DISSERTATION

CHARACTERIZATION OF THE EFFECTS OF DIBUTYL PHTHALATE ON GROWTH AND MALE REPRODUCTION IN FROGS AND RABBITS

Submitted by

Ty T. Higuchi

Department of Physiology

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TY T. HIGUCHI ENTITLED CHARACTERIZATION OF THE EFFECTS OF DIBUTYL PHTHALATE ON GROWTH AND MALE REPRODUCTION IN FROGS AND RABBITS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Adviser Veeramachaneni

Department Head

ABSTRACT OF DISSERTATION

CHARACTERIZATION OF THE EFFECTS OF DIBUTYL PHTHALATE ON GROWTH AND MALE REPRODUCTION IN FROGS AND RABBITS

Dibutyl phthalate (DBP), used extensively in consumer and industrial products, is known to adversely affect development and reproduction in rodents. Because DBP could be potentially ubiquitous and that studies using amphibians or mammalian animal models that closely mimic events in human reproductive development (e.g., rabbits) have not been performed, we studied its effects on growth and male reproduction in *Xenopus laevis* and Dutch-Belted rabbits.

Frogs were exposed from gastrulation to 12 wk of life to 0.1, 0.5, 1, 5, 10, or 15 ppm DBP in 0.01% DMSO and DMSO alone or FETAX solution only (controls). Cumulative mortality rates for FETAX solution, DMSO, 0.1, 0.5, 1, 5, 10, and 15 ppm DBP were 9, 6, 5, 6, 44, 30, 59, and 100% at 1 wk and 35, 32, 29, 38, 60, 76, 98% by 68 wk. The incidence of malformations was increased in 5, 10 and 15 ppm DBP groups. Metamorphosis, a thyroid-dependent event, was delayed in 1, 5, and 10 ppm DBP groups. Larynx, a secondary sexual organ required for mating calls, was regressed in 1 and 5 ppm DBP. Reflective of this change, the percentage of time producing the mating call and amplexus response were decreased. Furthermore, deleterious changes in spermiogenesis and structure of excurrent ducts were observed.

Rabbits were exposed orally to 0 or 400 mg DBP/kg/day during gestation (gestation days 15-29), adolescence (post-natal weeks 4-12), or after puberty (for 12 wk). Male reproductive function was compromised to varying degrees in all DBP-exposed groups. The most pronounced effects were in rabbits exposed *in utero* where severe malformations of reproductive tract and poor semen quality were observed. In all DBP exposures, incidence of morphologically normal sperm was decreased and occasional atypical germ cells resembling carcinoma *in situ* of the testis were observed. In adolescent exposure group, the hypothalamic-pituitary-gonadal axis was affected and thyroid hormone concentrations were elevated.

Collectively, these results indicate that: DBP is detrimental to frogs at relatively low concentrations; DBP affects non-rodent mammals as well as rodents; young animals are more susceptible to DBP; and DBP-mediated toxicity may involve disruption of thyroid hormone-, as well as androgen-dependent cascades.

Ty T. Higuchi
Department of Physiology
Colorado State University
Fort Collins, CO 80523
Fall, 2002

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DEDICATION

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ABBREVIATIONS

AAALAC	Association for assessment and accreditation of laboratory animal care
AI	Artificial insemination
6-AN	6-aminonicotinomide
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CIS	Carcinoma *in situ*
CPM	Counts per minute
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di-(2-ethylhexyl) phthalate
DES	Diethylstilbestrol
DGEL	Degree of germinal epithelial loss
DHBA	Dihydroxybenzoic acid
DHT	Dihydrotestosterone
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DPP	Dipentyl phthalate
DSP	Daily sperm production
EC_{50}	Effective concentration to induced 50% malformations
EPA	Environmental protection agency
ESR	Epididymal sperm reserve
FETAX	Frog embryo teratogenesis assay – *Xenopus*
FSH	Follicle stimulating hormone
g	Gram(s)
GD	Gestation day
GnRH	Gonadotropin-releasing hormone
H&E	Hematoxylin and eosin staining
hCG	Human chorionic gonadotropin
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
hr	Hour(s)
Hz	Hertz(s)
IT	Incubator temperature (24 ± 2°C)
IU	International unit(s)
kg	Kilogram(s)
L	Liters(s)
LC_{50}/LD_{50}	Lowest concentration/dose to cause 50% mortality
LH	Luteinizing hormone

LOAEL	Lowest-observable-adverse-effects-level
M	Mole(s) per liter
m^3	Cubic meter
MBP	Monobutyl phthalate
MCIG	Minimum concentration to inhibit growth
MEHP	Mono-(2-ethylhexyl) phthalate
μg	Microgram(s)
mg	Milligram(s)
ml	Milliliter(s)
μl	Microliter(s)
μM	Micromole(s) per liter
mM	Millimole(s) per liter
MIS	Mullerian inhibiting substance
MPS	Morton's pickling salt
ng	Nanogram(s)
NIOSH	National institute for occupational safety and health
NOAEL	No-observable-adverse-effects-level
NOEL	No-observable-effects-level
ppb	part(s) per billion
ppm	part(s) per million
PND	Post-natal day
PNW	Post-natal week
PPAR	Peroxisome proliferator activated receptor
PTU	6-*n*-propyl-2-thiouracil
RAR	Retinoic acid receptor
RIA	Radioimmunoassay
RR	Relative risk
StAR	Steroid acute regulatory protein
T_2	3,5 diiodothyronine
T_4	3,5,3',5' tetraiodothyronine, thyroxine
T_3	3,5,3' triiodothyronine
rT_3	3,3',5' triiodothyronine, reverse triiodothyronine
TI	Teratogenic index
TPO	Thyroid peroxidase
TR	Thyroid receptor
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
VDR	Vitamin D_3 receptor
UVB	Ultraviolet-B radiation
RfD	Reference dose
RT	Room temperature (19-21°C)
RXR	9-cis-retinoic acid receptor
wk	Week(s)

CHAPTER 1

GENERAL INTRODUCTION

Several studies report deterioration in reproductive function in the human male starting in the 1940's continuing through the present day (Adami *et al.*, 1994; Carlsen *et al.*, 1992; Power *et al.*, 2001; Swan *et al.*, 2000). It is likely that a similar trend exists in domestic animals, but data are not available due to the nature of animal husbandry practices – culling of reproductively inefficient food- and fiber- producing animals or castration at an early age of pet animals (Veeramachaneni, 2001a). There have also been reports of amphibian populations declining since the late 1950's (Houlahan *et al.*, 2000) with a recent increase in the incidence of reported field malformations – extra, missing, or malformed limbs. (Northern Prairie Wildlife Research Center.1997.North American Reporting Center for Amphibian Malformations, 2002). It is not known whether these trends are due to anthropogenic or environmental stresses.

The post World War II era brought a rapid development in the synthetic chemical industry leading to an increased use of several agrochemical and industrial products (reviewed by Autian, 1973; Ecobichon, 1996). Many of these chemicals persist in the environment and are capable of mimicking hormones and thus can disrupt normal endocrine and reproductive function (Colborn *et al.*, 1993; Jensen *et al.*, 1995; Sharpe and Skakkebaek, 1993). Phthalates used as plasticizers to produce polymeric materials and a variety of consumer products were first invented in the 1930's (Houlihan and Wiles, 2000). Since that time there has been steady increases in the number of

applications and production rates (Autian, 1973). Currently, they are among the highest-volume chemicals produced with an estimated 2-8 million tons per annum (WHO, 1992). Environmental contamination or exposure occurs as a result of the manufacture, use, disposal, by leaching out of plastic products or directly through one of the numerous consumer products (cosmetics, pharmaceuticals, etc.) (Autian, 1973; Jaeger and Rubin, 1970; 1972; Perwack *et al.*, 1981). One form of phthalates, dibutyl phthalate (DBP) has been found in the sediment, water, air, biota, and even stored in foods (Aurela *et al.*, 1999; Bove *et al.*, 1978; Giam *et al.*, 1980; Petersen and Breindahl, 2000). In a recent study, urine samples from a human reference population of 289 adults contained 7 to 294 ng/ml monobutyl phthalate (MBP) reflecting chronic exposure to higher levels of DBP (Blount *et al.*, 2000).

DBP is known to be a developmental and reproductive toxicant. In rodents, teratogenic response to DBP was observed only at maternally toxic doses (~1 g DBP/kg/day) from gestation day (GD) 6 to 15 (Ema *et al.*, 1993; 1994; 1995). This exposure window does not include the period of sexual differentiation, and when treatment was extended to include this critical period of reproductive development (GD 12-21) marked effects on male rats were observed at 100-750 mg DBP/kg/day. The macroscopic sequelae included: testicular atrophy, multinuclear gonocytes, Leydig cell hyperplasia, reduced anogenital distance, malformed epididymis and vas deferens, hypospadias, cryptorchidism, and retained thoracic nipples (Foster *et al.*, 2001; Mylchreest *et al.*, 1998; 1999; 2000). In spite of the well-characterized spectrum of effects caused by phthalates, the mechanism of phthalate-mediated reproductive toxicity is unclear. Many hypothesized this chemical acts as an anti-androgen, by disrupting

androgen signaling (Foster *et al.*, 2001; Gray *et al.*, 1999; 2001; Mylchreest *et al.*, 1998; 1999; 2000) or decreasing fetal testosterone levels (Mylchreest *et al.*, 2002; Parks *et al.*, 2000), yet no conclusive data exist.

