant doses (Rice et al., 1998; Rice, 1999) and (2) the effects of TCDD and TCDD-like PCB congeners on learning and memory might be distinguishable from effects of ortho-substituted PCB congeners that are not Ah receptor agonists (Schantz et al., 1996; Rice et al., 1999). These hypotheses have not been fully resolved. To the extent that effects of individual ortho-substituted PCB congeners on neurobehavior depend on reductions in thyroid hormone concentrations during the perinatal period (Collins et al., 1980; Ness et al., 1993), it is important to note that TCDD and coplanar PCBs do not reduce thyroid hormone concentrations to the same extent as the ortho-substituted PCBs (Seo et al., 1995). Difficulty in resolving these hypotheses also occurs because different laboratories, using the same testing methods, have not always obtained similar results. In addition, different testing paradigms that appear, at least superficially, to test similar phenomena can arrive at discordant conclusions. Where the results of testing by different methods are not in agreement, the differences are not easy to resolve, in part because the relative sensitivities of the different measures are not always clear.

Despite these difficulties, in utero and lactational exposure to TCDD-like Ah receptor agonists have affected endpoints that measure learning and memory, discrimination reversal learning, transitional behavior, avoidance behavior, neurotransmitter function, and locomotor activity. Perinatal exposure to Ah receptor agonists have inhibited long-term potentiation (LTP)

in the visual cortex, but not in the hippocampus evaluated in vitro (Altmann et al., 1995, 1998). In some cases, effects observed with Ah receptor agonists are similar to those of ortho-substituted PCB congeners (Schantz et al., 1995, 1996, 1997; Seo et al., 1999). Therefore, the available data obtained following in utero and lactational exposure of rats to these compounds tend to support the notion that TCDD and coplanar PCBs can affect neurobehavioral endpoints by a variety of mechanisms, only one of which is Ah receptor mediated. The results of neurobehavioral tests following perinatal exposure to TCDD and various PCBs are summarized below.

5.2.3.4.3.1. ***Spatial learning*****.** Female rats were administered 3,3',4,4'-TCB (PCB 77, 2 and 8 mg/kg/day), 3,3',4,4',5-PCB (PCB 126, 0.25 and 1 μg/kg/day), and TCDD (0.25 and 1 μg/kg/day) by gavage on gestational days 10-16 (Schantz et al., 1996). Beginning on postnatal day 80, spatial learning was evaluated in male and female offspring by using the radial arm maze. While no effects on overt toxicity were found, it was observed that the exposed rats made fewer errors than controls. This result was different from that of a previous study in which exposure to the ortho-substituted PCBs 2,4,4'-TCB (PCB 28), 2,3,4,4',5-PCB (PCB 118), and 2,2',4,4',5,5'-HCB (PCB 153) had no effect on the number of errors (Schantz et al., 1995).

The ability of in utero and lactational TCDD exposure to reduce the number of errors made by male rat offspring in the radial arm maze test were confirmed, even at a reduced exposure level (0.1 μg/kg/day) on gestational days 10-16 (Seo et al., 1999). In addition, no significant decreases in the error rate were found in female rats. However, further statistical analysis of the data suggested that the affected male rats were using a response strategy whereby they tended to enter adjacent arms of the maze. Because of this strategy, there might not be an effect of TCDD on working memory. The lack of an effect of TCDD on the Morris Water Maze test supports this interpretation (Seo et al., 1999). However, the test for adjacent arm selection behavior, which detects the use of a response strategy, had not been significant in the original study at the higher level of TCDD exposure (Schantz et al., 1996).

The low-dose TCDD exposure decreased latency in male rats in the radial arm maze test (Seo et al., 1999). This suggests an apparent feminization of this parameter similar to that caused by PCB 153 (Schantz et al., 1995). In addition, there may be other similarities between TCDD and at least some of the ortho-substituted PCBs, because a similar evaluation of 2,2',3,5',6-PCB (PCB 95) demonstrated an exposure-associated decrease in the error rate of male rat offspring (Schantz et al., 1997). Similar to the original result with the low-exposure dose of TCDD (Seo et al., 1999), the test for use of a response strategy was negative with PCB 95, but unlike the results with TCDD and PCB 153, there was no gender-related decrease in latency. In contrast to the decrease in the error rate that was caused by the perinatal exposure of Sprague-Dawley rats to

PCB 77, PCB 95, PCB 126, and TCDD (Schantz et al., 1996), there was no effect of perinatal exposure to PCB 77 or the diortho- substituted 2,2',4,4'-TCB (PCB 47) on the error rate of male Wistar rats that were tested on a similar radial arm maze (Weinand-Harer et al., 1997).

The male and female rat offspring that were exposed to the ortho-substituted PCB 28, PCB 118, and PCB 153 and tested on the radial arm maze were subsequently evaluated for delayed spatial alternation on the T-maze beginning after postnatal day 135 (Schantz et al., 1995). Exposed females learned this task more slowly than exposed males, and all compounds tested caused a decrease in the number of correct responses. There was no effect on the number of correct responses in exposed male offspring, but their latency to enter the maze was decreased compared with that of control male offspring. This effect on latency again suggests a more female-like pattern of response in male offspring exposed to the ortho-substituted PCBs, even though distinct gender differences remained for the delayed spatial alternation response. In contrast, in utero and lactational exposure to the coplanar PCBs produced no effect on the errors made by male or female offspring, or in their latency in the T-maze test (Schantz et al., 1996; Seo et al., 1999). In addition, another group also found that delayed spatial alternation was unaffected in both male and female Long Evans rat offspring exposed to PCB 126 and tested in an operant chamber setting (Rice, 1999). In this case, even prolonged dietary exposure of female rats to PCB 126, which began 7 weeks before mating to an unexposed male and continued until the offspring were weaned, caused no treatment-related differences in performance (Rice, 1999). However, the impression that delayed spatial alternation is selectively affected by the ortho-substitited PCBs is again offset by the results with PCB 95. Unlike the other ortho-substituted PCBs, PCB 95 did not affect the response (Schantz et al., 1997). Differences between the activities of PCB 95 and PCB 126 have also been found on parameters relevant to neurological function in vitro (Wong et al., 1997). Because effects of the triortho-substituted PCB 95 on delayed spatial alternation are similar to those of PCB 126, a coplanar PCB, but different from those of other ortho-substituted PCBs it remains possible that this effect of PCB 126 is non-Ah receptor mediated. However, not all ortho-chlorinated PCBs are equivalent (Schantz et al., 1995; Schantz et al., 1997), suggesting the existence of structural selectivity in the mechanism.

5.2.3.4.3.2. ***Visual discrimination reversal learning*****.** No effect was observed when the T-maze was used to assess spatial discrimination reversal learning following in utero and lactational exposure of male and female rat offspring to maternal doses of 0.1 μg TCDD/kg/day on gestational days 10-16 (Seo et al., 1999). Visual discrimination reversal learning was tested by placing electric light stimuli onto the cross-arms of the same T-maze used to evaluate spatial discrimination reversal learning. Male and female rat offspring were exposed to maternal doses of 0.1 μg TCDD/kg/day administered on gestation days 10-16 (Seo et al., 1999). When evaluated

beginning at approximately 100 days of age, the exposed offspring performed similar to controls during the original learning phase of the trial. However, TCDD-exposed offspring were slower to reach the testing criterion of 10 correct trials in a 12-trial session. This effect occurred equally in males and females and was most evident during the first and second reversal period. In the following reversal periods, no further differences were evident between the TCDD and vehicle exposure groups. Similarly, PCB 118 and PCB 126 were reported to impair visual discrimination learning in male rat offspring evaluated in an operant chamber (Holene et al., 1995). However, the authors of this study used more than one male offspring per litter, and they appear to have evaluated all rats from the same litter as if they were independent observations. Therefore, the results of this study have been considered to be uninterpretable on statistical grounds (Rice and Hayward, 1998; Rice, 1999), based on the criteria established by Holson and Pearce (1992).

One study using mixtures evaluated visual discrimination learning in the offspring of female rats exposed to Clophen A30 (32 mg total PCBs/kg diet) or a normal diet for 60 days prior to mating, and through pregnancy (Lilienthal et al., 1991). After birth some offspring exposed to each diet were cross-fostered to dams exposed to the other diet. When male and female offspring were evaluated at 120 to 180 days of age, there was no effect of PCB exposure during the acquisition phase of the paradigm (visual discrimination learning tested on a jumping stand). However, performance in all PCB-exposed groups was inferior during the retention phase, relative to their performance at the end of the acquisition phase. Because the effect was more pronounced in the prenatal-only and prenatal + lactational exposure groups, compared with the lactational-only exposure group, the results indicate that prenatal-only exposure to PCBs is all that is required to alter visual discrimination learning.

5.2.3.4.3.3. *Transitional behavior*. Female Long Evans rats were exposed to PCB 126 (0.25 and 1 μg/kg/day) via dietary supplementation that began 35 days prior to mating, and continued through pregnancy and lactation (Rice and Hayward, 1999). After postnatal day 400, transitional behavior was tested in male and female offspring by using a concurrent random interval-random interval reinforcement schedule in an operant chamber. In this test offspring of both sexes apportioned their responses less accurately than control offspring with respect to the pattern of scheduled reinforcements on the two levers. However, the treated rats perceived the reward offered by the reinforcements similarly to the control rats, because testing by a progressive ratio reinforcement schedule resulted in no treatment-related differences in the relative strength of the reinforcing event (Rice and Hayward, 1999). No treatment-related differences had previously been found in the same rats tested on postnatal day 220 by using a multiple fixed interval-fixed ratio reinforcement schedule (Rice and Hayward, 1998). Therefore, the results obtained on the

concurrent random interval-random interval schedule of reinforcement may indicate a selective effect of PCB exposure on adaptive ability in the offspring (Rice and Hayward, 1999).

5.2.3.4.3.4. ***Behavioral responses to CNS drugs*.** Haloperidol-induced catalepsy was evaluated in male Wistar and Long Evans rat offspring exposed to the diortho-substituted PCB 47 (1 mg/kg/day) or the coplanar PCB 77 (1 mg/kg/day) from days 7 to 18 of gestation (Weinand-Harer et al., 1997; Hany et al., 1999). At 100 and 180 days of age, catalepsy was induced in the male offspring by the dopaminergic antagonist haloperidol. Developmental exposure to PCB 77, but not to PCB 47, caused an increase in the time required for the affected rat to move its paw after the paw had been placed into certain positions by the experimenter. The effects of similar exposure to PCB 77 on dopaminergic function have also been tested in Long Evans rats by evaluating their ability to discriminate between the dopaminergic agonist apomorphine and saline (Lilienthal et al., 1997). As a positive control, the antithyroid drug propylthiouracil (PTU) given to adult control animals just prior to testing blocked their ability to discriminate between apomorphine and saline, whereas no effect was found on this discrimination in rats exposed to PCB 77 in utero and via lactation. However, the administration of buspirone to the adult animals just prior to testing blocked the ability of vehicle-exposed rats to recognize apomorphine much more than it blocked this ability in the PCB 77-exposed offspring, or PTU-dosed groups. As buspirone is a mixed serotonin receptor agonist and partial dopamine receptor antagonist, the authors suggested that perinatal exposure to PCB 77 may produce long-lasting effects on the interaction between dopaminergic and serotonergic processes in the CNS (Lilienthal et al., 1997).

Since PCB 77 was effective in prolonging haloperidol-induced catalepsy, whereas PCB 47 was not, it is interesting that perinatal exposure to coplanar PCBs and ortho-substituted PCBs also produce opposite effects on dopamine synthesis in the brain (Seegal et al., 1990, 1997). Perinatal exposure to ortho-substituted PCB congeners decreases dopamine synthesis in adulthood, whereas perinatal exposure to coplanar PCBs causes persistent elevations in brain dopamine and metabolite concentrations (Seegal et al., 1997). An increase in endogenous brain dopamine concentrations could be related to the ability of PCB 126 to alter the recognition of exogenous apomorphine. Dopaminergic function is one area where the effects of coplanar and ortho-substituted PCB congeners may be distinguishable. However, only a few PCB congeners have been evaluated.

Female Long Evans rats were exposed to PCB 126 (0.25 and 1.0 μg/kg/day) by dietary supplementation that began 35 days prior to mating and continued through pregnancy and nursing (Bushnell et al., 1999). After postnatal day 112, a chlordiazepoxide (CDP, 0, 3, 5, and 8 mg/kg) challenge test was used to evaluate neurobehavior. In control offspring all doses of CDP reduced performance. This result suggested that the control offspring were affected by an

increase in the visual threshold. Rats exposed in utero and via lactation to the low dose of PCB 126 were unaffected by CDP, whereas those exposed to the high dose exhibited less of a decrement in their performance than did the control offspring. Since additional test results demonstrated that PCB 126 exposure did not cause deficits in attention, the altered performance of these rats after the administration of CDP suggests that perinatal exposure to PCB 126 may affect γ-aminobutyric acid (GABA)-mediated pathways in the CNS during development (Bushnell and Rice, 1999).

5.2.3.4.3.5. ***Passive avoidance behavior*****.** Male Wistar rats were exposed to the coplanar PCB 77 or the diortho-substituted PCB 47 on gestational days 7 to 18. Passive avoidance behavior was tested on a step-down platform when the rats were 220 days old (Weinand-Harer et al., 1997). The latency of male rat offspring to step onto a grid that had previously given them an electric shock was used to evaluate the effects of perinatal PCB exposure. Latency was decreased by both PCBs, compared with control rats, up to 24 hours after the initial shock. However, only the effect of PCB 77 was significant when evaluated at a single time (5 min, 4 hours, and 24 hours). A similar paradigm was used to evaluate the effects of perinatal exposure to PCB 77, PCB 47, and a combination of both PCBs in Long Evans rats on postnatal day 85 (Hany et al., 1999). Under these conditions the most significant effect observed was a decreased latency in the PCB 77 and combined exposure groups at the 5-minute time. No significant effect was found when rats were exposed to PCB 47 only. These results suggest that differences in passive avoidance behavior may exist following perinatal exposure to non-ortho- and ortho-substituted PCBs, but only one congener of each type has been tested.