Considerable attention has been given to the teratological and reproductive toxicity of DBP. However, a majority of the studies investigating these endpoints used rodents in standard teratology or acute reproductive toxicology studies at high doses. Virtually nothing is known about the teratological or reproductive effects of phthalates in amphibians. Furthermore, studies on the effects of DBP on gametogenesis at critical periods of development and in adult life using a non-rodent-mammalian animal model such as the rabbit that mimics human reproductive development and facilitates longitudinal evaluation of seminal parameters have not been performed. Therefore, the overall objective of this study was to characterize the effects of DBP on growth and male reproduction using African clawed frogs and Dutch-Belted rabbits as animal models.

CHAPTER 2

LITERATURE REVIEW

2.1 Phthalates

Phthalates, diesters of 1,2-benzenedicarboxylic acid, were invented in the 1930's (Houlihan and Wiles, 2000). A diester is formed by reacting phthalic anhydride with a specific alcohol (Autian, 1973). These ubiquitous chemicals are used in a wide range of applications – adhesives, plastics, aerosols, cosmetics, and pharmaceuticals – where they may constitute 0.1 to 50% of the total weight (Autian, 1973; Brandt, 1985; Houlihan and Wiles, 2000). The worldwide production of phthalates is estimated to be 2-8 million tons per year (WHO, 1992). Environmental contamination occurs as a result of production, disposal and transportation (reviewed by ASTDR, 1990).

The toxicities of phthalates have been extensively studied over the past 50 years. However, a vast majority of the studies deal with di-(2-ethylhexyl) phthalate (DEHP). Recently it was found that the level of DBP, a reported developmental and reproductive toxicant in rodents, was higher than previously anticipated in a human reference population (Blount *et al.*, 2000).

2.2 Dibutyl Phthalate

The chemical and physical properties of DBP can be found in Appendix A. DBP is used in a variety of industrial and consumer products, by far the largest use is as plasticizing agent (Autian, 1973). Other products include: cosmetics, pharmaceuticals, adhesives, paints, glue, insect repellant, and rocket fuel (ASTDR 1990; Autian, 1973;

Brandt, 1985; Houlihan and Wiles, 2000). The number of applications and production of DBP have steadily increased since World War II (Autian, 1973).

2.2.1 Environmental Contamination and Exposure

2.2.1.1 Environmental Contamination

Phthalates are dispersed in the matrix of plastics to impart flexibility. Therefore, they are not chemically bound to the final product (Autian, 1973) and easily leach into the surrounding medium (Jaeger and Rubin, 1970; 1972). This phenomenon was originally reported for plastic blood storage bags that leached DEHP into the surrounding medium resulting in the exposure of at least two blood transfusion patients (Jager and Rubin, 1970; 1972). One study determined that 1.7 to 4.2 mg DEHP/kg and 0.2 to 0.7 mg mono-(2-ethylhexyl) phthalate (MEHP)/kg were unwittingly transfused to newborn infants (Sjoberg *et al.*, 1985). The migration coefficient of DEHP from hemodialysis tubes into plasma was determined to be 7.7 μg/ml/hr (Nassberger *et al.*, 1987).

Environmental contamination occurs as a result of production, disposal and transportation (reviewed by ASTDR, 1990). It is estimated that 6 million kg of DBP was disposed into landfills, 0.2 million kg incinerated, and 0.6 million kg released into water in 1977 (Perwack *et al.*, 1981). It was determined that in 2000, the total environmental release of DBP for all industrial facilities in the United States was ~129,642 kg (TRI, 2002).

Global distribution in the air was determined to be 1 to 5.7 ng/m^3 (Bove *et al.*, 1978; Giam *et al.*, 1980). Across the United States, the level of DBP in rivers and canals ranges from 0.5 to 0.15 μg/L, while levels for drinking water from 10 cities is 0.1 to 5

first-morning voids from a human reference population of 289 adults (Blount *et al.*, 2000). DBP had one of the highest levels (mean = 41.5 ng/ml, 36.9 μg/g creatinine) of the corresponding monoester, MBP in the urine (range 2.2 to 294 ng/ml, 1.6 to 276 μg/g creatinine) and the greatest levels of MBP were found in women of childbearing age (20-40 years). In another study, serum concentrations of DBP ranging from 15 to 276 μg/L were detected in 28 of 41 (68%) of females (6 months to 8 years of age) diagnosed with premature thelarche (a physical change characteristic of puberty before age 8) versus none in controls (Colon *et al.*, 2000).

2.2.2 Toxicology

2.2.2.1 Mortality

Mortality is observed following acute exposure to DBP by oral, dermal and intraperitoneal routes (ASTDR, 1990; Brandt, 1985). The acute toxicity for DBP is considered to be low, since values for the lethal dose causing 50% mortality (LD_{50}) for experimental animals is greater than 3 g DBP/kg for oral, dermal and intraperitoneal routes of exposure (ASTDR, 1990; Autian, 1973; Brandt, 1985; WHO, 1997) (Table 1).

2.2.2.2 Toxicokinetics

A. Absorption

DBP is readily absorbed by dermal, inhalation, or oral routes of exposure (reviewed by ASTDR, 1990; WHO, 1997). In rats exposed to 157 μM DBP/kg on a shaved area on their back, 50-60% of the dose was recovered in the urine and feces after one wk, and ~33% of the dose remained at the application site (Elsisi *et al.*, 1989). Rats chronically exposed to 50 mg/m^3 DBP for various periods of time had DBP in the brain, lung, liver, and testis (reviewed by WHO, 1997). Furthermore, after 6 months of chronic

TABLE 1
Acute effects of DBP on mortality

Route of Exposure	Animal	LD_{50}
Oral	Mouse	5 to 16 g/kg
	Rat	8 to 23 g/kg
	Rabbit	...
Dermal	Mouse	...
	Rat	...
	Rabbit	20.86 g/kg
Intraperitoneal	Mouse	3.13 to 6.26 g/kg
	Rat	4.17 to 7.30 g/kg
	Rabbit	...

Adapted from ASTDR, 1990; Autian, 1973; Brandt, 1985; WHO, 1997

exposure to 4.4 ppm DBP, there were relatively low concentrations of DBP in the lungs suggesting DBP was rapidly absorbed (reviewed by ASTDR, 1990). The gastrointestinal tract rapidly absorbs DBP. Following oral exposure to 0.27 or 2.31 g [^{14}C] DBP/kg, 87.6 and 61.3% of the dose was recovered in urine and feces by 24 hr, respectively (Williams and Blanchfield, 1975). At 48 hr, these values were 94.8 and 87.5%, respectively. Similarly, pregnant rats administered 0.5 or 1.5 g [^{14}C] DBP/kg on GD 14 had 84.3 and 54.6% of the doses recovered at 24 hr and 90.0 and 77.0% by 48 hr (Saillenfait *et al.*, 1998). The later recovery of DBP in the higher doses for both studies was attributed to a slower rate of absorption from the gastrointestinal tract (Saillenfait *et al.*, 1998; Williams and Blanchfield, 1975).

Bulk of DBP ingested is rapidly metabolized to MBP by intestinal esterases and absorbed; therefore a majority of the toxic effects are governed by the monoester and other metabolites (Lake *et al.*, 1977).

B. Distribution

Following absorption, DBP is rapidly distributed throughout the body with the highest concentrations found in the lung, kidney, testis, and liver (reviewed by ASTDR, 1990). DBP does not accumulate in any organs. There was no accumulation of DBP or MBP in tissues or organs after rats were fed a diet containing 1 g [^{14}C] DBP/kg for 12 wk (Williams and Blanchfield, 1975). DBP is transferred to the fetus. Following oral administration of 5 or 1.5 g [^{14}C] DBP/kg to pregnant rats on GD 14 only 0.12 to 0.15% of the total dose was transferred to embryonic tissues (Saillenfait *et al.*, 1998).

C. Metabolism

The primary metabolites identified in urine are MBP-glucuronide> unconjugated MBP>various oxidative products of MBP>DBP (Albro and Moore, 1974; Foster *et al.*, 1983; Lake *et al.*, 1977; Williams and Blanchfield, 1975) (Figure 1). Experimentally, phthalates are metabolized in rats, baboons and ferrets (Lake *et al.*, 1977) and the metabolism of phthalate esters is unaffected by the route of administration (Kluwe, 1982). Some species-difference in metabolic pathway has been reported (Foster *et al.*, 1983). Whereas rats typically excrete unconjugated MBP in the urine, hamsters have substantially lower levels of unconjugated MBP and high levels of MBP-glucuronide, indicating an increased activity of glucuronidase compared to rats (Foster *et al.*, 1983). This difference in metabolism may account for species differences in toxic responses (Foster *et al.*, 1983).