5.2.3.4.3.6. ***Open field locomotor activity*****.** When open field activity was tested in male offspring on postnatal day 25, rats exposed to PCB 57 in utero and via lactation had a significantly higher activity level than rats similarly exposed to PCB 77. However, there were no significant differences between the PCB-exposed groups and the unexposed control group (Weinand-Harer et al., 1997). When tested on postnatal day 340, offspring exposed to PCB 47, PCB 77, and a combination of both PCBs were hyperactive when compared with controls (Hany et al., 1999).

Locomotor activity has also been tested in an operant chamber setting in rats exposed to PCBs only during lactation. Female DA/OLA/HSD female rats were mated to Lewis male rats, and the pregnant dams were administered vehicle, 2,2',4,4',5,5'-HCB (PCB 153, 5 mg/kg), or PCB 126 (2 μg/kg) (Holene et al., 1998). Dosing of the female rats was accomplished on every second day from postnatal days 3 to 13. PCB-exposed, 112-day-old male rats were found to be hyperactive during both the fixed interval and extinction components of the reinforcement

schedule. In addition, the PCB 153-exposed offspring displayed a behavior pattern similar to that of spontaneous hypertensive (SHR) rats, which are used as an animal model of attention-deficit hyperactivity disorder (ADHD) in children. With the results of only one congener of each type being tested, the SHR-like pattern of activity appeared to be selective for the ortho-substituted PCB. However, with both congeners, the activity level was increased by exposure solely during the postnatal period. In contrast to all results that show that in utero and/or lactational PCB exposure causes hyperactivity, perinatal exposure to PCB 95 caused hypoactivity in offspring (Schantz et al., 1997).

5.2.3.4.4. ***Neurobehavior in monkeys.*** Schantz and Bowman (1989) and Bowman et al. (1989b) have conducted a series of studies on the long-term behavioral effects of perinatal TCDD exposure in monkeys. Because these were the first studies to evaluate the behavioral teratology of TCDD, monkeys exposed to TCDD via the mother during gestation and lactation were screened on a broad selection of behavioral tests at various stages of development (Bowman et al., 1989b). At the doses studied (5 or 25 ppt in the maternal diet), TCDD did not affect reflex development, visual exploration, locomotor activity, or fine motor control in any consistent manner (Bowman et al., 1989a). However, the perinatal TCDD exposure did produce a specific, replicable deficit in cognitive function (Schantz and Bowman, 1989). TCDD-exposed offspring were impaired on object learning, but were unimpaired on spatial learning. TCDD exposure also produced changes in the social interactions of mother-infant dyads (Schantz et al., 1986). TCDD-exposed infants spent more time in close physical contact with their mothers. The pattern of effects was similar to that seen in lead-exposed infants and suggested that mothers were providing increased care to the TCDD-exposed infants (Schantz et al., 1986).

5.2.3.4.5. ***Neurobehavior in humans.*** The intellectual and behavioral development of Yu-Cheng children transplacentally exposed to PCBs, CDFs, and PCQs was studied through 1985 by Rogan et al. (1988). In Yu-Cheng children matched to unexposed children of similar age, area of residence, and socioeconomic status, there was a clinical impression of developmental or psychomotor delay in 12 (10%) Yu-Cheng children compared with 3 (3%) control children and of a speech problem in 8 (7%) Yu-Cheng children versus 3 (3%) control children. Also, except for verbal IQ on the Wechsler Intelligence Scale for Children, Yu-Cheng children scored lower than control children on three developmental and cognitive tests (Rogan et al., 1988). Neurobehavioral data on Yu-Cheng children obtained after 1985 shows that the intellectual development of these children continues to lag somewhat behind that of matched control children. In addition, Yu-Cheng children are rated by their parents and teachers as having a higher activity level; more health, habit, and behavioral problems; and a temperamental

clustering closer to that of a "difficult child." It is concluded that in humans, transplacental exposure to halogenated aromatic hydrocarbons can affect CNS function postnatally. However, which congeners, TCDD-like versus non-TCDD-like, are responsible for the neurotoxicity is unknown.

Further research on the mechanism of these postnatal neurobehavioral effects, dose-response relationships, and reversibility of the alterations is needed before the role of TCDD-like congeners versus non-TCDD-like congeners in causing this toxicity can be understood. Mechanisms that respond uniquely to TCDD-like congeners may not necessarily be involved, as three lightly chlorinated, ortho-substituted PCB congeners, 2,4,4'-TCB, 2,2',4,4'-TCB, and 2,2',5,5'-TCB, have been detected in monkey brain following dietary exposure to Aroclor 1016 and appear to be responsible for decreasing dopamine concentrations in the caudate, putamen, substantia nigra, and hypothalamus of these animals (Seegal et al., 1990). These nonplanar PCB congeners are believed to cause these effects by acting through a mechanism that does not involve the Ah receptor. On the other hand, the results presented for mice and monkeys suggest that TCDD-like congeners could be involved in producing the observed postnatal neurobehavioral effects in humans.

5.2.3.5. *Thermoregulation*

In adult rats TCDD-induced reductions in body temperature are associated with reduced serum thyroxin levels and a decrease in basal metabolism (Potter et al., 1983, 1986). More recently the offspring of rats exposed in utero and via lactation to a maternal diet that contained Aroclor 1254 were affected by decreased body core temperature, reduced metabolic rate, and marked reductions in serum thyroxin up to an age of 14 days (Seo et al., 1995). As part of a larger study, male rats that had been exposed to vehicle or a single maternal dose of 1 μg/kg of TCDD on gestational day 15 were castrated (for reasons unrelated to the study of thermoregulation) at approximately 8 months of age (Gordon et al., 1995). When evaluated at 15.4 to 17.7 months of age, TCDD-exposed animals exhibited significantly lower core body temperatures than controls when the ambient temperature was varied between 10° and 28°C. However, the metabolic rate was not affected by TCDD, which indicates that the effector regulating body core temperature during cold exposure was unaffected. In addition, in utero and lactational TCDD exposure had no effect on evaporative heat loss or on skin blood flow when the rats were anesthetized so that this parameter could be measured. These results suggest that perinatal exposure to TCDD can decrease core temperature set point and cause a reduction in the regulated body temperature.

In a subsequent study pregnant Long Evans rats were administered a single maternal dose of vehicle or 1 μg TCDD/kg on gestational day 15, and their male offspring were implanted with

transmitters to monitor core temperature and motor activity (Gordon et al., 1998). At various ages these TCDD-exposed male rats were affected by a nocturnal hypothermia that was accompanied by decreased motor activity. These effects were especially pronounced at 7 and 11 months of age, did not occur at 3 months of age, and were reduced at 16 months of age. In addition, TCDD-exposed animals exhibited a greater febrile response compared with vehicle-exposed control rats when challenged with lipopolysaccharide (LPS) to induce fever. However, when 8-month-old rats were placed in a temperature gradient and allowed to select their own most favored ambient temperature, vehicle- and TCDD-exposed offspring selected the same ambient temperatures. This suggests that hypothalamic thermoregulatory centers were not permanently altered and that there was not a change in body temperature set point.

Similar alterations in thermoregulation have been produced in hamsters exposed to TCDD in utero and via lactation. When monitored by radiotelemetry, like the rats cited above, these offspring exhibited a persistent hypothermia in spite of normal metabolic responses to cold exposure (Gordon et al., 1996). In addition, there was no effect of TCDD exposure on the selection of an ambient temperature when hamsters were placed in a temperature gradient for 22 hours. These results are important because the adult hamster has an unusually high resistance to the lethal and thyrotoxic effects of TCDD. However, the rat and hamster have approximately the same sensitivity to perinatal TCDD-induced reproductive dysfunction and thermoregulatory dysfunction. The mechanisms for these responses have not yet been determined.

5.2.3.6. *Auditory Function and Thyroid Hormones*

Long Evans rats were exposed to daily maternal doses of 0, 1, 4, and 8 mg Aroclor 1254/kg/day administered from gestational day 6 to weaning. Low-frequency auditory thresholds evaluated in these male and female offspring beginning on postnatal day 85 were increased at the two largest levels of PCB exposure (Goldey et al., 1995a). This effect was statistically significant at 1 kHz, but not at 4 kHz or higher test frequencies. Since similar low-frequency hearing loss can be produced by perinatal exposure to the antithyroid drug propylthiouracil (PTU), the effect of Aroclor 1254 is believed to be associated with neonatal hypothyroidism (Goldey et al., 1995b). Indeed, there were dramatic decreases in total and free plasma thyroxin (T4) concentrations at all doses of Aroclor 1254 and at all times evaluated between postnatal days 7-42 (Goldey et al., 1995a, 1998). Plasma total and free triiodothyronine (T3) concentrations were decreased only by the largest two exposure levels of Aroclor 1254, and statistically significant effects were observed only on postnatal days 21 and 28, with no decrease on postnatal day 42.

Perinatal exposure to a daily maternal dose of 1 μg/kg/day of 3,3'4,4',5-PCB (PCB 126) administered to the dam for 7 weeks prior to breeding and throughout breeding, gestation, and

lactation to weaning decreased the auditory threshold in Long-Evans rat offspring to 0.5 and 1 kHz (Crofton et al., 1999). However, the 0.25 μg PCB 126/kg/day maternal dose did not affect the auditory threshold in exposed offspring. Thus, it is plausible that AhR agonists within the Aroclor 1254 PCB mixture caused the hearing loss.

While the daily maternal dose of 1 mg Aroclor 1254/kg/day did not significantly increase the auditory threshold to 1 kHz, it did cause alterations in brain stem auditory-evoked responses in exposed male and female offspring that were evaluated at 1 year of age (Herr et al., 1996). This result is consistent with the hypothesis that developmental exposure to Aroclor 1254 can damage the peripheral auditory system. It is believed by the authors that this apparently irreversible damage might occur at the level of the cochlea and/or auditory nerve. This result is important because offspring exposed to a maternally administered level of 1 mg Aroclor 1254/kg/day were affected by less substantial reductions in plasma thyroxin concentration than were those exposed to 4 mg/kg/day or 8 mg/kg/day doses of Aroclor 1254, which increased the auditory threshold (Goldey et al., 1995a; Goldey and Crofton, 1998). Thus, it is possible that less of a thyroid deficit during development could result in hearing loss. In support of the relationship between the chemical-induced auditory deficit and thyroid hormone status, the auditory deficit was partially alleviated by daily doses of thyroxin given to the Aroclor 1254-exposed pups from postnatal day 4 to postnatal day 21 (Goldey and Crofton, 1998).

Even as early as gestational day 20 brain thyroxin levels in the forebrain and cerebellum of fetal rats can be depressed after maternal exposure to Aroclor 1254 (Morse et al., 1996). However, in late-gestation fetuses, induction of the brain type II thyroxin 5'-deiodinase results in compensation for the decrease in thyroxin levels so that brain triiodothyronine levels are maintained. Similar alterations occur after exposure to the non-ortho-substituted PCB congeners 3,3'4,4'-TCB (PCB 77) and 3,3',4,4',5,5'-HCB (PCB 169) that are Ah receptor agonists (Morse et al., 1993). The authors suggest that increases in deiodinase activity could be indicative of a local hypothyroidism occurring in the brains of fetal and neonatal rats exposed to these PCBs.

In utero and lactational exposure to a daily maternal dose of 0.1 μg TCDD/kg/day administered on gestational days 10-16 can produce a statistically significant decrease in plasma thyroxin concentration in female offspring evaluated on postnatal day 21 (Seo et al., 1995). However, this reduction in plasma thyroxin concentration in Sprague-Dawley rat offspring was less than those associated with no effect on auditory threshold in Long Evans rat offspring (Goldey et al., 1995a). Therefore, it has not been demonstrated that perinatal exposure to TCDD will decrease plasma thyroxin concentrations enough to evoke an increase in the auditory threshold. It may turn out that TCDD doses larger than those already tested may be required to cause these effects. While it is possible that prenatal TCDD exposure might produce a functional hypothyroidism prior to birth or that mechanisms other than perinatal hypothyroxinemia may

play a role in producing the hearing loss, it is also possible that PCBs and/or their metabolites could affect auditory functional development by decreasing plasma thyroxin concentrations via non-Ah receptor-related mechanisms (Brouwer et al., 1995). Paradoxically, Aroclor 1254 and a single maternal dose of 50 ng TCDD/kg administered to Long-Evans rats on day 15 of gestation can accelerate eye opening (Goldey et al., 1995a; Gray et al., 1997a). This effect of Arochlor 1254 is exacerbated by thyroxin replacement in the pups (Goldey and Crofton, 1998), whereas hypothyroidism is typically associated with a delay in this developmental landmark (Comer et al., 1982; Goldey et al., 1995b). This suggests that some developmental effects of Aroclor 1254 can resemble those of hyperthyroidism, rather than hypothyroidism. Additional mechanistic work on the ability of Ah receptor agonists to induce hypothyroidism early in development, and their ability to decrease auditory function and cause postnatal hearing loss, appears to be required.