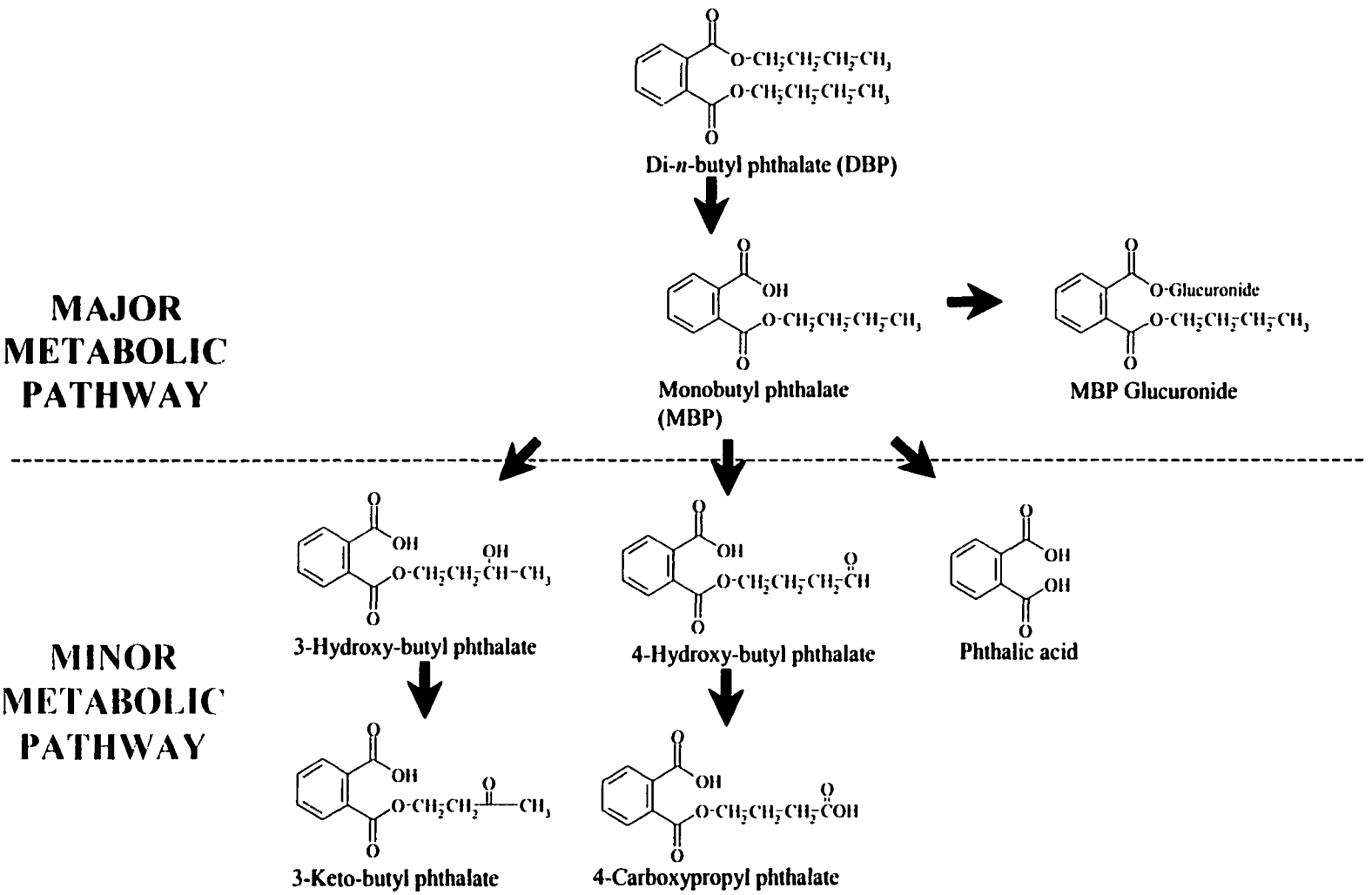


FIG. 1. Metabolic pathway of DBP. Adapted from Albro and Moore, 1974; Foster *et al.*, 1983; Lake *et al.*, 1977; Williams and Blanchfield, 1975.

2.2.2.3 Genotoxic and Carcinogenic Effects

A. Genotoxic Effects

A majority of the evidence available for the genotoxic effects of DBP from *in vitro* studies indicates it is not genotoxic. DBP alone induced a small dose-dependent increase in the mutational activity in various *Salmonella typhimurium* strains. However, when DBP is incubated with Aroclor-induced S-9 liver fractions, which metabolizes DBP to MBP, no mutational activity was detected. Numerous other *in vitro* tests were negative for chromosomal aberrations and transformations (reviewed by ASTDR, 1990; WHO, 1997).

Some *in vivo* studies support the *in vitro* data, while others do not. There is no phthalate-induced oxidative DNA damage, as evidenced by the unchanged 7,8-dihydro-8-oxo-2'-deoxyguanosine levels in liver, kidney, and testis (Wellejus *et al.*, 2002). However, in human mucosal cells and lymphocytes, DBP induced significant increases in DNA strand breaks (Kleinsasser *et al.*, 2001). The relevance of the genotoxicity observed *in vitro* and *in vivo* with DBP is questionable (WHO, 1997), since DBP is rapidly metabolized to MBP irrespective of the route of exposure (Kluwe, 1982).

B. Carcinogenic Effects

Although, no tumors have been observed in long-term rodent bioassays (WHO, 1997), the carcinogenic potential of DBP has not been studied (ASTDR, 1990; WHO, 1997).

2.2.2.4 Organ Effects

A. Liver

Hepatomegaly with severe alterations in liver function has been observed in rats orally administered DBP (Murakami *et al.*, 1986a; 1986b; NTP, 1995). Rats exposed to 250 or 2500 mg DBP/kg/day for 34 to 36 days have decreased mitochondrial respiration with a reduction in glutamate dehydrogenase, succinate dehydrogenase, and pyruvate dehyrodogenase activity. In another study, rats treated with 1413 or 2923 mg DBP/kg/day for 13 wk had clinical chemistry indicative of cholestasis (hypocholesterolemia, hypotriglyceridemia, increased levels of alkaline phophatase activity and concentration of bile acids) (NTP, 1995). Ultrastructural changes revealed degenerative processes associated with single cell and zonal necrosis; proliferation of lysosomes, peroxisomes and mitochondria; and deposition of lipofuchsin in the cytoplasm (Murakami *et al.*, 1986a; 1986b). Similar results were observed in mice treated with 812, 1601, or 3689 mg DBP/kg/day for 13 wk (NTP, 1995).

Similar to the hepatic effects of DBP, rats treated with DEHP had hepatomegaly with alterations in liver function. However, an increased incidence of hepatocellular carcinoma was observed in DEHP-exposed animals (NTP, 1982; Rao *et al.*, 1990). The incidence of hepatocellular carcinoma in rats fed a diet containing 20 g DEHP/kg for 108 wk was 78% compared to 10% in controls (Rao *et al.*, 1990). Furthermore, rats treated with 3 or 6 g DEHP/kg/day for 103 wk had an increased incidence of hepatocellular carcinoma with 35% metastasizing to the lungs (NTP, 1982).

The mechanism of DEHP-induced hepatocellular carcinoma has been characterized. Following DEHP exposure, proliferation of hepatic peroxisomes causes an

increase in fatty acid metabolism (reviewed by ASTDR, 1993; WHO, 1992). This effect is mediated by peroxisome proliferator-activated receptor α (PPARα), since phthalates bind to PPARα (Hasmall *et al.*, 1999) and PPARα-null mice do not have DEHP-induced lesions of the liver (Lee *et al.*, 1995, Ward *et al.*, 1998). DEHP exposure also decreases the levels of enzymes that detoxify the by-products of fatty acid metabolism (catalase, glutathione peroxidase) and other reactive oxygen species (superoxide dismutase, glutathione-S-transferase). Collectively, these DEHP-induced hepatic effects led to an increase in membrane damage and 8-hydroxydeoxy-guanosine levels (reviewed by ASTDR, 1993; WHO, 1992) and the damage is promoted by stimulation of mitosis and suppression of apoptosis (reviewed by Roberts *et al.*, 1997).

B. Kidney

Following exposures to DBP, increases in kidney weight have been reported by some (Murakami *et al.*, 1986a) but not by others (Nikonorow *et al.*, 1973). Rats fed a diet containing 5% DBP or MBP for 34 to 36 days had a significant decrease in kidney weight (Murakami *et al.*, 1986a), while rats exposed to 120 or 1200 mg DBP/kg/day for 120 days had no changes in kidney weight or histological features (Nikonorow *et al.*, 1973). No alterations in urine analysis (albumin, hippuric acid or sediment) were observed in rats fed a diet containing the equivalent of 10 mg DBP/kg/day for 7 months (WHO, 1997).

C. Thyroid

In a chronic exposure study, rats treated with 1000 mg DEHP/kg/day had alterations in thyroid structure consistent with acinar hyperactivity (increased lysosomes and dilation and hypertrophy of the Golgi apparatus) (Price *et al.*, 1988). At this time

there was a decrease in serum thyroxine (T_4) levels that persisted for 9 months, yet levels of triiodothyronine (T_3) were unaffected. Significant levels of phthalates were found in the environment, where a high incidence of human endemic goiter was detected (Kentucky and western Columbia) (Gaitan, 1989) indicating that there may be a direct effect of phthalates on thyroid function. It is speculated that this may be a result of degradation of the parent phthalate into dihydroxybenzoic acid (DHBA), a compound that may block I^{131} uptake and is a potent inhibitor of thyroid peroxidase (TPO) (Gaitan, 1989). Supporting animal studies have not been performed yet.

2.2.2.5 Developmental Effects

DBP has been shown to be toxic to developing fetuses in a number of animal studies (Bower *et al.*, 1970; Ema *et al.*, 1993; 1994; 1995; 1997; Hardin *et al.*, 1987; Lamb *et al.*, 1987; Nikonorow *et al.*, 1973; Singh *et al.*, 1972). Chick embryos injected with a single dose of 520 or 104 mg DBP at 72 hr had 67 and 79% mortality (Bower *et al.*, 1970). The surviving chicks had no gross defects. Pregnant mice treated with 600 mg DBP/kg/day from GD 0-21 had an increase number of resorptions (Nikonorow *et al.*, 1973). Similarly, intraperitoneal injections of 0.61 or 1.017 ml DBP/kg to pregnant rats on GD 5, 10 and 15 increased the number of resorptions and incidence of skeletal malformations while decreasing the number of live fetuses (Singh *et al.*, 1972). Administration of 2500 or 4000 mg DBP/kg to pregnant mice from GD 6-13 caused 10 and 54% mortality and no viable litters in surviving animals (Hardin *et al.*, 1987). A continuous breeding study for 98 days determined that diets containing 1.0% DBP reduced the number of litters per pair (1.8), live pups per litter (1.7), and the proportion of pups born alive (0.5) compared to controls (4.9, 12 and 1.0) (Lamb *et al.*, 1987).

Pregnant rats treated with 0.63 or 0.75 g DBP/kg/day from GD 7-15 had a significant increase in the post-implantation loss and decrease in the body weight gain during pregnancy (Ema *et al.*, 1993). At 1.0 g DBP/kg/day, maternal death (18%) and complete resorption of all implanted embryos was observed in surviving animals. A single dose of 2500 mg DBP/kg from GD 6, 8-10 or 12-16 increased the incidence of postimplantation loss by GD 20 (Ema *et al.*, 1997).

Numerous studies indicate DBP is teratogenic (Ema *et al.*, 1993; 1994; 1995; 1996; 1997; Saillenfait, 2001; Shiota, 1980). GD 18 fetuses from mice administered 1.0% DBP in the diet from GD 0-18 showed a borderline increased (p=0.05) incidence of neural tube defects (exencephaly and myeloschisis) (Shiota, 1980) A significant increase in the incidence of cleft palate was observed in offspring from rats administered 0.75 g DBP/kg/day from GD 7-15 (Ema *et al.*, 1993). However it was uncertain if the increased incidence of cleft palate was a result of maternal toxicity since weight gain of pregnant animals was decreased, and decreased weight gain alone has been associated with an increased incidence of cleft palate (Hemm *et al.*, 1977). GD 20 fetuses from rats exposed to 0.75 or 1.0 g DBP/kg from GD 7-9 had an increased incidence (32%) of deformities of the cervical and thoracic vertebral column and ribs, while treatment from GD 13-15 increased the incidence (63%) of cleft palate and fusion of the sternebrae (Ema *et al.*, 1994). The production of these teratogenic effects may be the result of the metabolism of DBP to MBP, since identical results were obtained using lower levels of MBP (625 and 750 mg MBP/kg) (Ema *et al.*, 1995; 1996). Even a single administration of 1500 mg/ DBP/kg on GD 8, 9, or 15 increases the incidence of malformations in GD 20 fetuses

(Ema *et al.*, 1997). Likewise, decreased growth and an increased incidence of malformations were observed in GD 12 fetuses following a single administration of 3.6 mM DBP or MBP/kg on GD 10 (Saillenfait *et al.*, 2001).

2.2.2.6 Reproductive Effects

DBP has adverse effects on the adult male reproductive system in some animal species (rats, guinea pigs, and mice) but not others (hamsters) (Gray *et al.*, 1982). Administration of 2 g DBP/kg/day for 7 to 9 days induced severe atrophy of seminiferous epithelium in rats and guinea pigs, focal atrophy in mice and no changes in hamsters (Gray *et al.*, 1982). Administration of 2 g DBP/kg/day to adult rats for 4 days decreased the testicular weights and caused severe atrophy of the seminiferous epithelium with complete loss of spermatocytes and spermatids (Cater *et al.*, 1976; 1977; Foster *et al.*, 1980). Adult rats fed a diet containing 2% DBP had decreased testicular weights and marked reduction in spermatogenesis with desquamation of spermatocytes 7 days after the initiation of treatment (Oishi and Hiraga, 1980). However, the adverse effects of DBP on adult testis appear to be in part reversible. Discontinuation of treatment for two to three weeks following administration of 2.4 g DBP/kg/day for 7 days led to partial regeneration of the seminiferous epithelium and active spermatogenesis in most tubules (Tanino *et al.*, 1987). However, vacuolization of seminiferous epithelium was still observed.

Numerous studies indicate phthalate-mediated reproductive toxicity in adult animals is caused by damage to Sertoli cells. A single dose of 2200 mg dipentyl phthalate (DPP)/kg to adult rats caused ultrastructural changes in smooth endoplasmic reticulum, mitochondria, ectoplasmic specializations and cell junctions in Sertoli cells with shedding

of morphologically normal germ cells (Creasy *et al.*, 1983, 1987; Foster *et al.*, 1982). When treatment with DPP was continued for three days the production of seminiferous tubule fluid and androgen binding protein were decreased (Gray and Gangolli, 1986). Phthalates also affect Sertoli cells *in vitro*. MEHP, MBP and monopentyl phthalate but not monoethyl, monomethyl or monopropyl phthalates inhibit follicle stimulating hormone (FSH)-stimulated cyclic AMP (cAMP) accumulation in Sertoli cells (Heindel and Chapin, 1989; Heindel and Powell, 1992).

Many metabolic pathways in the testis are affected by DBP. Oral administration of 2 g DBP/kg/day to adult rats for 4 days increased the urinary zinc excretion and reduced the testicular zinc content (Cater *et al.*, 1976; 1977; Foster *et al.*, 1980), while zinc supplementation protects the testis from the effects of DBP (Cater *et al.*, 1977). Zinc is known to be an essential element for the maintenance of normal testicular function and any metabolic alteration leads to testicular atrophy (reviewed by Foster *et al.*, 1980). Decreased succinate dehydrogenase and iron levels and increased lactate dehyrodogenase have been observed following acute DBP exposure (Fukuoka *et al.*, 1989; 1990; 1993; 1994; 1995; Zhou *et al.*, 1990).

Damaged Leydig cells also contribute to phthalate-mediated reproductive toxicity in adult animals. Administration of 2 g DEHP/kg to adult male rats for two days caused mitochondrial swelling and focal dilation and vesiculation of smooth endoplasmic reticulum (Jones *et al.*, 1993). *In vitro*, Leydig cells treated with 1000 μM MEHP have decreased luteinizing hormone (LH)-stimulated testosterone secretion (Jones *et al.*, 1993).

Limited data exist on the effect of DBP on the female reproductive system. Female mice fed a diet containing 1.0% DBP for 105 days and mated with untreated males had a significant decrease in the percentage of fertile pairs, number of live pups per liter, proportion of pups born alive and live pup weight compared to controls indicating female reproduction is altered by DBP (Lamb *et al.*, 1987). However, no adverse effects on estrous cyclicity were observed in adult female rats fed a diet containing 1.0% DBP for 14 wk (Wine *et al.*, 1997). Pseudopregnant rats exposed to 2000 mg DBP/kg/day from GD 0-8 had no change in decidual cell response compared to controls, (Cummings and Gray, 1987). Similarly, treatment with DBP in ovariectomized rats had no effect on uterine weight, vaginal cell cornification or lordosis behavior (Gray *et al.*, 1999; Zacharewski *et al.*, 1998). However, 1000 mg MBP/kg/day administered to pseudopregnant rats from GD 0-8 significantly decreased uterine decidualization (Ema *et al.*, 2001).

Continuous breeding of Sprague-Dawley rats provided strong evidence that DBP induced a more marked effect on the reproductive system of F_1 offspring than the F_0 parental generation (Wine *et al.*, 1997). No adverse effects (sperm counts, estrous cyclicity) were identified in both sexes of the F_0 parental generation fed a diet containing 1% DBP throughout a 14 wk continuous breeding period. However, in the F_1 males epididymal and testicular sperm head counts were decreased by ~50% compared to controls and histopathological evaluation of F_1 males revealed 8 out of 10 had degeneration of the seminiferous epithelium and 5 out of 10 had underdeveloped or defective epididymides. No lesions in female reproductive tract were observed (Wine *et al.*, 1997). This observation has been confirmed by several studies (Foster *et al.*, 2001;

Mylchreest *et al.*, 1998; 1999; 2000) where male offspring exposed to 500 or 750 mg DBP/kg/day from GD 12-21 had severe alterations – decreased anogenital distance, missing or underdeveloped epididymis, hypospadias, missing prostate and seminal vesicles, cryptorchidism, Leydig cell hyperplasia, testicular atrophy and germ cell exfoliation. These effects were irreversible and at PND100 the weights of the paired testes, epididymides, seminal vesicle and prostate were significantly reduced (Mylchreest *et al.*, 1998). Closer examination of the testis on GD 21 revealed multinuclear gonocytes (Mylchreest *et al.*, 2002). The no-observable-adverse-effects-level (NOAEL) and lowest-observable-adverse-effects-level (LOAEL) for androgen-dependent alterations of the male reproductive tract is 50 and 100 mg DBP/kg/day, respectively (Mylchreest *et al.*, 2000).