5.2.3.7. *Night Vision*

Pregnant Long Evans rats were exposed to the ortho-chlorinated 2,2',4,4'-tetrachloro-biphenyl (PCB 47) and/or the coplanar 3,3',4,4'-tetrachlorobiphenyl (PCB 77) on days 7-18 of gestation. Daily doses 1.5 mg PCB 47/kg/day, 1.5 mg PCB 77/kg/day, a combination of 1.0 mg PCB 47/kg/day + 0.5 mg PCB 77/kg/day, or an equivalent volume of vehicle were administered subcutaneously to each dam (Kremer et al., 1999). The effects of PCB exposure on visual processes were then assessed in male and female offspring at 200 days of age. The scotopic b-wave, maximum potential, and oscillatory potentials were recorded on the electroretinogram after the rats were adapted to the dark. Perinatal exposure to PCB 77 reduced the amplitudes of these potentials in female offspring in adulthood, but not their male littermates. Exposure to PCB 47 alone was without effect; however, many of the decreases that resulted from PCB 77 appeared to be alleviated after simultaneous exposure to PCB 47. While this suggests that functional antagonism between these ortho-substituted and coplanar PCBs can occur in the endpoints measured, it is also possible that this apparent antagonism resulted from the lower level of PCB 77 administered in the combination. These results indicate that in utero and lactational exposure to PCB 77, but not PCB 47 exposure, can produce long-lasting effects on night vision in female rat offspring (Kremer et al., 1999). Interestingly, the susceptibility to this effect was congener-specific, suggesting that the effect may be Ah receptor mediated. In addition, it was gender dependent.

5.2.4. Cross-Species Comparison of Effect Levels

TCDD exposure levels that cause a variety of developmental effects in different species are summarized for fish in Table 5-5, birds in Table 5-6, and mammals in Table 5-7. Fertilized lake trout eggs and Japanese medaka eggs were exposed to different waterborne concentrations

of ^{3}H-TCDD. Estimates of the amount of TCDD in these eggs were then made from measurement of the TCDD-derived radioactivity within them. Fertilized rainbow trout, chicken, ring-necked pheasant, and eastern bluebird eggs were injected directly with the indicated doses of TCDD. Thus, the doses of TCDD given in Tables 5-5 and 5-6 for all fish and bird species represent TCDD egg burdens where a significant portion of the dose may be present within the yolk of the egg rather than the developing embryo.

Mammalian embryo/fetuses, on the other hand, were exposed via administration of TCDD to the pregnant female. Therefore, the doses given in Table 5-7 are maternal TCDD doses, where a significant portion of the dose may be retained by the mother and never actually reach the embryo/fetus. In some studies, pregnant rats and rhesus monkeys were exposed to TCDD on a chronic or subchronic basis, respectively. The doses given in Table 5-7 for these particular studies represent the calculated maternal body burdens at the time of conception. In rats, the duration of chronic exposure was much longer than the whole body elimination half-life for TCDD in rats. Therefore, the body burden of TCDD given for the rat is 92.8% of the calculated steady-state body burden. In rhesus monkeys, the half-life for whole body elimination of TCDD is longer than the duration of exposure prior to conception. Therefore, the steady-state body burdens that would be expected for rhesus monkeys exposed to the different levels of dietary TCDD intake are approximately three times greater than the maternal body burdens estimated at the time of conception (Table 5-7).

In both rats and rhesus monkeys, the maternal body burdens are calculated using a one-compartment open model, assuming 86.1% bioavailability for TCDD. The bioavailability used for TCDD was determined in rats (Rose et al., 1976). As no estimate for TCDD bioavailability has been reported in rhesus monkeys, the same 86.1% value was used. The whole body elimination half-life used for TCDD in the rat is 23.7 days (Rose et al., 1976).

McNulty et al. (1982) estimated a half-life of approximately 1 year for TCDD elimination from adipose tissue in the rhesus monkey, and for calculation of the body burdens estimated in Table 5-7, this half-life was rounded to 400 days for whole body elimination. The maternal body burden given for chronic exposure in the rat was calculated from the data of Murray et al. (1979). The maternal body burden given for subchronic exposure in the rhesus monkey was calculated from data obtained from Dr. R. E. Bowman (personal communication), which included the daily dietary TCDD exposure level for each pregnant female used in the studies reported by Bowman et al. (1989a,b) and Schantz and Bowman (1989). Dr. Bowman's results indicate that the range of TCDD half-lives in these monkeys was 200 to 600 days, which is consistent with the results of McNulty et al. (1982). The body burdens estimated for rhesus monkeys used in these studies are averages based on the average daily TCDD consumption of all pregnant females used at a particular level of maternal TCDD exposure.

As summarized in Table 5-5, lake trout and rainbow trout sac fry and Japanese medaka embryos are similarly affected by a spectrum of lesions that includes hemorrhage, edema, collapse of the yolk sac, cessation of blood flow, and embryo mortality. Estimates of the NOAEL and LOAEL are given in Table 5-5 for the appearance of these lesions in Japanese medaka embryos and for embryo mortality in the two trout species. Although fertilized lake trout eggs and Japanese medaka eggs were exposed to various TCDD concentrations dissolved in static water, and fertilized rainbow trout eggs were injected directly with TCDD, the egg doses given in Table 5-5 represent the concentration of TCDD within the eggs themselves. Therefore, the different NOAELs and LOAELs for developmental toxicity in different fish species probably represent species differences in susceptibility to TCDD-induced developmental toxicity rather than differences in method of TCDD exposure. Of the three fish species, lake trout sac fry are the most sensitive to TCDD-induced mortality. However, based on the LOAELs shown in Table 5-5, the difference in susceptibility between fish species may be less than tenfold.

Based on the LOAELs shown in Table 5-6, the sensitivity of different bird species to TCDD-induced embryo mortality varies by more than fortyfold. The chicken embryo is more susceptible to TCDD-induced mortality than are embryos of the ring-necked pheasant and eastern bluebird. In addition, chicken embryos are highly sensitive to the formation of TCDD-induced structural defects in the heart and aortic arch. The incidence of cardiac malformations in the chicken embryo is increased at an egg exposure level as low as 9 ng TCDD/kg egg. However, such cardiac malformations have not been found in any other bird species that has been examined.

Table 5-7 summarizes the levels of TCDD exposure that cause certain structural malformations, functional alterations, and prenatal mortality in the embryo/fetus of different mammalian species. Based on the LOAELs given for rats and monkeys in Table 5-7, functional

alterations in learning behavior and the male reproductive system occur at lower TCDD doses than those required to produce structural malformations. Maternal doses of TCDD between 19 and 160 ng/kg decreased object learning in monkeys, accelerated eye opening, produced adverse effects on the male reproductive system, and altered sexual behavior in rats. Developmental toxicity to the female reproductive tract in rats occurred at TCDD doses between 200 and 800 ng/kg. Although TCDD-induced developmental toxicity has been extensively studied in mice and rats, the LOAELs in Table 5-7 indicate that the embryo/fetus of rodent species is generally not as sensitive to TCDD-induced prenatal mortality as is the embryo/fetus of the rhesus monkey. The sensitivity of the embryo/fetus to TCDD-induced prenatal mortality in different mammalian species varies approximately 240-fold. This is in contrast to the 1,000- to 5,000-fold variation in the LD_{50} of TCDD when adult animals of these same species are exposed. The agreement between studies with respect to the LOAEL in Table 5-7 for prenatal mortality in rats and monkeys is particularly striking. The 500 ng/kg dose of TCDD on gestational days 6 to 15 that caused prenatal mortality in rats (Sparschu et al., 1971) agrees with the maternal TCDD body burden of 270 ng/kg calculated from the chronic exposure of rats by Murray et al. (1979) to within a factor of 2. Similarly, the TCDD dose of 111 ng/kg that was given to rhesus monkeys nine times during the first trimester of pregnancy (McNulty, 1984) agrees with the maternal body burden of 97 ng/kg that increased prenatal mortality in rhesus monkeys following subchronic dietary exposure (Schantz and Bowman, 1989).

5.3. REPRODUCTIVE TOXICITY

5.3.1. Female

5.3.1.1. *Reproductive Function/Fertility*

TCDD and its approximate isostereomers have been shown to affect female reproductive endpoints in a variety of animal studies. Among the effects reported are reduced fertility, reduced litter size, and effects on the female gonads and menstrual/estrous cycle. These studies are reviewed below. Other TCDD effects on pregnancy maintenance, embryo/fetotoxicity, and postnatal development are covered in Section 5.2 of this chapter.

5.3.1.1.1. *Rats.* The study by Murray et al. (1979) employed a multigenerational approach, examining the reproductive effects of exposure of male and female rats over three generations to relatively low levels of TCDD (0, 0.001, 0.01, and 0.1 μg/kg/day). There was variation in the fertility index in both the control and the exposed groups, and a lower than desirable number of impregnated animals in the exposed groups. Nevertheless, the results showed exposure-related effects on fertility, an increased time between first cohabitation and delivery, and a decrease in litter size. The effects on fertility and litter size were observed at 0.1 μg/kg/day in the F_0

generation and at 0.01 μg/kg/day in the F_1 and F_2 generations. Additionally, in a 13-week exposure to 1 to 2 μg/kg/day of TCDD in nonpregnant female rats, Kociba et al. (1976) reported anovulation and signs of ovarian dysfunction, as well as suppression of the estrous cycle. However, at exposures of 0.001 to 0.01 μg/kg/day in a 2-year study, Kociba et al. (1978) reported no effects on the female reproductive system.

5.3.1.1.2. ***Monkeys.*** Allen and colleagues reported on the effects of TCDD on reproduction in the monkey (Allen et al., 1977, 1979; Barsotti et al., 1979; Schantz et al., 1979). In a series of studies, female rhesus monkeys were fed 50 or 500 ppt TCDD for ~9 months. Females exposed to 500 ppt showed obvious clinical signs of TCDD toxicity and lost weight throughout the study. Five of the eight monkeys died within 1 year after exposure was initiated. Following 7 months of exposure to 500 ppt TCDD, seven of the eight females were bred to unexposed males. The remaining monkey showed such severe signs of TCDD toxicity that she was not bred due to her debilitated state. Of the seven females that were evaluated for their reproductive capabilities, only three were able to conceive and, of these, only one was able to carry her infant to term (Barsotti et al., 1979). When females exposed to 50 ppt TCDD in the diet were bred at 7 months, two of eight females did not conceive and four of six that did conceive could not carry their pregnancies to term. As one monkey delivered a stillborn infant, only one conception resulted in a live birth (Schantz et al., 1979). As described in an abstracted summary, these results at 50 and 500 ppt TCDD are compared with a group of monkeys given a dietary exposure to PBB (0.3 ppm, Firemaster FF-1) in which seven of seven exposed females were able to conceive, five gave birth to live, normal infants, and one gave birth to a stillborn infant (Allen et al., 1979). Although the effects at 500 ppt TCDD may be associated with significant maternal toxicity, this would not appear to be the case at the lower dose. After administration of 50 ppt TCDD, no overt effects on maternal health were observed, but the ability to conceive and maintain pregnancy was reduced (Allen et al., 1979).

In a similar series of experiments, female rhesus monkeys were fed diets that contained 0, 5, and 25 ppt TCDD (Bowman et al., 1989a; Schantz and Bowman, 1989). Reproductive function was not altered in the 5 ppt group, as seven of eight females mated to unexposed males after 7 months of dietary exposure to TCDD were able to conceive. Six of these females gave birth to viable infants at term and one gave birth to a stillborn infant. This was not significantly different from the results of the control group, which was fed a normal diet that contained no TCDD. All seven of the monkeys in this control group were able to conceive and give birth to viable infants. The 25 ppt dietary exposure level, however, did affect reproductive function. Only one of the eight females in this group that was mated gave birth to a viable infant. As in the 50 ppt group from earlier studies, there were no serious health problems exhibited by any females

exposed to 0, 5, or 25 ppt TCDD. Therefore, the results in the 25 and 50 ppt groups suggest that maternal exposure to TCDD before and during pregnancy can result in fetomortality without producing overt toxic effects in the mother.

McNulty (1984) examined the effect of a TCDD exposure during the first trimester of pregnancy (gestational age 25 to 40 days) in the rhesus monkey. At a total dose of 1 μg/kg given in nine divided doses, three of four pregnancies ended in abortion and two of these abortions occurred in animals that displayed no maternal toxicity. At a total dose of 0.2 μg/kg, one of four pregnancies ended in abortion. This did not appear to be different from the control population, but the low number of animals per group did not permit statistical analysis. McNulty (1984) also administered single 1 μg/kg doses of TCDD on gestational days 25, 30, 35, or 40. The number of animals per group was limited to three, but it appeared that the most sensitive periods were the earlier periods, days 25 and 30, and that both maternal toxicity and fetotoxicity were reduced when TCDD was given on later gestational days. For all days at which a single 1 μg TCDD/kg dose was given (gestational day 25, 30, 35, or 40), 10 of 12 pregnancies terminated in abortion. Thus, of 16 monkeys given 1 μg TCDD/kg in single or divided doses between days 25 and 40 of pregnancy, only three normal births occurred (McNulty, 1984, 1985).

5.3.1.2. *Ovarian Function*

Signs of ovarian dysfunction in rats and monkeys such as anovulation and suppression of the estrous cycle had been reported previously (Kociba et al., 1976; Barsotti et al., 1979; Allen et al., 1979), and Li et al. (1995a,b) recently extended these studies. In their initial study (Li et al., 1995a), adult female rats were given a single oral dose of 10 μg TCDD/kg BW and observed for changes in estrous cyclicity and ovulation. The number of ova ovulated per female and the number of females ovulating were decreased by 75%, and the estrous cycle was also altered, with a significant increase in the time spent in diestrus and a decrease in proestrus and estrus. However, these findings were clouded by the fact that the exposure caused a body weight loss in the females. A subsequent dose-response study in immature hypophysectomized/eCG-primed females provided support that the effects on ovulation were dose-dependent (Li et al., 1995b). However, the effects were only statistically significant at exposure levels that also caused a significant loss in body weight over the experimental period.

5.3.1.3. *Reproductive Capability of Ah Receptor Knockout Mice*

Reproductive success is adversely affected in some Ah receptor null mouse lines (in the absence of TCDD exposure). Ah receptor null female mice (Fernandez-Salguero et al., 1995) become pregnant at similar rates and implant similar numbers of embryos as control females. However, these Ah receptor null dams experience increased prenatal loss of conceptuses and

difficulty in surviving the stress of lactation, and their pups show poor survival during lactation and shortly after weaning (Abbott et al., 1999c). In contrast, offspring from a different Ah receptor null mouse line (Schmidt et al., 1996) exhibit low neonatal mortality. Possible reasons for this and other phenotypic differences between offspring of these two Ah receptor null mouse lines is unclear (Schmidt et al., 1996).