Studies into the mechanism of phthalate-mediated toxicity on sexual differentiation and the developing reproductive system led many to hypothesize that this chemical acts as an anti-androgen (Foster *et al.*, 2001; Gray *et al.*, 1999; 2001; Mylchreest *et al.*, 1998; 1999; 2000), since many of the effects of DBP on the developing male reproductive tract were similar to the reported effects of flutamide, a potent androgen receptor antagonist (Imperato-McGinley *et al.*, 1992). However, flutamide and DBP disrupt androgen-dependent development with a different pattern of effects. Whereas exposure to flutamide causes high incidence of prostate agenesis, hypospadias with vaginal pouch, and inguinal testes, exposure to DBP causes low incidence of prostate agenesis, hypospadias with no vaginal pouch, and intraabdominal testes (Mylchreest *et al.*, 1999). Furthermore, neither DBP nor MBP interact directly with the androgen receptor *in vitro* (Foster *et al.*, 2001) or *in vivo* (Paganetto *et al.*, 2000). This prompted investigation into

the effects of DBP on fetal testosterone synthesis. Treatment with 500 mg DBP/kg/day from GD 12-21 significantly decreased testicular testosterone concentrations by reducing the expression of steroidogenic enzymes – cytochrome P450 side chain cleavage, cytochrome P450c17 and steroid acute regulatory protein (StAR), while inducing Leydig cell hyperplasia by up-regulating the cell survival proteins – testosterone-repressed prostate message-2, bcl-2 and proliferating cell nuclear antigen (Mylchreest *et al.*, 2002; Shultz *et al.*, 2001).

Phthalate-induced alterations in Sertoli cells may contribute to the adverse effects on the developing reproductive system. One study found that treatment with 1000 mg DEHP/kg/day from PND 6-11 decreased the number of Sertoli cells by 35% which returned to normal by PNW 6, yet a decrease in the number of testicular spermatids was observed at PNW 19 (Dostal *et al.*, 1988). Similarly, neonatal Sertoli cells and gonocytes cultured with MEHP and FSH had decreased proliferation (Li *et al.*, 1998). Metabolic alterations were observed after phthalate exposure. Sertoli cells isolated from 18-day-old-rats exposed to MEHP had decreased mitochondrial succinate dehydrogenase activity, cellular ATP, and pyruvate levels and increased levels of lactate and intracellular lipid (Chapin *et al.*, 1988). Multinuclearity of gonocytes in GD 21 testes are thought to occur as a consequence of abnormal proliferation related to Sertoli cell dysfunction (Mylchreest *et al.*, 2002).

Other mechanisms of phthalate-mediated reproductive toxicity may contribute to the adverse effects in young and adult animals. DBP was found to be weakly estrogenic by some (Harris *et al.*, 1997; Jobling *et al.*, 1995; Zacharewski *et al.*, 1998) but not by others (Blair *et al.*, 2000; Paganetto *et al.*, 2000). Furthermore, in frogs, DBP increased the

incidence of female phenotype when genetically male tadpoles were exposed during sexual differentiation (Ohtani *et al.*, 2000). Phthalates disrupted retinoic acid-induced nuclear localization of retinoic acid receptor α (RARα) with concomitant decreases in the retinoic acid-induced transcriptional activity of RARα (Vo *et al.*, 2001). Phthalates increase the incidence of hepatocellular carcinoma in rodents by activating PPARα, which increases peroxisomal proliferation (Hasmall *et al.*, 1999), oxidative damage (reviewed by ASTDR, 1993; WHO, 1992) and mitosis (Robertson *et al.*, 1997). PPARα-null (-/-) and wild type (+/+) adult mice had testicular lesions when a diet containing 1200 mg DEHP/kg was fed for 24 wk (Ward *et al.*, 1998). However, PPARα-null (-/-) mice had normal spermatogenesis after 4-8 wk of exposure and severe tubular atrophy at 24 wk, while wild type (+/+) mice develop tubular atrophy after 8-16 wk. These differences in the timing of the testicular toxicity suggests phthalate-mediated toxicity occurs through PPAR-independent and dependent pathways.

2.2.2.7 Effects on Aquatic Organisms

DBP is toxic to a number of aquatic organisms (Table 2). The lowest concentration to cause 50% mortality (LC_{50}) for 8 aquatic organisms is between 0.40 and 6.29 mg/L (Adams *et al.*, 1995). Full life cycle (21 days) exposure of waterfleas (*Daphnia magna*) to 2.5 mg/L DBP reduces the survival and the mean number of offspring per living female at the end of exposure (Rhodes *et al.*, 1995). Furthermore, the growth rate of rainbow trout (*Oncorhynchus mykiss*) decreased following exposure to 0.19 mg DBP/L for ~60 days posthatch (Rhodes *et al.*, 1995).

As mentioned above (*Section: 2.2.2.6: Reproductive Effects*), DBP increased the incidence of female phenotype in *Rana rugosa* when genetically male tadpoles were

exposed during sexual differentiation (Ohtani *et al.*, 2000). After exposure to 0.1, 1, or 10 μM DBP during sexual differentiation 0, 7, and 17% of tadpoles were phenotypically female compared to 18, 63, and 100% of tadpoles exposed to 0.01, 0.1, or 10 μM 17-β-estradiol. These results suggest DBP is about 1,000-fold less potent than 17-β-estradiol on sexual differentiation in frogs.

TABLE 2
Effects of DBP on aquatic organisms

Animal	Acute toxicity (mg/L)		Chronic toxicity (mg/L)		
	96hr LC_{50}	96hr NOEC	LOEC	NOEC	Endpoint
Flathead minnow (*Pimephales promelas*)	0.92-1.54	0.32-.08	.....	.....	
Rainbow trout (*Oncorhynchus mykiss*)	1.60	0.50	0.10	0.19	Growth
Bluegill sunfish (*Lepomis macrochirus*)	0.48	0.42	.....	.....	
Waterflea (*Daphnia magna*)	2.99	1.70	2.50	0.96	Survival and Reproduction
Midge (*Paratanytarsus parthenogeneitca*)	6.29	2.35	.....	.....	
Mysid shrimp (*Mysidopsis bahia*)	0.50	0.26	.....	.....	
Sheepshead minnow (*Cyprinodon variegatus*)	>0.60	0.60	.....	.....	
Green Algae (*Selenastrum capricornutum*)	0.40	0.21	.....	.....	

LC_{50}= lethal concentration to cause 50% mortality

NOEC= no-observed-effect concentration

LOEC= lowest-observed-effect concentration

Adapted from Adams *et al*.,1995; Rhodes *et al*.,1995

CHAPTER 3

CHARACTERIZATION OF THE EFFECTS OF DIBUTYL PHTHALATE IN XENOPUS LAEVIS

3.1 Introduction

As discussed in Chapter 2, the effects of DBP on development and the male reproductive system of rodents have been well characterized. However, similar studies have not been conducted in amphibians. Furthermore, a majority of the data on aquatic organisms utilize acute toxicity test to determine mortality (reviewed by WHO, 1997). In view of the possible ubiquitous contamination of water by phthalates, it is important to study effects in amphibians following long-term chronic exposure to phthalates. Therefore, we studied the developmental and reproductive effects of DBP using the African clawed frog, *Xenopus laevis*.

3.1.1 Amphibian Declines and Increased Incidence of Malformations

Over the past several years, numerous reports indicate that amphibian populations are undergoing range reduction, population decline and even extinction in certain geographical regions (reviewed by Blaustein, 1994b). However, due to several limitations (length of study, topography, etc.) none of these reports provided concrete evidence on the status of global amphibian populations. In an effort to overcome these limitations, Houlahan *et al.* (2000) analyzed data from 936 amphibian populations from around the world and determined that a global decline in amphibian populations started in the late 1950's and continues to present day.

Little information is available with regard to the etiology of declining amphibian populations, yet several factors have been implicated – disease, changes in climate, xenobiotics – as contributing to these trends. Pox virus-like particles caused an unusual mortality of adult *Rana temporaria* at 222 sites in the United Kingdom (Cunningham *et al.*, 1993). Changes in climate of Costa Rica's Monteverde Clouds Forest Preserve led to unusually warm and dry conditions, which led to the disappearance of the Golden toad (*Bufo periglenes*) and the Harlequin frog (*Atelopus varius*) (Pounds *et al.*, 1994). Acid rain decreasing the pH (< 4.5) of breeding ponds led to the decline of the Natterjack Toad, *Bufo clamita* (Bebee *et al.*, 1990). Exposure to ultraviolet-B radiation (UV-B) increases mortality and decreases hatching success of Long-toed salamanders (*Ambystoma macrodactylum*), Western Toad (*Bufo boreas*) and Cascades frog (*Rana* cascadae) but not Pacific treefrog (*Hyla regilla*) or Red-legged frog (*Rana aurora*) (Blaustein 1994a; 1997; 1998). The differences in susceptibility are thought to be due to the ability of embryos to inactivate cytotoxic and mutagenic by-products of increased UV-B exposure (Blaustein, 1994a). Furthermore, changes in climate and UV-B exposure may act synergistically on amphibian populations. Changes in climate in the western United States led to a reduction in precipitation, which decreased the water depth of breeding ponds. This decreased water depth led to an increased exposure of embryos to damaging UV-B radiation and, consequently a fatal infection by the fungus, *Saprolegnia ferax* (Kiesecker *et al.*, 2001).

Man-made factors such as xenobiotics are suspected to be contributing factors to amphibian declines because of the widespread geographical distribution and the rapid rate at which they have occurred. Environmental xenobiotics may alter amphibian

populations by causing direct mortality, increasing the susceptibility of young to disease, retarding tadpole development, altering reproductive development, or by decreasing the ability of amphibians to avoid predation (Carey and Bryant, 1995). For example, Kirk (1988) noted a higher incidence of mortality in Western spotted frogs (*Rana pretiosa*) following an application of dichlorodiphenyltricholorethane (DDT) to control the Douglas fir tussock moth in Oregon. In laboratory experiments, Burkhardt *et al.* (1998) found that water samples taken from several ponds in Minnesota caused a dose-dependent mortality and induced malformations in *Xenopus laevis*.

The incidence of reported field malformations in North America has increased dramatically. By March 27, 2002 the North American Reporting Center for Amphibian Populations had more than 900 verified reports of malformations from 52 species in 46 states and 4 provinces (Northern Prairie Wildlife Research Center.1997.North American Reporting Center for Amphibian Malformations, 2002). The reported malformations predominantly occurred in the hindlimb, although craniofacial malformations, abnormal internal organs and retained tails have been reported (Loeffler *et al.*, 2001). Whether this phenomenon is related to the worldwide decline in amphibian populations is unknown.

Several field and laboratory studies indicate the factors implicated in the decline in amphibian populations may also be contributing to the increased incidence of field malformations. Northern leopard frog (*Rana pipiens*) tadpoles exposed to UV light during early limb bud development have a high percentage (~50%) of hindlimb malformations (Ankley *et al.*, 1998). These malformations included amelia, phocomelia or ectromelia, and ectrodactyly or brachydactyly. Johnson *et al.*(1999) found that Pacific treefrogs exposed to *Ribeiroroia* cercariae have a dose-dependent increase in the

incidence of hindlimb malformations, reaching 100% in the highest dose. The specific abnormalities recorded by Johnson *et al.* (1999) account for 95% of the abnormalities observed in 1086 Pacific tree frogs in the field. Frogs naturally exposed to water contaminated with sewage or industrial (pulp mill) effluents had developmental anomalies and tumor-like dysplasia of osteochondrous tissue (Mizgireuv *et al.*, 1984). Other studies suggest that the agrochemicals with anti-thyroid activity may interfere with normal limb development (Fort *et al.*, 2000a).

3.1.2 *Xenopus laevis* as an Animal Model for Toxicological Studies

In spite of the rapidly accumulating data on amphibian declines and increased incidence of field malformations relatively few amphibian species are utilized for toxicological studies. The diversity of amphibians makes it difficult in choosing the appropriate animal for certain toxicological endpoints (terrestrial vs. aquatic species, time to metamorphose, etc.). Fortunately, the African clawed frog overcomes many of these problems because it is easily bred and raised in the laboratory (Hilken *et al.*, 1995) and exclusively aquatic, which facilitates chronic exposure. Furthermore, the chronology of *Xenopus* development has been meticulously characterized into 66 stages based on external morphology (Nieuwkoop and Faber 1967). The development of *Xenopus laevis* is known to be dependent on thyroid hormones (reviewed by Dodd and Dodd, 1976). This is one of the few animal models in which macroscopic perturbations in thyroid-dependent development can be monitored and correlated with other developmental processes (brain, liver, gut, lung) (Tata, 1993; 1998). These attributes make *Xenopus* an ideal research animal for studies ranging from molecular endocrinology (Tata, 1993; 1998) to neurophysiology (Kelley, 1996) and developmental toxicology (Bantle, 1995).

3.1.2.1 Frog Embryo Teratogenesis Assay – *Xenopus*

As of 1997 there were approximately 75,500 man-made chemicals listed in the Toxic Substance Control Act Inventory, of which 121 chemicals were identified to need further toxicological testing (Endocrine Disrupting Screening and Testing Advisory Committee, 1998). Furthermore, it has been estimated that approximately 90% of these chemicals are toxic to animals and plants (Wilson and Frazer, 1991). Several short-term assays, such as the Ames test and mammalian *in vivo* cytogenetic assays (reviewed by Hoffman 1996) are employed as quick and cost-effective methods to determine the genotoxic and carcinogenic effects. Unfortunately, follow up studies in mammals to determine developmental ramifications are time-consuming, expensive and raise concern for animal welfare due to the large number of animals often needed (Dawson *et al.*, 1989). The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) was initially designed to address many of these issues and serve as an indicator of potential human developmental health hazards (Bantle *et al.*, 1994a; 1994b; 1995; 1996; 1999a, Dawson *et al.*, 1989, Fort *et al.*, 1998).

FETAX is 96-hr whole animal assay used to evaluate the developmental and teratogenic effects of chemicals by exposing *Xenopus laevis* embryos from gastrulation until primary organogenesis is complete (Bantle, 1995). The reliability and utility of FETAX in screening potentially toxic chemicals is evident by the number of publications using this method (Bantle *et al.*, 1994a; 1994b; 1995; 1996; 1999a, Dawson *et al.*, 1989, Fort *et al.*, 1998). Recently the predictive validity of FETAX was compared to Sprague-Dawley rats using 12 blind-coded chemicals, previously characterized as having high teratogenic potential, low teratogenic potential yet embryolethal, or little to no hazard.

Results indicate that FETAX accurately predicted the toxicity hazards of the 12 chemicals and the range of malformations induced by each chemical mimicked those in Sprague-Dawley rats (Fort, 2000b).

FETAX is one of a few amphibian assays with the potential to correlate the declining amphibian populations and increased incidence of field malformations with environmental pollutants (Bantle *et al.*, 1994a), but is limited by the short period of development studied. This assay does not include sensitive periods of development following primary organogenesis, such as limb development, gonadal differentiation, thyroid-dependent metamorphosis, androgen-dependent laryngeal development, and reproductive competence in adult animals.

3.1.2.2 Current Toxicological Studies using *Xenopus laevis*

Similar to FETAX, other studies investigating the effects of xenobiotics on *Xenopus laevis* focus on specific periods of development and do not provide a comprehensive assessment of development and reproductive function. Ohtani *et al.* (2000) determined the immediate effects of DBP on sex ratios in *Rana rugosa*. Likewise, Hayes *et al.* (2002) determined the immediate effects of Atrazine on gonadal differentiation in tadpoles and estrogen synthesis in adult animals. A different group of studies focuses on using *Xenopus* to determine the effects of xenobiotics on vitellogenin synthesis *in vitro* (Kloas *et al.*, 1999) or *in vivo* (Palmer and Palmer, 1985). The contribution of these studies towards understanding the potential endocrine disrupting properties of xenobiotics cannot be overlooked. However, their inability to determine the long-term effects of these chemicals on development and reproduction makes it difficult

to ascertain whether xenobiotics may be contributing to the global declines in amphibian populations by altering reproductive function in adult animals.

3.1.3 General Development of *Xenopus laevis*

As indicated above (*Section: 3.1.2: Xenopus laevis as an Animal Model for Toxicological Studies*), the development of *Xenopus laevis* has been defined into 66 stages (Nieuwkoop and Faber, 1967). At 6 hr post-fertilization (stage 8) embryos are blastulae, hollow ball of cells just about to enter primary organogenesis. At stage 11 gastrulation, formation of the embryonic gut and neural tube commences (Bantle, 1995). By 96 hr (stage 46) primary organogenesis is complete, the free-swimming tadpole is ready to feed, and hindlimb development begins (Bantle, 1995). Subsequent development is hallmarked by thyroid-dependent metamorphosis, where free-swimming tadpoles transform into four-legged frogs (reviewed by Dodd and Dodd, 1976).

Metamorphosis transforms most, if not all, tissues in the tadpole by selective proliferation/differentiation and apoptosis (reviewed by Dodd and Dodd, 1976). Gudernatsch (1912) provided some of the first experimental evidence that hormones participated in the regulation of tadpole development, by feeding different horse tissues and recording the results. He determined that feeding horse thyroid gland increased the rate at which tadpoles metamorphosed. Later, as a consequence of several studies and the availability of pure 3,5,3',5' tetraiodothyronine (T_4) it was determined that metamorphosis is dependent on thyroid hormones (reviewed by Shi *et al.*, 1996).

Tadpole development has been classified based on metamorphic changes (Dodd and Dodd, 1976) and concentrations of thyroid hormones (Leloup and Buscaglia, 1977). Thyroid hormones are primarily secreted (~90%) as T_4 and converted to more active

3,5,3'triiodothyronine (T_3) by 5'-deiodinases present in target tissues (reviewed by Genuth, 1998). 5-deiodinases convert T_4 and T_3 to the inactive 3,3',5' reverse T_3 (rT_3) and 3,5 diiodothryonine (T_2). During premetamorphosis (stages 37-54) the concentrations of thyroid hormones are low (T_4 = 51 ng/dl, T_3 = not detectable) and the limited hindlimb development is independent of their secretion. Stage 55 marks the start of thyroid-dependent metamorphosis called prometamorphosis. Thyroid hormone concentrations gradually increase from stage 55 to 58 (T_4 = 210-384 ng/dl, T_3 = 0-122 ng/dl) as the hindlimb undergoes differentiation, growth, and maturation from a stump to a five-digit limb. During metamorphic climax (stages 59-62) concentrations of thyroid hormones continue to increase (T_4 = 384-750 ng/dl, T_3 = 122-478ng/dl) and tail regression begins. From stage 62 to 66, concentrations of thyroid hormone (T_4 = 750-459 ng/dl, T_3 = 478-43 ng/dl) and body length decrease, while tail regression progresses rapidly. At stage 66 metamorphosis is complete and tadpoles are completely transformed into frogs.