5.3.1.4. *Endometriosis*

5.3.1.4.1. *Humans*

Endometriosis is characterized by endometrial cell growth outside the uterus and can be associated with infertility and pain. Of increasing interest is the initial report that women with endometriosis in Germany are more likely to have elevated concentrations of PCBs in their blood (Gerhard and Runnebaum, 1992). While this report did not provide sufficient methodological detail (reviewed in Ahlborg et al., 1995), Koninckx and coworkers (1994) reported that Belgium also has a high incidence of endometriosis and that TCDD concentrations in breast milk in Belgian women are among the highest in the world. Similarly, a larger number of women in Israel with endometriosis were found to have measurable blood levels of TCDD when compared to age-matched control women that had tubal infertility but no endometriosis (Mayani et al., 1997). More recently in Belgian women, high serum TCDD-like toxic equivalent concentrations (TEQs) were associated with a greater risk for endometriosis (Pauwels et al., 1999), but no association was found between endometriosis and total serum PCB concentrations in this study. This suggests that only TCDD-like PCBs may be capable of producing the response in women.

A recent study has demonstrated the occurrence of certain TCDD-induced biochemical changes that facilitate the ectopic growth of human endometrial tissue (Bruner-Tran et al., 1999). When the human tissue is exposed to TCDD *in vitro* and implanted into immunologically impaired nude mice, TCDD exposure inhibits the ability of progesterone to decrease the expression of matrix metalloproteinase enzymes. This effect, which is associated with a TCDD-induced decrease in the ability of human endometrial organ cultures to produce TGF-β_2, enhances ectopic growth of the endometrial lesions. These results begin to provide a biochemical basis for the ability of TCDD exposure to facilitate the expression of endometriosis in women, and they strengthen the association between elevated exposure to TCDD-like AhR agonists and the increased incidence and severity of this disease.

5.3.1.4.2. *Monkeys*

An association between TCDD exposure and endometriosis has found some experimental support in studies using the rhesus monkey. However, the association between PCB exposure

and endometriosis in monkeys is less clear. Rier and coworkers chronically exposed rhesus monkeys to TCDD in their diet for 4 years and then maintained the monkeys for an additional 10 years. These monkeys were then compared to similar unexposed animals in the same colony (Rier et al., 1993; Rier et al., 1995). In monkeys exposed to dietary levels of 5 ppt and 25 ppt TCDD, the incidence of endometriosis was 43% and 71%, respectively, whereas the incidence in control monkeys was 33%. Moreover, the severity of endometriosis was TCDD dose-dependent. Monkeys in the studies by Rier and coworkers appeared to be quite sensitive to TCDD-induced increases in the incidence and severity of endometriosis. It has been calculated that the female monkeys exposed to 5 ppt TCDD in the diet for 4 years had accumulated a TCDD body burden of 69 ng/kg (DeVito et al., 1995). However, another study found no association between the incidence and severity of endometriosis and exposure to Aroclor 1254 when rhesus monkeys were exposed for up to 6 years. Unlike the Rier studies, these monkeys were not held for evaluation a long time after exposure (Arnold et al., 1996). Interestingly, both the Rier and the Arnold studies reported a similar high background incidence (33% -37%) of endometriosis in unexposed monkeys. When taken together the results of these studies indicate that it may take some time for a TCDD-induced increase in endometriosis to become manifest above the background level, that sensitivity to halogen aromatic hydrocarbon-induced increases in endometriosis may be more readily detected when TCDD equivalent concentrations (TEQs) rather than total PCB concentrations are considered, and that the effect, if produced by PCBs at all in monkeys, could be PCB congener-specific. In this last sense, the results in monkeys correspond to those recently obtained by Pauwels et al. (1999) in women, which also suggest that the effect on endometriosis is congener specific for those halogenated aromatic congeners with AhR agonist activity.

5.3.1.4.3. ***Rats and mice.*** An animal model has been developed in the rat and mouse to evaluate the effects of TCDD exposure on the development of endometriosis (Cummings et al., 1995, 1996). While rodents do not spontaneously develop endometriosis, the surgical implantation of uterine tissue at ectopic sites in the abdominal cavity is a way of mimicking aspects of the disease. The formation of clear vesicles, fibrosis, inflammation, and adhesions are common to the disease in primates and to the rodent model of endometriosis (Cummings et al., 1996). Female rats and mice were administered 0, 3, or 10 μg TCDD/kg 3 weeks before, at the time of, and at 3, 6, and 9 weeks after surgery to induce endometriosis (Cummings et al., 1996). At 3, 6, 9, and 12 weeks following surgery, there were dose-dependent increases in lesion diameter in both species if all time points were pooled. In addition, rats showed a decrease in body weight and ovarian weight at 9 and 12 weeks, accompanied by an increase in the time spent in vaginal estrus, and histology of the ovary at 12 weeks indicated ovulatory arrest. These effects on body

weight and the ovary were not observed in the mouse, but the mouse seemed more susceptible to the TCDD-increase in lesion diameter than the rat at 9 and 12 weeks postsurgery.

Additional studies done to assess the effects of TCDD in the mouse model of endometriosis used slightly different methodology and resulted in different results. In these studies mice were first subjected to the surgery to induce endometriosis and then were exposed chronically to daily doses of 0, 10, 50, or 100 ng TCDD/kg for 28 days. When the effects of TCDD exposure on the endometriosis lesions were assessed 2 days after the last dose, it was found that there was a dose-dependent decrease in lesion diameter (Yang et al., 1997). In addition, uterine tissue implant survival and growth was decreased in ovariectomized mice and restored by estrogen replacement (Foster et al., 1997). Exposure to TCDD inhibited the ability of estrogen replacement to promote implant survival and growth, suggesting that TCDD acts as an antiestrogenic compound in this form of the model. The authors of this study noted the difference between their results and those of Cummings et al., and they suggested that when TCDD is administered prior to the induction of endometriosis in ovary-intact mice, immune suppression facilitates the growth of the endometriosis implants, or that factors of ovarian origin other than steroids may play a role in the establishment, maintenance, and growth of endometriosis. In contrast, when TCDD is administered after surgically induced endometriosis is established, the antiestrogenic effects of TCDD inhibit lesion growth. In spite of these suggestions, it seems possible that insufficient time is allowed when this model is used for the severity of surgically-induced endometriosis to be increased by TCDD exposure subsequent to the initial inhibition.

Unlike TCDD, the Ah receptor agonists 3,3',4,4',5-PCB (PCB 126), 1,3,6,8-tetrachlorodibenzo-*p*-dioxin, and 2,3,4,7,8-pentachlorodibenzofuran were not able to alter lesion diameter or lesion weight in the mouse model (Johnson et al., 1997). This is reminiscent of the ability of TCDD, but not Aroclor 1254, to increase the incidence of endometriosis in rhesus monkeys. Interestingly, the dose-response relationship for TCDD in the mouse model was U-shaped, with low doses promoting endometriosis and larger doses resulting in a decreased response. Therefore, the effects of Ah receptor agonists on endometriosis may depend on a complex interplay between immune suppression and antiestrogenicity.

5.3.1.5. *Mammary Gland*

5.3.1.5.1. ***Postnatal mammary gland development.*** The mammary gland of weanling rats and mice is a system of branching ducts that terminate in actively growing terminal end buds (TEBs). Elongation of the mammary ducts and penetration of the epithelium into the surrounding adipose stroma results from the rapid cellular proliferation of the TEBs (Williams et al., 1983). The density of TEBs (number of TEB/mm^2) increases steadily after birth until it reaches a maximum

value in the rat on postnatal day 21 (Russo et al., 1978). This is accompanied by a concomitant increase in the total area of the mammary gland. After postnatal day 21 numerous lateral buds develop along the growing ducts as further growth of the gland occurs. During this time septation and cleavage in the TEBs and lateral ducts result in the formation of 3-5 smaller buds per structure, the alveolar buds (ABs) (Russo and Russo, 1978). With the initiation of estrus cycling (postnatal days 35-42), alveoli form from the ABs, and until cessation of mammary growth occurs, these branched structures increase progressively in number with each successive estrus cycle resulting in the formation of lobules. TEBs that do not further differentiate in this manner regress into finger-shaped structures called terminal ducts (TDs).

5.3.1.5.2. ***Effects of postnatal TCDD exposure in vivo*.** Female rats that were orally administered daily doses of 2.5 μg TCDD/kg or vehicle on postnatal days 24, 26, 28, and 30 were affected by decreased cellular proliferation within their mammary glands and by decreased mammary gland development (Brown et al., 1995). When evaluated 18 hours after the last TCDD dose, body weight in the treated rats was slightly but not significantly reduced. However, the combined uterine-ovarian weights were less than half, and mammary gland size was only 61% that of vehicle-treated control rats. TCDD treatment caused a statistically significant 59% reduction in the number of TEBs without significantly affecting the numbers of ABs, lobules, and TDs. Therefore, the postnatal TCDD exposure-induced inhibition of mammary growth is accompanied by a selective size reduction within the most rapidly dividing portion of the mammary ducts, the TEBs. While TCDD exposure did not decrease the percentage of TEB cells that are proliferating (PCNA labeling index) or percentage of TEB cells in S-phase, it decreased these parameters in TDs and lobules. Consistent with the decrease in the number of TEBs in TCDD- treated rats, the total numbers of PCNA-labeled and S-phase cells were decreased (even though percentages were not) compared with the values obtained in vehicle-exposed control rats. In addition, TCDD exposure decreased the total numbers of PCNA-labeled and S-phase cells in TDs and lobules. These results are consistent with the finding that early postnatal TCDD exposure in the rat causes an inhibition of mammary epithelial cell proliferation, but the mechanism for this effect remains to be determined. It may be a consequence of the antiestrogenic properties of TCDD (Harris et al., 1990; Safe et al., 1991).

5.3.1.5.3. ***Expression of Ah receptor and effects of the Ah receptor null mutation.*** Mammary glands from estrous cycling C57BL/6J mice express high levels of Ah receptor mRNA and protein (Hushka et al., 1998). Lower or undetectable levels of the Ah receptor mRNA and protein were found during late pregnancy and during mammary gland involution immediately after the cessation of nursing. Transgenic female mice heterozygous for the Ah receptor null

mutation were mated with transgenic heterozygous males. Comparative analysis of mammary gland development in 6- to 8-week-old female Ah receptor wild-type and Ah receptor null littermates demonstrated that there was a 50% reduction in TEBs and an increase in TDs in the Ah receptor null females. In most Ah receptor null females the ductal architecture, branching patterns, and overall organization of specific cell types in the mammary epithelium did not appear to be altered. However, a small percentage of mammary glands from Ah receptor null females exhibited little or no branching. These findings support the conclusion that Ah receptor-dependent processes may play a role in TEB development even in the absence of endogenous ligand. However, as indicated below, the effects of the Ah receptor null mutation and Ah receptor activation by exogenous ligand in the mouse turn out to be similar rather than opposite.

5.3.1.5.4. ***Response of organ-cultured mammary gland to exogenous ligand.*** Nulliparous C57BL/6J mice were primed with 15 daily injections of estradiol and progesterone (Hushka et al., 1998). Mammary glands were removed 24 hours after the last priming and cultured in the presence of 0.1% DMSO or 1 to 100 nM 2,3,7,8-TCDF for 5 days and prepared for histology and immunohistochemistry. Lobule size after culturing was suppressed by TCDF in a dose-related manner, such that lobules in mammary glands exposed to the largest dose of TCDF were less than one-half the size of vehicle-exposed lobules. The ^{3}H-thymidine labeling index was also reduced in the TCDF-exposed lobules. Therefore, the growth and development of TEBs into lobules appeared to be suppressed by TCDF, but the effects of TCDF on TEB number after organ culture were not reported, and therefore it is not known whether TCDD exposure caused the expected increase in the number of TEBs. Overall, these results are consistent with the effects of TCDD on mammary growth and development that were previously mentioned for the rat. In addition, they suggest that toxicity results from direct effects of TCDF on the mammary gland.

5.3.1.5.5. ***Effects of prenatal TCDD exposure.*** Pregnant rats were orally administered 1 μg TCDD/kg or vehicle on gestational day 15 (Brown et al., 1998). Mammary development was evaluated in female offspring at 21 and 50 days of age. While body weight of the TCDD-exposed female offspring, compared with that of vehicle-exposed female offspring, was reduced at both time points, liver weight was reduced only on postnatal day 50. TCDD exposure delayed the time of vaginal opening and caused a disruption in the estrus cycle. However, uterus weight and mammary gland size were unaffected by in utero and lactational TCDD exposure. Nevertheless, the number of TEBs was increased in the mammary glands of TCDD-exposed female offspring, and there was a corresponding decrease in the number of lobules. Cellular proliferation (BrdU labeling index) was not affected by TCDD exposure in TDs at either time

point, but the results indicate that the differentiation pathway from TEBs to lobules was inhibited.

When the carcinogen DMBA is administered to 50- to 60-day-old female rats, the differentiation pathway of the mammary gland epithelium is disrupted (Russo and Russo, 1978). Between 14 and 21 days post-DMBA inoculation, TEBs increase markedly in size and do not differentiate into lobules. Instead, these TEB-derived larger structures, termed intraductal proliferations (IDPs), may progress along an alternate pathway to form microtumors that have the characteristics of rat mammary adenocarcinomas. These structures are derived exclusively from TEBs. In utero and lactational exposure to a single maternal dose of 1 μg TCDD/kg administered on gestational day 15 increased the number of TEBs in 50-day-old female offspring (Brown et al., 1998). In addition, these glands were rendered more susceptible to the formation of DMBA-induced mammary tumors. While neither epidemiological data nor occupational studies provide clear support for an association between TCDD and the occurrence of breast cancer in women, it is interesting to note that prenatal and postnatal exposure to TCDD can have opposite effects in laboratory animals. Postnatal exposure to TCDD decreased the incidence of DMBA-induced mammary tumors in female rats (Holcomb et al., 1994). This latter effect was believed to be due to the antiestrogenic properties of TCDD. The mechanism whereby in utero and lactational TCDD exposure alters TEB differentiation and promotes DMBA-induced mammary tumorigenesis is not yet known.