Regulation of thyroid hormone concentrations during metamorphosis has been characterized. During premetamorphosis, melatonin inhibits secretion of T_4 (Wright *et al.*, 1991; 2000) and prolactin blocks the effects of thyroid hormone in the limb (Wright *et al.*, 1994) by inhibiting the upregulation of thyroid hormone receptors (TR) (Tata, 1998). Melatonin secretion begins to decrease during prometamorphosis (Wright *et al.*, 1997) and the levels of thyrotropin-releasing (TRH), thyroid-stimulating (TSH) and thyroid hormones increase (Bray and Sicard, 1982). During this time, the negative peripheral effects prolactin are not observed (Wright *et al.*, 1994, 2000) and the hindlimb has increased levels of 5'-deiodinase (Huang *et al.*, 2001) and decreased 5-deiodinase

(Shi *et al.*, 1996) compared to the tail. It is speculated that environmental cues (temperature, light, nutrition) (Tata, 1993; 1998) and increased levels of corticotropin-releasing factor (Gancendo *et al.*, 1992) contribute to this change. However, the precise mechanism regulating the endocrine changes at prometamorphosis is not understood. The concentration of thyroid hormone peaks during metamorphic climax (Leloup and Buscaglia, 1977) and corticosterone potentiates the action of thyroid hormones by increasing the number of TR and 9-cis retinoic acid receptors (RXR) in the tail (Tata, 1993, 1998). During this time, the tail has increased levels of 5'-deiodinase (Huang *et al.*, 2001) and decreased 5-deiodinase (Shi *et al.*, 1996) compared to the hindlimb. Due to the high levels of thyroid hormones at metamorphic climax, further secretion of thyroid hormone is inhibited and metamorphosis is complete (Huang *et al.*, 2001).

3.1.4 Reproductive Development of *Xenopus laevis*

Xenopus laevis belongs to the ZZ-ZW classification of chromosomes where males are homogametic (ZZ) and females are heterogametic (ZW) (Chang and Witschi, 1955; 1956; Mikamo and Witschi, 1963; 1964; 1966; Weiler and Ohno, 1962). Tadpoles can be sex-reversed by either administration of estradiol (Chang and Witschi, 1956) to produce fertile females or testicular transplant (Mikamo and Witschi, 1963) to produce fertile males. However, the actions of testicular transplant cannot be duplicated by administration of testosterone alone (Mikamo and Witschi, 1963), suggesting that a testis-specific factor, such as mullerian inhibiting substance (MIS) may be vital during gonadal differentiation (Kelley, 1996). Other studies indicate that estradiol may be responsible for gonadal differentiation in *Xenopus*, since treatment with estradiol (Villalpando and Merchant-Larios, 1990; Miyata and Kubo, 2000) induces female

phenotype, while treatment with an aromatase inhibitor causes male phenotype (Miyata and Kubo, 2000). Therefore, the determination of gonadal sex is not determined by genotype, but rather by the endocrine environment in which the germ cells develop (Mikamo and Witschi, 1964).

The precise mechanism of gonadal differentiation in *Xenopus* is still unknown. However, the hormone-sensitive stages for gonadal differentiation in *Xenopus* have been determined (Villapando and Merchant-Larious, 1990). Typically at stage 56, the gonads are morphologically distinct where phenotypic females have primordial germ cells located in the cortex, while phenotypic males have primordial germ cells located in the medulla. Stage 44-48 tadpoles, in which no genital ridges are present, or stages 49-50 in which genital ridges are developed and primordial germ cells are just arriving, when exposed to 100 μg/L estradiol benzoate develop into 100% female phenotype. Treatment with estradiol benzoate at stages 51-53 (undifferentiated gonad with primordial germ cells remaining in the surface epithelium and some cells of the coelomic epithelium migrating towards the interior of the genital ridge to form the gonadal medulla) or stages 54-55 (when primordial germ cells are migrating from the cortex to the medulla in males) leads to 50% of survivors with normal ovaries and 50% with ovotestes (primordial germ cells in cortex and medulla). Treatment with estradiol benzoate at stages 55-56 (all primordial germ cells completely translocated present in the medullary region of presumptive genetic males) leads to an ovary to testis ratio close to 1:1.

Testicular development beyond gonadal differentiation has not been well characterized. Whereas Nieuwkoop and Faber (1967) identified spermatocytes at stage 59, Witschi (1971) reported that these cells were not present until two to three months

post-metamorphosis. Spermatozoa have been found as early as six months post-metamorphosis (Mikamo and Witschi, 1963).

The development of male reproductive tract of *Xenopus laevis* is unlike that of mammals. During early development, the Wolffian ducts differentiate into the primary nephric duct and later into the mesonephric duct of both sexes (Lofts, 1974). In females, Wolffian ducts serve exclusively as an excretory duct; however in males they serve as urogenital ducts. Spermatozoa produced by testes are conveyed to the kidneys through the excurrent ducts and excreted through the Wolffian ducts to the cloaca (Lofts, 1974). Luminal expansion of the excurrent ducts occurs in response to gonadotropins to allow the contents of the seminferous tubules to be transported to the Wolffian ducts (Kobayashi and Iwasawa, 1989). However, the precise development of the excurrent ducts has not been characterized.

While information about development of testes and excurrent ducts is lacking, normal development of the vocal system, the larynx and laryngeal motor neurons has been extensively characterized (Kelley, 1996). The larynx is sexually dimorphic organ, where adult males have 6-7 times the number of laryngeal muscle fibers (Sassoon and Kelley, 1986), a greater number of axons innervating laryngeal muscle (Kelley and Dennison, 1990), and different twitch characteristics of the muscle fibers (Tobias *et al.*, 1991). This sexual dimorphism allows sexually active males to produce a distinct advertising call to initiate courtship with sexually receptive females (Tobias and Kelley, 1987). The masculine phenotype becomes evident at tadpole stages 59-62, corresponding to the peak in thyroid hormone concentrations, when the number of axons innervating male larynges increases by 119% per nerve (Kelley and Dennison, 1990). Treatment

with the potent goitrogen, 6-*n*-propyl-2-thiouracil from stage 50 (when the larynx is unable to respond to androgen) for 50 or 100 days blocked normal and androgen-stimulated development of the masculine phenotype compared to controls (Robertson and Kelley, 1996). Conversely, by treating stage 50 tadpoles with exogenous thyroid hormones, precocious androgen competency is observed (Cohen and Kelley, 1996). Therefore thyroid hormones set the stage for androgen-induced laryngeal development later in life.

A majority of laryngeal differentiation occurs post-metamorphically. Muscle fiber proliferation occurs in male larynges between 0 and 6 months post-metamorphosis, due to an increase in androgen secretion by males (Marin *et al.*, 1990). Also during this time the twitch characteristic of muscle fibers change in males and females. Muscle fibers of the adult male larynx are exclusively fast twitch, while those of females are mostly (~70%) slow twitch (Tobias *et al.*, 1991).

3.1.5 Spermatogenesis in *Xenopus laevis*

Spermatogenesis in *Xenopus laevis* is of the cystic variety (Lofts, 1974). The development of germ cells is coordinated, occurring in capsules called germinal cysts (also called nest, follicle) where each cyst is ensheathed in a membranous capsule of Sertoli cells for most of its development (Lofts, 1974). The germ cells follow normal progression of primary spermatogonia to mature spermatozoa to produce a haploid germ cell. Primary spermatogonia divide mitotically, either into renewing spermatogonia or divide a number of times to produce a germinal cyst filled with secondary spermatogonia. Germinal cysts of secondary spermatogonia divide approximately eight times, indicating a single primary spermatogonium can produce ~200 germ cells (Lofts, 1974). This

pattern of spermatogenesis is suited for amphibians since the males and females only associate for a brief time during breeding and a large number of spermatozoa are needed to account for the hazards and fertilization rates associated with external fertilization (Lofts, 1974). During spermiogenesis, the germinal cyst ruptures and the Sertoli cells remain associated with the basal lamina of the seminiferous tubule allowing the maturing spermatid heads to remain embedded in the cytoplasm and the tail segments to grow luminally (Lofts, 1974). Gonadotropin-induced spermiation leads to depolymerization of mucopolysaccharides in the Sertoli cell; fluid secretion into the lumen causing tubular expansion; and sperm bundles are released and transported to the excurrent ducts (Lofts, 1974). The excurrent ducts undergo tubular expansion in response to gonadotropins to allow the contents of the seminiferous tubule fluid to flow into the kidney and ultimately out of the cloaca (Kobayashi and Iwasawa, 1989). Spermatogenesis in adult male *Xenopus* is continuous throughout the year and that the duration of spermatogenesis is 41-42 days (Kalt, 1976).

3.2 Objective

The objective of this study was to characterize the effects of exposure to DBP from 0-12wk of life on development and male reproductive competence in *Xenopus laevis*.

3.3 Preliminary Studies

To accomplish the objective of this study, several preliminary studies were conducted to determine sensitivity of *Xenopus laevis* to DBP, end points/parameters used, appropriate doses, and the optimal environmental conditions for rearing *Xenopus laevis* for toxicological studies.