5.3.1.5. Summary. The primary effects of TCDD on female reproduction appear to be decreased fertility, inability to maintain pregnancy for the full gestational period, and in the rat, decreased litter size. It is likely that ovarian dysfunction and alterations in normal estrous cyclicity also result from TCDD exposure. The growing body of data also indicate that TCDD exposure may lead to or favor the appearance of endometriosis. Koninckx et al. (1994) have noted that Belgium has a high incidence of endometriosis and that TCDD concentrations in breast milk in Belgium women are among the highest in the world, and a similar relationship between measurable blood levels of TCDD and endometriosis has been reported in women in Israel (Mayani et al., 1997). Mammary gland development in rats and mice can be adversely affected by in utero and lactational TCDD exposure potentially leading to the formation of tumors. Effects of TCDD exposure on the formation of mammary gland tumors can depend on the life-stage at which exposure occurs. Continued attention to these and other effects on the female reproductive system, especially in the nonpregnant state, will be important to determining the potential female reproductive toxicity of TCDD.

5.3.1.6. *Alterations in Hormone Levels*

The potential for TCDD to alter circulating female hormone levels has been examined, but only to a very limited extent. In monkeys fed a diet that contained 500 ppt TCDD for ~9 months, the length of the menstrual cycle, as well as the intensity and duration of menstruation, were not appreciably affected by TCDD exposure (Barsotti et al., 1979). However, there was a decrease in serum estradiol and progesterone concentration in five of the eight exposed monkeys, and in two of these animals the reduced steroid concentrations were consistent with anovulatory menstrual cycles. In summary form, Allen et al. (1979) described the effects of dietary exposure of female monkeys to 50 ppt TCDD. After 6 months of exposure to this lower dietary level of TCDD, there was no effect on serum estradiol and progesterone concentrations in these monkeys. Thus, the presence of these hormonal alterations is dependent on the level of dietary TCDD exposure.

5.3.1.7. *Antiestrogenic Action*

5.3.1.7.1. ***In vivo.*** Estrogens are necessary for normal uterine development and for maintenance of the adult uterus. The cyclic production of estrogens partially regulates the cyclic production of FSH and LH that results in the estrous cycling of female mammals. In addition, estrogens are necessary for the maintenance of pregnancy. Any effect that causes a decrease in circulating or target cell estrogen levels can alter normal hormonal balance and action.

Early experimental results in rats and monkeys indicated that TCDD may have an antiestrogenic action. Following administration of 1 μg TCDD/kg/day to rats for 13 weeks, Kociba et al. (1976) reported morphologic changes in the ovaries and uterus that were interpreted as being due to a suppression or inhibition of the estrous cycle. Rhesus monkeys exposed to 500 ppt of TCDD in the diet for 6 months developed hormonal irregularities in their estrous cycles that were associated with reduced conception rates as well as a high incidence of early spontaneous abortions (Allen et al., 1977; Barsotti et al., 1979).

In rhesus monkeys, the severity of the TCDD-associated reproductive alterations was correlated with decreased plasma levels of estrogen and progesterone (Barsotti et al., 1979). Thus, one possible mechanism for these effects would be increased metabolism of estrogen and progesterone due to induction by TCDD of hepatic microsomal enzymes and/or a decrease in the rate at which these steroids are synthesized. On the other hand, serum concentrations of 17β-estradiol are not significantly affected when TCDD is administered to pregnant rats (Shiverick and Muther, 1983). Thus, an alternative mechanism for TCDD-associated reproductive dysfunction could involve effects of TCDD on gonadal tissue itself, such as a decrease in its responsiveness to estrogen. In support of this latter mechanism, the administration of TCDD to CD-1 mice results in a decreased number of cytosolic and nuclear estrogen receptors in

hepatocytes and uterine cells. Although TCDD treatment induces hepatic cytochrome P-450 levels in these animals, it has no effect on serum concentrations of 17β-estradiol (DeVito et al., 1992). This indicates that the antiestrogenic effect of TCDD in CD-1 mice is not caused by a decrease in circulating levels of estrogen.

Effects of estrogen on the uterus include a cyclic increase in uterine weight, increased activity of the enzyme peroxidase, and an increase in the tissue concentration of progesterone receptors. Antiestrogenic effects of TCDD administration to female rats include a decrease in uterine weight, decrease in uterine peroxidase activity, and a decrease in the concentration of progesterone receptors in the uterus (Safe et al., 1991). In addition, when TCDD and 17β-estradiol are coadministered to the same female rat, the antiestrogenic action of TCDD diminishes or prevents 17β-estradiol-induced increases in uterine weight, peroxidase activity, progesterone receptor concentration, and expression of EGF receptor mRNA (Astroff et al., 1990; Safe et al., 1991). Similarly, in mice TCDD administration decreases uterine weight and antagonizes the ability of 17β-estradiol to increase uterine weight (Gallo et al., 1986).

The ability of TCDD to antagonize the effects of exogenously administered estrogen in the rat is dependent on the age of the animal. In 21-day-old rats, TCDD does not affect 17β-estradiol-induced increases in uterine weight or progesterone receptor concentration. On the other hand, in 28-day-old intact rats and 70-day-old ovariectomized rats, both of these 17β-estradiol-mediated responses are attenuated by TCDD (Safe et al., 1991). Previously, it had been reported that TCDD administration does not alter the dose-dependent increase in uterine weight due to exogenously administered estrone in sexually immature rats (Shiverick and Muther, 1982). The later work by Safe et al. (1991) suggests that this apparent lack of an antiestrogenic effect of TCDD may have been due to the young age of the rats used.

5.3.1.7.2. ***In vitro.*** Both TCDD and progesterone can affect a decrease in the nuclear estrogen receptor concentration in rat uterine strips (Romkes and Safe, 1988). However, the effect of progesterone is inhibited by actinomycin D, cycloheximide, and puromycin, whereas the effect of TCDD is inhibited only by actinomycin D. The reasons that the TCDD-induced decrease in nuclear estrogen receptors is blocked by a transcription inhibitor, but not by protein synthesis inhibitors, are not understood. However, these results indicate that TCDD and progesterone decrease the nuclear estrogen receptor concentration by different mechanisms. In addition, the antiestrogenic actions of TCDD can be demonstrated in cell culture, and two prominent mechanisms could potentially be involved. They are (1) increased metabolism of estrogen due to Ah receptor-mediated enzyme induction and (2) a downregulation of estrogen receptors within the target cell.

In MCF-7 cells, which are estrogen-responsive cells derived from a human breast adenocarcinoma, antiestrogenic effects caused by the addition of TCDD to the culture medium include a reduction of the 17β-estradiol-induced secretion of a 160 kDa protein, 52 kDa protein, and 34 kDa protein (Biegel and Safe, 1990). These last two proteins are believed to be procathepsin D and cathepsin D, respectively. In addition, treatment of MCF-7 cells with TCDD suppresses the 17β-estradiol-enhanced secretion of tPA and inhibits estrogen-dependent postconfluent cell proliferation (Gierthy et al., 1987; Gierthy and Lincoln, 1988). Thus, cultured MCF-7 cells have several estrogen-dependent responses that are inhibited by TCDD; this characteristic makes them a useful model system for studying the antiestrogenic actions of dioxin.

In cultured MCF-7 cells, TCDD treatment induces aryl hydrocarbon hydroxylase (AHH) activity, the hallmark response of Ah receptor binding, and increases hydroxylation of 17β-estradiol at the C-2, C-4, C-6α, and C-15α, positions (Spink et al., 1990). It turns out that the particular cytochrome P-450 that catalyzes the C-2, C-15α, and C-6α hydroxylations of 17β-estradiol is cytochrome P-4501A1, which is identical to AHH (Spink et al., 1992). TCDD treatment also results in reduced levels of occupied nuclear estrogen receptors (Harris et al., 1990). These results indicate, in MCF-7 cells, that the antiestrogenic effect of TCDD could result from (1) an increased metabolism of estrogens due to Ah receptor-mediated enzyme induction and/or (2) a decreased number of estrogen receptors in the nucleus. Safe and his colleagues have published TCDD-concentration-response information for both the TCDD-induced decrease in occupied nuclear estrogen receptors (Harris et al., 1989) and the induction of AHH and EROD activities in MCF-7 cells (Harris et al., 1990). In addition, they have reported that TCDD causes a decreased number of cytosolic and nuclear estrogen receptors in Hepa 1c1c7 cells, which are a mouse hepatoma cell line (Zacharewski et al., 1991).

Independent analysis of the data suggests that the EC_{50} values for these effects are not dissimilar enough to distinguish between the proposed mechanisms. Instead, it appears as though TCDD induces the enzymes AHH and EROD over the same concentration range that it causes a decreased concentration of occupied nuclear estrogen receptors in MCF-7 cells. In Hepa 1c1c7 cells, the lowest concentration used was 10 pM. Although exposure to 10 pM TCDD resulted in a statistically significant downregulation of estrogen receptors, Israel and Whitlock (1983) reported that this concentration is the approximate EC_{50} for the induction of cytochrome P-4501A1 mRNA and enzyme activity in these cells. Therefore, in Hepa 1c1c7 cells as well as in MCF-7 cells, it would appear that the TCDD concentrations required to produce enzyme induction and reduction in occupied nuclear estrogen receptor levels are not dissimilar enough to distinguish between the two proposed mechanisms.

More recently, Safe and his colleagues have used an analog of TCDD, MCDF, which inhibits the 17β-estradiol-induced secretion of the 34, 52, and 160 kDa proteins and downregulates estrogen receptors in MCF-7 cells. These effects occurred at concentrations of MCDF at which there is no detectable induction of EROD activity (Zacharewski et al., 1992). In addition, it has been stated that the downregulation of estrogen receptors in Hepa 1c1c7 cells can be detected as early as 1 hour after exposure of the cell cultures to 10 nM TCDD (Zacharewski et al., 1991). This time is slightly less than the 2 hours that was required for Israel and Whitlock (1983) to detect an increase in cytochrome P-4501A1 mRNA levels after exposure of Hepa 1c1c7 cells to 10 pM TCDD. After exposure of Hepa 1c1c7 cells to a maximally inducing concentration of 1 nM TCDD, however, there are significant increases in the cellular concentration of cytochrome P-4501A1 mRNA after 1 hour, whereas the induction of AHH activity takes slightly longer (Israel and Whitlock, 1983).

Gierthy et al. (1987) reported that exposure of MCF-7 cells to 1 nM TCDD caused suppression of the 17β-estradiol-induced secretion of tPA. This effect of TCDD, however, occurred in the absence of any measurable decrease in the whole cell concentration of estrogen receptors, even though the cultures were pretreated with serum-free medium to reduce cell proliferation and maximize the cellular content of estrogen receptors. Gierthy's group pretreated their cultures with serum-free medium, which was done to reduce cell proliferation and maximize the cellular content of estrogen receptors. The disparity between this result of Gierthy et al. (1987), which suggests no effect of TCDD on the estrogen receptor content of MCF-7 cells, and the results of Safe and his colleagues to the contrary in this same cell line remains largely unexplained. Overall, it appears as though no obvious distinction between the two proposed mechanisms can be made at the present time. Therefore, it seems that the antiestrogenic effect of TCDD results from both an increased metabolism of estrogen and a decreased number of estrogen receptors. It is important to note that TCDD does not compete with radiolabeled estrogens or progesterone for binding to estrogen or progesterone receptors and that these steroids do not bind to the Ah receptor or compete with radiolabeled TCDD for binding (Romkes et al., 1987; Romkes and Safe, 1988).

5.3.1.7.3. *Evidence for an Ah receptor mechanism*

5.3.1.7.3.1. *Ah receptor mutants*. Although the precise cellular mechanism by which TCDD produces its antiestrogenic effect is subject to a discordance between two primary schools of thought, there is agreement that the response is mediated by the Ah receptor. Thus, the antiestrogenic effects of TCDD in cultured cells appear to involve an Ah receptor-mediated alteration in the transcription of genes. This is indicated by studies using wild-type Hepa 1c1c7 cells and mutant Hepa 1c1c7 cells in culture (Zacharewski et al., 1991). In wild-type cells,

TCDD reduces the number of nuclear estrogen receptors, and this response can be inhibited by cycloheximide and actinomycin D. However, in class 1 mutants, which have relatively low Ah receptor levels, TCDD has only a small effect. Similarly, in class 2 mutants, which have a defect in the accumulation of transcriptionally active nuclear Ah receptors, there was no effect of TCDD on the number of nuclear estrogen receptors. Taken together, these results indicate that the downregulation of estrogen receptors in Hepa 1c1c7 cells involves an Ah receptor-mediated effect on gene transcription. As previously noted, TCDD induces cytochrome P-4501A1 mRNA transcription and enzyme activity in Hepa 1c1c7 cells (Israel and Whitlock, 1983). This effect is also Ah receptor mediated (Nebert and Gielen, 1972).

5.3.1.7.3.2. ***Structure-activity relationships in vivo.*** The relative potencies of halogenated aromatic hydrocarbon congeners as inhibitors of uterine peroxidase activity in the rat are similar to their relative Ah receptor-binding affinities (Astroff and Safe, 1990). Only limited relative potency information is available for the reduction of hepatic and uterine estrogen receptor concentrations per se by these substances in rats. TCDD and 1,2,3,7,8-PeCDD both exhibit high affinity for the Ah receptor. At an 80 μg/kg dose of either of these two substances, hepatic estrogen receptor concentrations are reduced 42% and 41%, whereas uterine estrogen receptor concentrations are reduced 53% and 49% by TCDD and 1,2,3,7,8-PeCDD, respectively. On the other hand, 1,3,7,8-TCDD and 1,2,4,7,8-PeCDD bind less avidly to the Ah receptor. At a 400 μg/kg dose of either of these two substances, hepatic estrogen receptor concentrations are reduced 36% and 40%, whereas uterine estrogen receptor concentrations are reduced 21% and 24% by 1,3,7,8-TCDD and 1,2,4,7,8-PeCDD, respectively (Romkes et al., 1987). As the potency of these congeners for reducing estrogen receptor concentrations correlates with their Ah receptor-binding affinities, these in vivo results provide evidence that the antiestrogenic effect of TCDD is mediated by the Ah receptor.

5.3.1.7.3.3. ***Genetic evidence.*** Consistent with the interpretation based on structure-activity relationships, there is a greater reduction in the number of hepatic estrogen receptors when Ah^bAh^b C57BL/6 mice are exposed to TCDD than when Ah^dAh^d DBA/2 mice are similarly exposed (Lin et al., 1991). To date, however, the antiestrogenic effects have not been studied in the progeny of test crosses between Ah^bAh^b and Ah^dAh^d mouse strains that respectively produce Ah receptors with high- or low-binding affinity for TCDD. Therefore, the potential segregation of the antiestrogenic effects of TCDD with the Ah locus has not been verified by the results of genetic crosses.

5.3.1.7.3.4. ***Structure-activity relationships in vitro*.** The Ah receptor is detectable in MCF-7 cells, and AHH as well as EROD activities are both inducible in these cells (Harris et al., 1989). The relative abilities of TCDD and other CDD, CDF, and PCB congeners to suppress 17β-estradiol-induced secretion of tPA by MCF-7 cells are consistent with the structure-activity relationships for other Ah receptor-mediated responses (Gierthy et al., 1987). In addition, the rank order of potency for several Ah receptor agonists in reducing nuclear estrogen receptors in MCF-7 cells is TCDD > 2,3,4,7,8-PeCDD > 2,3,7,8-TCDF > 1,2,3,7,9-PeCDD > 1,3,6,8-TCDF (Harris et al., 1990). The rank order of potency for these substances is consistent with their relative activities as Ah receptor agonists. These results in vitro support a role for the Ah receptor in the antiestrogenic actions of TCDD.

5.3.2. Male

5.3.2.1. *Reproductive Function/Fertility*

TCDD and related compounds decrease testis and accessory sex organ weights, cause abnormal testicular morphology, decrease spermatogenesis, and reduce fertility when given to adult animals in doses sufficient to reduce feed intake and/or body weight. Certain of these effects have been reported in chickens, rhesus monkeys, rats, guinea pigs, and mice treated with overtly toxic doses of TCDD, TCDD-like congeners, or toxic fat that was discovered later to contain TCDD (Allen and Lalich, 1962; Allen and Carstens, 1967; Khera and Ruddick, 1973; Kociba et al., 1976; van Miller et al., 1977; McConnell et al., 1978; Moore et al., 1985; Chahoud et al., 1989; Morrisey and Schwetz, 1989). In testis of these different species, TCDD effects on spermatogenesis are characterized by loss of germ cells, the appearance of degenerating spermatocytes and mature spermatozoa within the lumens of seminiferous tubules, and a reduction in the number of tubules containing mature spermatozoa (Allen and Lalich, 1962; Allen and Carstens, 1967; McConnell et al., 1978; Chahoud et al., 1989). The lowest cumulative dose of TCDD to decrease spermatogenesis in the rat was 1 μg/kg/day administered 5 days a week for 13 weeks (Kociba et al., 1976). With this dosage regimen, which resulted in a TCDD body burden of approximately 20 μg/kg at the end of the dosing period (Rose et al., 1976), body weights and feed consumption of the rats also were significantly depressed. A similar 13-week dosing study using adult male mice found that 3 and 30 mg 3,3',4,4'-TCAOB/kg/day caused reductions in epididymal sperm number (van Birgelen et al., 1999). In adult male Sprague-Dawley rats a single dose of 25 μg TCDD/kg decreased epididymal sperm numbers, whereas testicular Leydig cell volume was decreased at 12.5 μg TCDD/kg (Johnson et al., 1992). By comparison, daily sperm production was not affected, even by 50 μg TCDD/kg. Thus, the suppression of spermatogenesis and reduction in epididymal sperm number are not highly sensitive effects when Ah receptor agonists are administered to adult animals.

In contrast, daily sperm production assessed on postnatal day 90 was significantly decreased in weanling Sprague-Dawley rats administered 1 μg TCDD/kg on postnatal day 21 (el-Sabeawy et al., 1998). In addition, testis histology revealed that a dose of 10 μg TCDD/kg caused a decrease in seminiferous tubule diameter compared with that in vehicle-dosed control rats. The spermatogonial population normally located in the basal area of the tubules was absent in the TCDD-treated rats. However, these effects on testis histology were not found at TCDD doses less than 10 μg TCDD/kg. Motility studies were performed on epididymal sperm, and dose-related decreases in sperm curvilinear velocity and beat cross frequency were found over the dose range from 0.1-5.0 μg TCDD/kg. Average path and straight line velocity were significantly decreased at 5 μg TCDD/kg (el-Sabeawy et al., 1998).

Effects of TCDD administration to 21-day-old rats on epidermal growth factor receptor-, protein tyrosine kinase-, protein kinase A-, protein kinase C-, mitogen-activated protein 2 kinase, and c-Src tyrosine kinase-mediated pathways in the testis were also examined (el-Sabeawy et al., 1998). Dose-related increases in the activity of c-Src kinase were found on postnatal days 34 and 90 over the dose range from 0.1-5.0 μg TCDD/kg. In addition, the administration of multiple doses of the c-Src kinase inhibitor geldanamycin over the time period from postnatal days 21-90 blocked the effects of TCDD on testis weight and daily sperm production. The authors conclude that these results provide evidence for the involvement of epidermal growth factor and Src kinasesignaling pathways in the mechanism by which TCDD disrupts testicular development and subsequently affects testis function.

5.3.2.2. *Alterations in Hormone Levels*

The effects of TCDD on the male reproductive system are believed to be due in part to an androgenic deficiency. This deficiency is characterized in adult rats by decreased plasma testosterone and DHT concentrations, unaltered plasma LH concentrations, and unchanged plasma clearance of androgens and LH (Moore et al., 1985, 1989; Mebus et al., 1987; Moore and Peterson, 1988; Bookstaff et al., 1990a). The ED_{50} of TCDD for producing this effect in adult male rats on day 7 after dosing is 15 μg/kg (Moore et al., 1985), and it can be detected within 1 day of treatment. As described in the following sections, the cause of the androgenic deficiency is decreased testicular responsiveness to LH and increased pituitary responsiveness to feedback inhibition by androgens and estrogens (Moore et al., 1989, 1991; Bookstaff et al., 1990a,b; Kleeman et al., 1990).

5.3.2.3. *Target Organ Responsiveness*

5.3.2.3.1. ***Inhibition of testicular steroidogenesis.*** Testicular steroidogenesis occurs within Leydig cells and is regulated primarily by plasma LH concentrations (Payne et al., 1985; Hall,

1988). Binding of LH to the LH receptor causes cAMP and possibly other second messengers to be formed (Cooke et al., 1989). In response, cholesterol is rapidly transported to the initial enzyme in the testosterone biosynthetic pathway, a cholesterol side-chain cleavage enzyme, which is a cytochrome P-450 (cytochrome P-450_{scc}) located on the inner side of the inner mitochondrial membrane that converts cholesterol to pregnenolone. The mobilization of free cholesterol rather than its conversion to pregnenolone and other metabolites is generally considered to be the rate-limiting step in testicular steroidogenesis. TCDD inhibits testosterone biosynthesis, predominantly if not exclusively by inhibiting the mobilization of free cholesterol that acts as a substrate for cytochrome P-450_{scc} (Moore et al., 1991). Thus, in the testes of TCDD-treated rats, cholesterol is provided to the cytochrome P-450_{scc} enzyme at too slow a rate to maintain androgenic homeostasis, even when the plasma LH concentration characteristic of "normal" androgen levels is present.

Leydig cell volume was significantly reduced 4 weeks after a single intraperitoneal injection of TCDD (Johnson et al., 1994). The effect was dose related and observed at the lowest dose tested, 12.5 μg TCDD/kg BW. This reduction in total cell volume resulted from both a reduced number of cells and a reduced size of individual cells. Wilker et al. (1995) were able to establish that this effect of TCDD can be prevented by hCG.

5.3.2.3.2. ***Altered regulation of pituitary LH secretion.*** In TCDD-treated male rats, the expected increase in plasma LH concentration that would facilitate testicular compensation for the decreased plasma androgens does not occur (Moore et al., 1989; Ruangwises et al., 1991). The failure of the plasma LH concentration to rise appropriately is not caused by an increase in the plasma clearance of LH or by a decrease in the maximal rate of pituitary LH synthesis or secretion (Bookstaff et al., 1990a,b). Rather, TCDD alters the feedback regulation of LH secretion in male rats by increasing the potency of testosterone and its metabolites (DHT and 17β-estradiol) as inhibitors of LH secretion. The ED_{50} of TCDD for enhancing the testosterone-mediated inhibition of LH secretion 7 days after treatment is the same as its ED_{50} for causing the androgenic deficiency (15 μg/kg). Also, both responses are detected within 1 day of TCDD dosing and are fully developed after 7 days when the ED_{50}s were determined.

Decreased plasma androgen concentrations normally result in compensatory increases in both the number of pituitary gonadotropin-releasing hormone (GnRH) receptors and the responsiveness of the pituitary to GnRH. TCDD treatment prevents the increases in GnRH receptor number and responsiveness that would be expected in the light of the decreased plasma androgen concentrations (Bookstaff et al., 1990b). The pituitary is thus a target organ for TCDD because its responsiveness to hormones secreted by the testis (testosterone) and hypothalamus (GnRH) is altered by TCDD.

If the plasma LH concentrations in TCDD-treated rats did increase appropriately in response to decreased plasma androgens, it is expected that plasma androgens would return to normal levels (Kleeman et al., 1990). This is because the testes of TCDD-treated rats are capable of synthesizing more testosterone than is needed to maintain androgen concentrations in the physiological range, although this would require significantly elevated levels of LH in TCDD-treated rats. The fact that there is a testicular reserve capacity to provide for sufficient amounts of androgen synthesis, even when compromised, underscores the importance of the effects of TCDD on pituitary LH secretion in producing the effects of TCDD on plasma androgen concentrations.

5.3.2.3.3. ***Differential responsiveness of androgen target organs.*** The dose-related reductions in plasma testosterone and DHT concentrations in intact adult rats are accompanied by similar dose-related reductions (ED_{50} 15 μg TCDD/kg) in seminal vesicle and ventral prostate weights measured 7 days after dosing (Moore et al., 1985). In contrast, TCDD has no effect on accessory sex organ weights (or plasma androgen concentrations) in castrated adult rats implanted with either testosterone- or DHT-containing capsules (Moore and Peterson, 1988; Bookstaff et al., 1990a,b). As trophic responsiveness of the seminal vesicles and ventral prostate to testosterone and DHT are unaffected by postpubertal TCDD treatment, it follows that TCDD can increase responsiveness of the pituitary to androgens without affecting responsiveness of the accessory sex organs to androgens.

5.3.2.3.4. ***Relative sensitivity.*** The male reproductive system in rats is ~100 times more susceptible to TCDD toxicity when exposure occurs perinatally (ED_{50} for the most sensitive effects, 0.16 μg/kg) rather than in adulthood (ED_{50} for the most sensitive effects, 15 μg/kg). To illustrate this sensitivity, a single maternal TCDD dose as low as 0.064 μg/kg given on day 15 of gestation significantly decreases epididymis and cauda epididymis weights, cauda epididymal sperm numbers, and daily sperm production in male offspring at various stages of sexual development. Decreases in ventral prostate weights in 32-day-old male offspring and in older males, increases in the number of mounts preceding ejaculation, and increases in intromission latency also are produced by maternal TCDD doses as low as 0.064 μg/kg. The 0.064 μg TCDD/kg dose is not maternally toxic and produces no signs of overt toxicity in male or female offspring. Other effects of perinatal exposure on the male reproductive system were detected at a maternal TCDD dose of 0.16 μg/kg or higher (Mably et al., 1991, 1992a,b,c). On the other hand, when exposure occurs in adulthood, relatively large doses in the overtly toxic range are required to cause decreases in spermatogenesis and in ventral prostate and caput epididymis weight (Kociba et al., 1976; Moore et al., 1985). Kociba et al. (1976) reported that accessory sex organ

weights and spermatogenesis are decreased in rats following exposure to 1 μg TCDD/kg/day, 5 days per week for 13 weeks. Using the parameters for TCDD half-life and bioavailability in the rat determined by Rose et al. (1976), this dosage regimen results in a TCDD body burden of approximately 20 μg/kg at the end of the dosing period.

In adult rats, the most sensitive toxic responses to TCDD have been observed following long-term, low-level exposure. In a three-generation reproduction study, Murray et al. (1979) reported that dietary administration of TCDD at doses as low as 0.01 μg/kg/day significantly affected reproductive capacity in female rats, with no effects seen at 0.001 μg/kg/day (NOAEL). The same NOAEL was found in a 2-year chronic toxicity and oncogenicity study in which an increased incidence of certain types of neoplasms was altered among rats given TCDD doses of 0.01 or 0.1 μg/kg/day (Kociba et al., 1978). Based on the pharmacokinetics of TCDD in the rat (Rose et al., 1976), the steady-state body burden of TCDD in these rats that were chronically dosed (>90 days) with either 0.01 or 0.001 μg TCDD/kg/day is approximately 0.29 μg/kg (LOAEL) and 0.029 μg/kg (NOAEL), respectively. Yet, Mably et al. (1991, 1992a,b,c) found that a single TCDD dose of 0.064 μg/kg given on day 15 of gestation produces a number of statistically significant effects on the reproductive system of male rat offspring. Because 0.064 μg TCDD/kg was the lowest dose tested, a NOAEL for developmental male reproductive toxicity, which is defined as the lowest dose used that has no statistically significant effect, could not be determined by Mably et al. (1991, 1992a,b,c). It is concluded that developmental effects on spermatogenesis occur at a maternal TCDD dose that is lower than any previously shown to produce toxicity in rats.

5.4. SUMMARY

This chapter has focused on the variety of effects that dioxin and dioxin-like agents can have on human reproductive health and development. The review is not exhaustive, and emphasis has been placed on the more recent reports. These have been put into context with previous reviews of the literature applicable in risk assessment (Kimmel, 1987) to develop a profile of the potential for dioxin and dioxin-like agents to cause reproductive or developmental toxicity based on the available literature. A portion of this report has been previously published (Peterson et al., 1993).

5.4.1. Human

The literature base with regard to potential human effects is detailed in Chapter 7. In general, there is no epidemiological evidence that makes a direct association between exposure to TCDD or TCDD-related agents and effects on human reproduction or development. However, the evidence that has been accumulated is suggestive of such an effect, at least with respect to

developmental toxicity. All four manifestations of developmental toxicity (reduced viability, structural alterations, growth retardation, and functional alterations) have been observed to some degree following presumed exposure to TCDD-related agents. The incidents at Yusho and Yu-Cheng resulted in increased perinatal mortality and low birthweight in infants born to women who had been exposed. Rocker bottom heal was observed in Yusho infants, and functional abnormalities have been reported in Yu-Cheng children.

Of particular interest is the ectodermal origin of many of the organs and tissues that are affected in the human. An ectodermal dysplasia syndrome has been clearly associated with the Yusho and Yu-Cheng episodes, involving hyperpigmentation, deformation of the fingernails and toenails, conjunctivitis, gingival hyperplasia, and abnormalities of the teeth. The developmental effects that can be associated with the nervous system are also consistent with this pattern because the nervous system is of ectodermal origin.

5.4.2. Experimental Animal

In developing a toxicological profile, it is rare to have sufficient data from human studies for quantitative analysis. Consequently, the risk assessment most often relies on data from experimental animal studies. For dioxin and the dioxin-related agents, the experimental animal database is fairly extensive with respect to reproductive and developmental toxicity. Dioxin exposure has been observed to result in both male and female reproductive effects, as well as effects on development. These latter effects are among the most responsive health endpoints to dioxin exposure. In general, the prenatal and developing postnatal animal is more sensitive to the effects of dioxin than the adult.

5.4.2.1. Developmental Toxicity

Dioxin exposure results in a wide variety of developmental effects and these are observed in three different vertebrate classes and in several species within each class. All four of the manifestations of developmental toxicity have been observed following exposure to dioxin, including reduced viability, structural alterations, growth retardation, and functional alterations. As summarized previously (Peterson et al., 1993), increased prenatal mortality (rat and monkey), functional alterations in learning and sexual behavior (rat and monkey), and changes in the development of the reproductive system (rat) occur at the lowest exposure levels.

Dioxin exposure results in reduced prenatal or postnatal viability in virtually every species in which it has been tested. Previously, increased prenatal mortality appeared to be observed only at exposures that also resulted in maternal toxicity. However, the studies of Olson and McGarrigle (1991) in the hamster and Schantz et al. (1989) in the monkey were suggestive that this was not the case in all species. Although the data from these two studies were limited,

prenatal death was observed in cases where no maternal toxicity was evident. In the rat, Peterson's laboratory (Bjerke et al.,1994a,b; Roman et al., 1995) reported increased prenatal death following a single exposure to TCDD during gestation that did not cause maternal toxicity, and Gray et al. (1995a) observed a decrease in postnatal survival under a similar exposure regimen. While identifying the presence or absence of maternal toxicity may be instructive as to the specific origin of the reduced prenatal viability, it does not alter the fact that pre- and postnatal death were observed. In either case, the Agency considers these effects as being indicators of developmental toxicity in response to the exposure (U.S. EPA, 1991).

Some of the most striking findings regarding dioxin exposure relate to the effects on the developing reproductive system. The findings are even more impressive with the understanding that only a single, low-level exposure during gestation is required to initiate these developmental alterations. Mably et al. (1992a,b,c) originally reported that a single exposure of the Holtzman maternal animal to as low as 0.064 μg/kg could alter normal sexual development in the male offspring. More recently, these findings have been further defined (Bjerke et al., 1994; Gray et al., 1995; Roman et al., 1995), as well as extended to females and another strain and species (Gray et al., 1995). In general, the findings of these later studies have produced qualitatively similar results that define a significant effect of dioxin on the developing reproductive system.

In the developing male, dioxin exposure during the prenatal and lactational periods results in the delay of the onset of puberty as measured by age at preputial separation. There is a reduction in testis weight, sperm parameters, and sex accessory gland weights. In the mature male exposed during the prenatal and lactational periods, there is an alteration of normal sexual behavior and reproductive function. Males exposed to TCDD during gestation are demasculinized. Feminization and a reduction in the number of implants in females mated with exposed males have also been reported, although these effects have not been consistently found. These effects do not appear to be related to reductions in circulating androgens, which were shown in the most recent studies to be normal. Most of these effects occur in a dose-related fashion, some occurring at 0.05 μg/kg and 0.064 μg/kg, the lowest TCDD doses tested (Mably et al., 1992c; Gray et al., 1997a).

In the developing female, Gray and Ostby (1995) have demonstrated altered sexual differentiation in both the Long Evans and Holtzman rat. The effects observed depended on the timing of exposure. Exposure during early organogenesis altered cyclicity, reduced ovarian weight, and shortened reproductive life span. Exposure later in organogenesis resulted in slightly lowered ovarian weight, structural alterations of the genitalia, and a slight delay in puberty. However, cyclicity and fertility were not affected with the later exposure. The most sensitive dose-dependent effects of TCDD in the female rat were structural alterations of the genitalia that occurred at 0.20 μg/kg (Gray et al., 1997b).

Structural malformations, particularly cleft palate and hydronephrosis, occur in mice. While these are not the most sensitive developmental endpoints, the findings indicate that exposure during the critical period of organogenesis can affect the processes involved in normal tissue formation. The TCDD-sensitive events appear to require the Ah receptor. Mouse strains that produce Ah receptors with relatively high affinity for TCDD respond to lower doses than strains with relatively low-affinity receptors. Moreover, congeners with a greater affinity for the Ah receptor are more developmentally toxic than those with a lower affinity.

5.4.2.2. Adult Female Reproductive Toxicity

The primary effects of TCDD on female reproduction appear to be decreased fertility, inability to maintain pregnancy for the full gestational period, and in the rat, decreased litter size. In some studies, signs of ovarian dysfunction such as anovulation and suppression of the estrous cycle have been reported (Kociba et al., 1976; Barsotti et al., 1979; Allen et al., 1979; Li et al., 1995a,b). Rier et al. (1993) reported TCDD-associated endometriosis in the monkey, and similar effects have now been reproduced in rats and mice (Cummings et al., 1996). Unfortunately, the amount of attention given to the female reproductive system, especially in the nonpregnant state, has been limited. Additional studies on the female reproductive system will be important to determining the potential female reproductive toxicity of TCDD.

5.4.2.3. Adult Male Reproductive Toxicity

TCDD and related compounds decrease testis and accessory sex organ weights, cause abnormal testicular morphology, decrease spermatogenesis, and reduce fertility when given to adult animals in doses sufficient to reduce feed intake and/or body weight. In the testis of these different species, TCDD effects on spermatogenesis are characterized by loss of germ cells, the appearance of degenerating spermatocytes and mature spermatozoa within the lumens of seminiferous tubules, and a reduction in the number of tubules containing mature spermatozoa (Allen and Lalich, 1962; Allen and Carstens, 1967; McConnell et al., 1978; Chahoud et al., 1989). This suppression of spermatogenesis is not a highly sensitive effect when TCDD is administered to postweanling animals, as an exposure of 1 μg/kg/day over a period of weeks appears to be required to result in these effects.

The effects of TCDD on the male reproductive system when exposure occurs in adulthood are believed to be due in part to an androgenic deficiency. This deficiency is characterized in adult rats by decreased plasma testosterone and DHT concentrations, unaltered plasma LH concentrations, and unchanged plasma clearance of androgens and LH (Moore et al., 1985, 1989; Mebus et al., 1987; Moore and Peterson, 1988; Bookstaff et al., 1990a). The cause of the androgenic deficiency is decreased testicular responsiveness to LH and increased pituitary

responsiveness to feedback inhibition by androgens and estrogens (Moore et al., 1989, 1991; Bookstaff et al., 1990a,b; Kleeman et al., 1990).

5.4.3. Conclusion

TCDD and related compounds have reproductive and developmental toxicity potential. It is assumed that the responses observed in animal studies are indicative of the potential for reproductive and developmental toxicity in humans. This is an established assumption in the risk assessment process for developmental toxicity (U.S. EPA, 1991). It is supported by the number of animal species and strains in which effects have been observed. The limited human data are consistent with an effect following exposure to TCDD or TCDD-like agents.

Many of the effects have been shown to be dose-response related. The effects on perinatal viability and male reproductive development are among the most sensitive effects reported, occurring at a single prenatal exposure range of as little as 0.05-0.075 μg/kg. In these studies, this was the lowest exposure level tested; thus a NOAEL has not been established for these endpoints.

In general, the structure-activity results are consistent with an Ah receptor-mediated mechanism for many of the developmental effects that are observed. The structure-activity relationship in laboratory mammals appears to be similar to that for Ah receptor binding. This is especially the case with cleft palate in the mouse. However, a direct relationship with Ah receptor binding is less clear for other effects, including those involving the nervous system.

Table 5-1. Relationship between maternal toxicity and prenatal mortality in laboratory mammals exposed to TCDD during gestation

Species/strain	Daily TCDD dose (μg/kg/day)	Cumulative TCDD dose (μg/kg)	Overt maternal toxicity[a]	Percent prenatal mortality[b]	Reference
Monkey/rhesus		0[c]	-	25	McNulty, 1984
		0.2	-	25	
		1	+[d]	81	
		5	+[d]	100	
Guinea pig/Hartley		0[e]	-	-	Olson and McGarrigle, 1991
		0.15	-	-	
		1.5	+	+	
Rabbit/New Zealand	0[f]	0	-	7	Giavini et al., 1982b
	0.1	1	-	12	
	0.25	2.5	+	42	
	0.5	5	+	22	
	1	10	+	100	
Rat/Wistar	0[f]	0	-	3	Khera and Ruddick, 1973
	0.125	1.25	-	1	
	0.25	2.5	-	2	
	0.5	5	-	9	
	1	10	±	8	
	1	10	+	36[g]	
	2	20	+	53[g]	
	4	40	+	100[g]	
Rat/Sprague-Dawley	0[f]	0	-	25	Sparschu et al., 1971
	0.03	0.3	-	21	
	0.125	1.25	-	15	
	0.5	5	+	41[g]	
	2	20	+	95[g]	
	8	80	+	100[g]	
Hamster/Golden Syrian		0[h]	-	-	Olson and McGarrigle, 1991
		1.5	-	-	
		3	-	-	
		6	-	-	
		18	-	58	
Mouse/CD-1	0[i]	0	-	7	Courtney, 1976
	25	250	-	6	
	50	500	-	13	
	100	1,000	-	14	
	200	2,000	+	87	
	400	4,000	+	97	

[a]Decreased body weight gain or marked edema compared with vehicle dosed controls. A (+) or (-) indicates the presence or absence of an effect.
[b]Percentage of absorptions plus late gestational deaths relative to all implantations. A (+) or (-) indicates the presence or absence of an effect.
[c]TCDD administered in single or divided doses between gestational days 20 and 40.
[d]Effects include thickening and reddening of the eyelids, weight loss, dryness and granularity of the skin, loss of hair, and in some cases anemia, purpura, and bleeding from the nose and mouth.
[e]Single dose of TCDD administered on gestational day 14.
[f]TCDD administered daily on days 6 to 15 of gestation.
[g]Significant at $p<0.05$.
[h]Single dose of TCDD administered on gestational days 7 or 9.
[i]TCDD administered daily on days 7 to 16 of gestation.

Source: Couture et al., 1990a.

Table 5-2. Developmental toxicity following gestational exposure to 2,3,7,8-TCDD

Species/Strain	Daily dose[a]	Treatment days[b]	Sacrifice day[b]	Maternal effects[c]	Embryo/fetal effects[c]	Reference
Mice/C57BL/6N	0, 1, or 3 μg/kg	10, 10-13	18	NR	Increase in cleft palate and hydronephrosis	Moore et al., 1973
Mice/C57BL/6N	0, 12, 17, or 22 μg/kg	10	18	Increase in liver-to-body weight ratio	Increase in cleft palate and hydronephrosis	Weber et al., 1985
Mice/C57BL/6N	0, 3, or 12 μg/kg	11, 10-13	18	Increase in liver-to-body weight ratio	Increase in cleft palate and hydronephrosis	Birnbaum et al., 1985
Mice/C57BL/6N	0 or 3 μg/kg	10-13	18	Increase in liver-to-body weight ratio	Increase in hydronephrosis	Birnbaum et al., 1986
Mice/C57BL/6N	0, 6, 9, 12, 15, or 18 μg/kg	10, 12	18	Increase in liver-to-body weight ratio and weight gain	Increase in cleft palate and hydronephrosis	Birnbaum et al., 1989
Mice/C57BL/6J	0 or 3 μg/kg (subcutaneous)	6-15	18	Increase in liver-to-body weight ratio	Increase in cleft palate and kidney anomaly	Courtney and Moore, 1971
Mice/C57BL/6J	20 μg/kg	10	17	Increase in liver-to-body weight ratio	Increase in cleft palate, hydronephrosis, and fetal body weight	Haake et al., 1987
Mice/C57BL/6J	0, 0.5, 1, 2, or 4 μg/kg	6-15	18	Increase in liver-to-body weight ratio	Increase in cleft palate, hydronephrosis, and fetal body weight	Silkworth et al., 1989
Mice/NMR	0.3, 3, 4, 5, or 9 μg/kg	6-15	18	NR	Increase in cleft palate and fetal mortality; decrease in fetal body weight	Neubert and Dillman, 1972
Mice/CF-1	0, 0.001, 0.01, 0.1, or 1.3 μg/kg	6-15	NR	None	Increase in cleft palate and hydronephrosis	Smith et al., 1976
Mice/DBA	0 or 3 μg/kg (subcutaneous)	6-15	18	Increase in liver-to-body weight ratio	Increase in cleft palate and kidney anomaly	Courtney and Moore, 1971
Mice/DBA	0, 0.5, 2, 4, or 8 μg/kg	6-15	18	Increase in liver-to-body weight ratio; decrease in thymus-to-body weight ratio	Increase in cleft palate and hydronephrosis	Silkworth et al., 1989

Table 5-2. Developmental toxicity following gestational exposure to 2,3,7,8-TCDD (continued)

Species/Strain	Daily dose[a]	Treatment days[b]	Sacrifice day[b]	Maternal effects[c]	Embryo/fetal effects[c]	Reference
Mice/CD-1	0, 1, or 3 μg/kg (subcutaneous)	6-15	17	None	Increase in cleft palate and kidney anomaly	Courtney and Moore, 1971
Mice/CD-1	0, 25, 50, 100, 200, or 400 μg/kg	6-15	17	Increase in liver-to-body weight ratio	Increase in cleft palate, hydronephrosis, and fetal mortality	Courtney, 1976
Rats/CD	0 or 0.5 μg/kg 2 μg/kg (subcutaneous)	6-15 9-10 or 13-14	20	None at 0.5 μg/kg NR at 2 μg/kg	Increase in kidney anomaly	Courtney and Moore, 1971
Rats/Sprague-Dawley	0, 0.125, 0.5, or 2 μg/kg	0-2	20	Decrease in weight gain	Decrease in fetal body weight	Giavini et al., 1982a
Rats/Sprague-Dawley	0.03, 0.125, 0.5, 2, or 8 μg/kg	6-15	21	Decrease in weight gain; toxicity	Increase in fetal mortality, resorptions, edema, and gastrointestinal hemorrhage	Sparschu et al., 1971
Rats/Wistar	0, 0.125, 0.25, 0.5, 1, 2, 4, 8, or 16 μg/kg	5-14	21	Toxicity	Increase in fetal mortality, edema and gastrointestinal hemorrhage; decrease in fetal weight	Khera and Ruddick, 1973
Guinea pigs/Hartley	0, 0.15, or 1.5 μg/kg	14	58	Increase in mortality; toxicity	Increase in fetal mortality	Olson and McGarrigle, 1990
Hamsters/Golden Syrian	0, 1.5, 3, 6, or 18 μg/kg	7, 9	15	Increase in liver-to-body weight ratio	Increase in fetal mortality, hydronephrosis, and renal congestion; decrease in thymus size	Olson and McGarrigle, 1990
Rabbits/New Zealand	0, 0.1, 0.25, 0.5, or 1 μg/kg	6-15	28	Decrease in weight gain; toxicity	Increase in fetal mortality and resorptions; extra ribs	Giavini et al. 1982b

Table 5-2. Developmental toxicity following gestational exposure to 2,3,7,8-TCDD (continued)

Species/Strain	Daily dose[a]	Treatment days[b]	Sacrifice day[b]	Maternal effects[c]	Embryo/fetal effects[c]	Reference
Monkeys/rhesus	0, 5, 25, 50, or 500 ppt 7 months before and during pregnancy	Chronic	--	Increase in mortality; toxicity	Increase in fetal mortality	Allen et al., 1979; Bowman et al., 1989
Monkeys/rhesus	0, 0.2[d], 1[d], 1[e], or 5[d] µg/kg	20-40	--	Increase in mortality; toxicity	Increase in fetal mortality	McNulty, 1985

[a]Oral exposure unless otherwise noted.
[b]All days adjusted to reflect plug day—gestation day 0.
[c]Effects reported are only those that were statistically significant.
[d]Cumulative dose divided into nine oral doses administered between days 20 and 40 of gestation; two to four monkeys/dose.
[e]Three animals given single oral dose, on either gestation days 25, 30, 35, or 40; 12 monkeys total.
NR = Not reported.

Source: Couture et al., 1990a.

Table 5-3. TCDD responsiveness of palatal shelves from the mouse, rat, and human in organ culture

Species	Molar concentration of TCDD		
	Induction of epithelial proliferation and prevention of epithelial-to-mesenchyme transformation		
	LOEL	EC_{100}	Cytotoxicity
Mouse	1×10^{-13}	5×10^{-11}	1×10^{-10}
Rat[a]	1×10^{-10}	1×10^{-8}	1×10^{-7}
Human[b]	5×10^{-11}	1×10^{-8}	1×10^{-7}

[a]At the highest concentration tested, 60% of the palatal shelves failed to undergo programmed cell death.
[b]One of four shelves responded by failing to undergo programmed cell death at 5×10^{-11} M.

Source: Birnbaum, 1991.

Table 5-4. Relative teratogenic potency of halogenated aromatic hydrocarbon congeners in C57BL/6 mice

Congener	Relative potency (ED_{50} TCDD/ED_{50} congener)	
	Cleft palate	Hydronephrosis
2,3,7,8-TCDD	1.000	1.000
2,3,7,8-TBDD	0.235	0.444
2,3,7,8-TBDF	0.100	0.333
2,3,4,7,8-PeCDF	0.095	0.057
2,3,7,8-TCDF	0.049	0.021
1,2,3,7,8-PeCDF	0.026	0.074
1,2,3,4,7,8-HxCDF	0.010	0.049
2,3,4,7,8-PeBDF	0.005	0.009
1,2,3,7,8-PeBDF	0.004	0.018
2,3,4,5,3′,4′-HxCB	0.0000287	0.0000894

Source: Weber et al., 1985; Birnbaum et al., 1987a,b, 1991.

Table 5-5. Cross-species comparison of NOAELs and LOAELs for TCDD developmental toxicity in fish

Species	Effect	Exposure	Egg dose	Effect level	Reference
Lake trout	Sac fry mortality	Static waterborne	34 ng/kg	NOAEL	Walker et al., 1991
Japanese medaka	Lesions[a]	Static waterborne	<100 ng/kg	NOAEL	Wisk and Cooper, 1990a
Rainbow trout	Sac fry mortality	Single injection	194 ng/kg	NOAEL	Walker et al., 1992
Lake trout	Sac fry mortality Sac fry mortality	Static waterborne Static waterborne	40 ng/kg 55 ng/kg	LOAEL LOAEL	Spitsbergen et al., 1991 Walker et al., 1991
Rainbow trout	Sac fry mortality	Single injection	291 ng/kg	LOAEL	Walker et al., 1992
Japanese medaka	Lesions[a]	Static waterborne	300 ng/kg	LOAEL	Wisk and Cooper, 1990a

[a]Consist of a spectrum of effects including hemorrhage in various areas, pericardial edema, collapse of the yolk sphere, cessation of blood flow throughout the animal, and embryo mortality.

Table 5-6. Cross-species comparison of NOAELs and LOAELs for TCDD developmental toxicity in birds

Species	Effect	Exposure	Egg dose	Effect level	Reference
Ring-necked pheasant	Embryo mortality	Single injection	100 ng/kg	NOAEL	Nosek et al., 1993
Eastern bluebird	Embryo mortality	Single injection	1,000 ng/kg	NOAEL	Thiel et al., 1988 Martin et al., 1989
Chicken	Cardiac malformations	Single injection	9 ng/kg[a]	LOAEL	Cheung et al., 1981a,b
Chicken	Embryo mortality	Single injection	240 ng/kg	LD_{50}	Allred and Strange, 1977
Ring-necked pheasant	Embryo mortality	Single injection	1,000 ng/kg	LOAEL	Nosek et al., 1993
Ring-necked pheasant	Embryo mortality	Single injection	1,354 ng/kg[b]	LD_{50}	Nosek et al., 1993
Ring-necked pheasant	Embryo mortality	Single injection	2,182 ng/kg[c]	LD_{50}	Nosek et al., 1993
Eastern bluebird	Embryo mortality	Single injection	10,000 ng/kg	LOAEL	Thiel et al., 1988

[a]Chi-square analysis of the data in Table 1 of Cheung et al. (1981b) demonstrated that the incidence of cardiac malformations in all embryos examined at dose levels of 1.6 pmol/egg or greater are significantly (p<0.05) increased compared to the incidence in the control group designated "all examined." Assuming a 55 g egg weight, 1.6 pmol/egg corresponds to a TCDD egg burden of 9 ng/kg.
[b]Injected into the egg albumin.
[c]Injected into the egg yolk.

Table 5-7. Cross-species comparison of NOAELs and LOAELs for TCDD developmental toxicity in mammals

Species	Effect	Exposure	Maternal dose	Effect level	Reference
Monkey	Prenatal mortality	Multiple dose	22 ng/kg, 9×, gd 20-40	NOAEL	McNulty, 1984
Rat	Prenatal mortality	1 ng/kg/day	27 ng/kg[a], chronic	NOAEL	Murray et al., 1979
Rat	Prenatal mortality	Multiple dose	30 ng/kg/day, gd 6-15	NOAEL	Sparschu et al., 1971
Mouse	Hydronephrosis	Multiple dose	100 ng/kg/day, gd 6-15	NOAEL	Smith et al., 1976
Mouse	Cleft palate	Multiple dose	300 ng/kg/day, gd 6-15	NOAEL	Neubert and Dillman, 1972
Monkey	Object learning	0.126 ng/kg/day	19 ng/kg[a], subchronic	LOAEL	Schantz and Bowman, 1989
Rat	Accelerated eye opening	Single	50 ng/kg, gd 15	LOAEL	Gray et al., 1997a
Rat	Reduced ejaculated sperm numbers	Single	50 ng/kg, gd 15	LOAEL	Gray et al., 1997a
Rat	Reduced ventral prostate weight	Single	64 ng/kg, gd 15	LOAEL	Mably et al., 1992a
Rat	Reduced cauda epididymal sperm numbers	Single	64 ng/kg, gd 15	LOAEL	Mably et al., 1992c
Rat	Partial feminization of sexual behavior (male)	Single	160 ng/kg, gd 15	LOAEL	Mably et al., 1992b
Rat	Vaginal thread malformation	Single	200 ng/kg, gd 15	LOAEL	Gray et al., 1997b
Rat	Hypospadias (female)	Single	200 ng/kg, gd 15	LOAEL	Gray et al., 1997b
Rat	Cleft phallus (female)	Single	800 ng/kg, gd 15	LOAEL	Gray et al., 1997b
Rat	Partial demascu-linization of sexual behavior (male)	Single	64 ng/kg, gd 15	LOAEL	Mably et al., 1992b
Monkey	Prenatal mortality	0.642 ng/kg/day Multiple dose	97 ng/kg[a], subchronic 111 ng/kg, 9×, gd 20-40	LOAEL LOAEL	Schantz and Bowman, 1989 McNulty, 1984
Rabbit	Extra ribs	Multiple dose	100 ng/kg/day, gd 6-15	LOAEL	Giavini et al., 1982b

Table 5-7. Cross-species comparison of NOAELs and LOAELs for TCDD developmental toxicity in mammals (continued)

Species	Effect	Exposure	Maternal dose	Effect level	Reference
Rat	Fetal growth	Multiple dose	125 ng/kg/day, gd 6-15	LOAEL	Sparschu et al., 1971
Rabbit	Prenatal mortality	Multiple dose	250 ng/kg/day, gd 6-15	LOAEL	Giavini et al., 1982b
Rat	Prenatal mortality	10 ng/kg/day Multiple dose	270 ng/kg[a], chronic 500 ng/kg/day, gd 6-15	LOAEL LOAEL	Murray et al., 1979 Sparschu et al., 1971
Mouse	Hydronephrosis	Multiple dose	500 ng/kg/day, gd 6-15	LOAEL	Silkworth et al., 1989
Guinea pig	Prenatal mortality	Single dose	1,500 ng/kg, gd 14	LOAEL	Olson and McGarrigle, 1992
Hamster	Thymic hypoplasia	Single dose	1,500 ng/kg, gd 7 or 9	LOAEL	Olson and McGarrigle, 1992
Mouse	Cleft palate	Multiple dose	3,000 ng/kg/day, gd 6-15	LOAEL	Courtney and Moore, 1971
Hamster	Prenatal mortality	Single dose	18,000 ng/kg/day, gd 7 or 9	LOAEL	Olson et al., 1990
Mouse	Prenatal mortality	Single dose	24,000 ng/kg/day, gd 6	LOAEL	Couture et al., 1990b

[a]Maternal body burdens of TCDD at the time of conception were calculated by assuming a one-compartment open model and half-life for whole body TCDD elimination of 400 days in the monkey (McNulty et al., 1982) and 23.7 days in the rat (Rose et al., 1976). A bioavailabilty of 86.1 percent was used in the monkey and rat (Rose et al., 1976). The daily dietary exposure levels in rhesus monkeys were approxiately 5 and 25 ppt at the NOAEL and LOAEL doses, respectively. Rhesus monkeys were exposed to these levels of TCDD for 7 months prior to conception. At this time (0.525 half-lives) the cumulative amount of TCDD in rhesus monkeys was 30.5 percent of the calculated steady-state level. Rats were exposed to the indicated daily doses of TCDD for a period of 90 days (3.8 half-lives) prior to conception. At this time the cumulative amount of TCDD in rats was 92.8 percent of the calculated steady-state level.
gd=gestational day.

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