3.3.1 Sensitivity of *Xenopus* to DBP and Selection of End Points/Parameters

Although the underlying mechanisms are yet to be identified, it has been observed that DBP exerts anti-androgenic effects in rodents (Foster *et al.*, 2001; Mylchreest *et al.*, 1998; 1999; 2000) and that sequelae may vary due to possible differences in how DBP is metabolized (Foster *et al.*, 1983). To determine if DBP also affects frogs, we studied the effects of DBP on *Xenopus laevis* tadpoles. *Xenopus laevis* tadpoles were exposed to 0 (n = 14) or 10 (n = 52) ppm DBP in a static alternate-day-water-renewal system beginning at 3 wk of age (stage 52). Mortality and time to complete metamorphosis (stage 66) were monitored weekly. Body weights were measured at 24 and 36 wk, and necropsy performed at 36 wk. The mortality rates in control and treated groups were 0% and 85% by wk 1 post-exposure, and 28% and 92% by wk 16 (Figure 2). Whereas 75% of controls metamorphosed by 12 wk of age and 100% by 14 wk, none of the treated tadpoles completed metamorphosis until 16 wk (Figure 3). Body weights did not differ ($p>0.1$) between groups at 24 wk, but decreased ($p<0.05$) in treated tadpoles at 36 wk. Larynx, a secondary sexual organ pivotal in mating calls, appeared to have regressed upon gross and microscopic evaluation at 36 wk (Figure 4), while paired testes (weighed as a unit with kidney) did not ($p>0.1$) (Table 3).

Based on the observations described above the following parameters were included in the definitive experiment: 1) periodic recording of mortality, 2) monitoring stage of development and metamorphic index (hindlimb:tail ratio), 3) periodic sampling of thyroid hormones during metamorphosis, 4) assessment of sexual competence of male frogs, 5) hypothalamic gonadotropin-releasing hormone (GnRH) content and

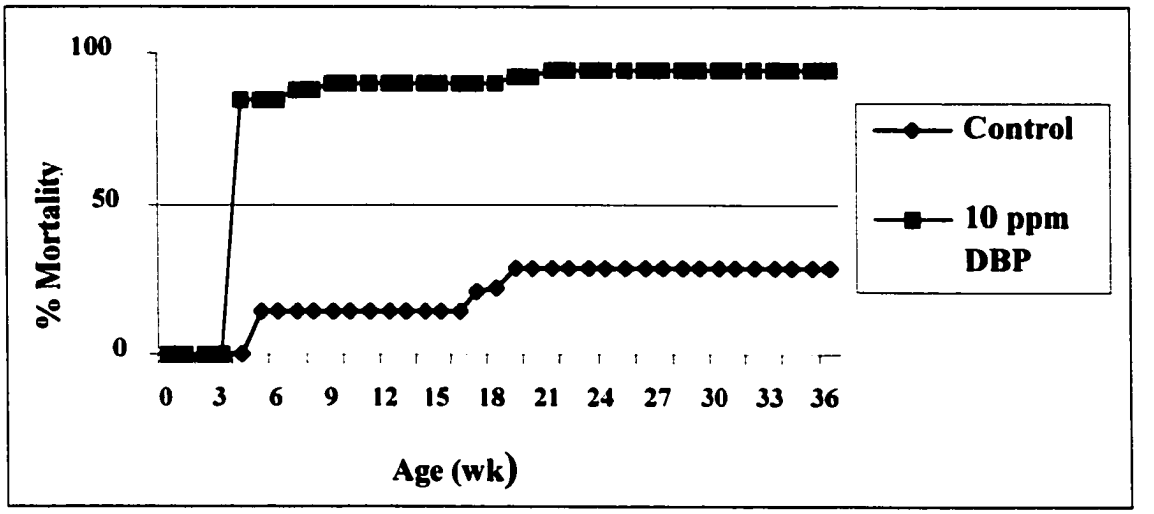


FIG. 2. Mortality rates of *Xenopus laevis* following treatment with 0 (Control) or 10 ppm DBP from 3 to 36 wk of age. No mortality was observed in controls (n = 14) after the 1st wk of treatment, but reached 28% mortality by the 16th wk. DBP group (n = 52) suffered an 85% mortality during the 1st wk of treatment leveling off by the 16th wk at 92%.

concentration of testosterone, 6) quantification of laryngeal development, and 7) evaluation of histopathological changes in testes and excurrent ducts.

3.3.2 Dose Response

The dose response of *Xenopus* embryos to DBP was determined using the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX). All procedures performed were according to ASTM (1991) standards are described below (*Section: 3.4.1.5: FETAX Procedures*). Prior to initiating the FETAX experiment, a preliminary range-finding test using a log scale (0.1-100 ppm DBP) was performed (data not presented) to determine where the 96-hr lowest concentration 50 (LC_{50}, the concentration of chemical that kills 50% of the animals), effective concentration 50 (EC_{50}, the concentration of chemical that causes at least one malformation in 50% of the animals) and minimum concentration to inhibit growth (MCIG, as reflected by a body length) occurred. Embryos were obtained

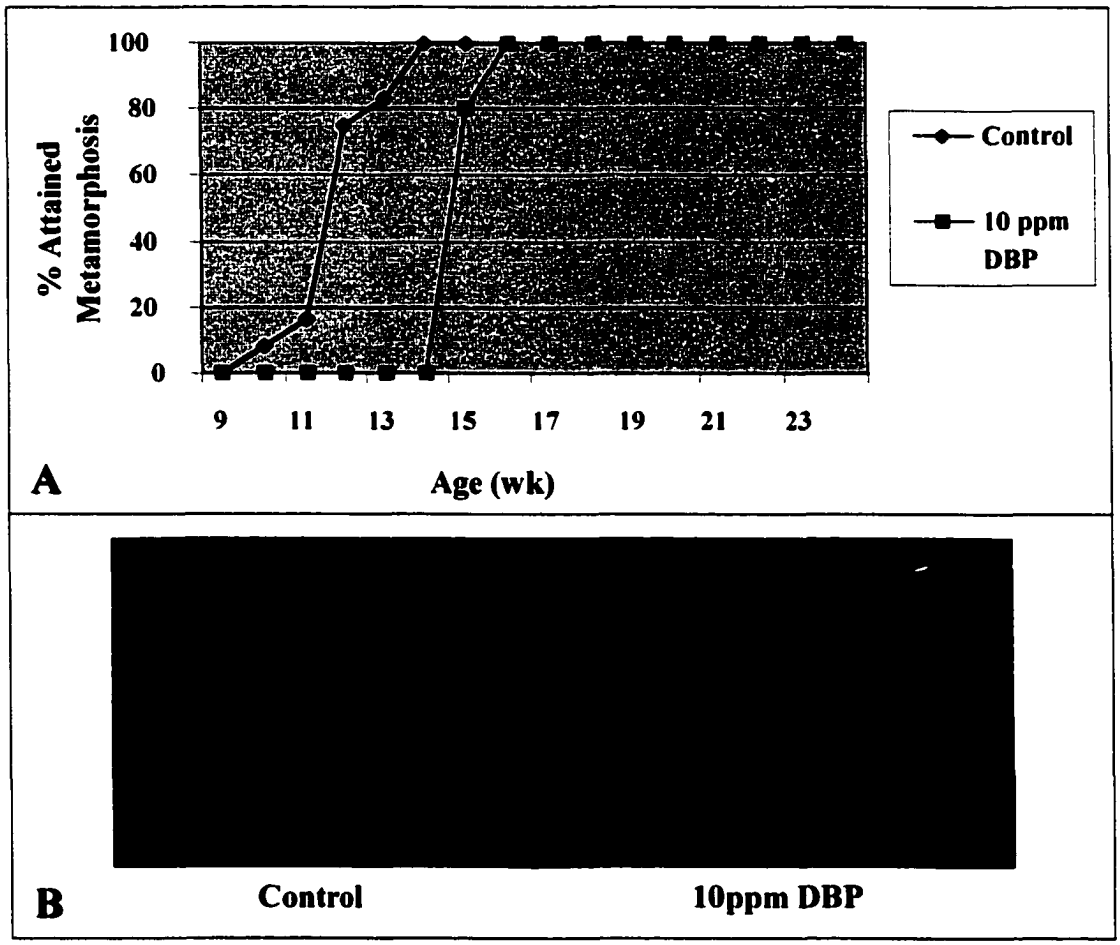


FIG. 3. Time to attain metamorphosis of *Xenopus laevis* following treatment with 0 (Control) or 10 ppm DBP from 3 to 36 wk of age. ***A.*** Metamorphosis began at 9 wk of age in controls and was completed by the 14 wk whereas DBP group did not begin metamorphosis until the 14 wk, and completed the process by the 16 wk. ***B.*** Photograph of two 14-wk-old *Xenopus*. Control froglet on the left has completed metamorphosis (stage 66), whereas the DBP-treated tadpole is approximately two weeks behind in development (stage 59).

TABLE 3

Effects of chronic exposure (3-36 wk of age) to DBP on organ weights of *Xenopus laevis* at 24 and 36 wk

		Organ			
		Body (g)		Kidney with gonad (mg)	Larynx (mg)
Treatment	n	24 wk	36 wk		
Control	8	2.66 ± 0.17	7.04 ± 0.47	37.0 ± 1.0	65.0 ± 20.0
10 ppm DBP	3	2.06 ± 0.55	5.07 ± 0.40*	47.0 ± 6.0	62.0 ± 3.0

Values represent mean ± SEM

*p<0.05 using t-test

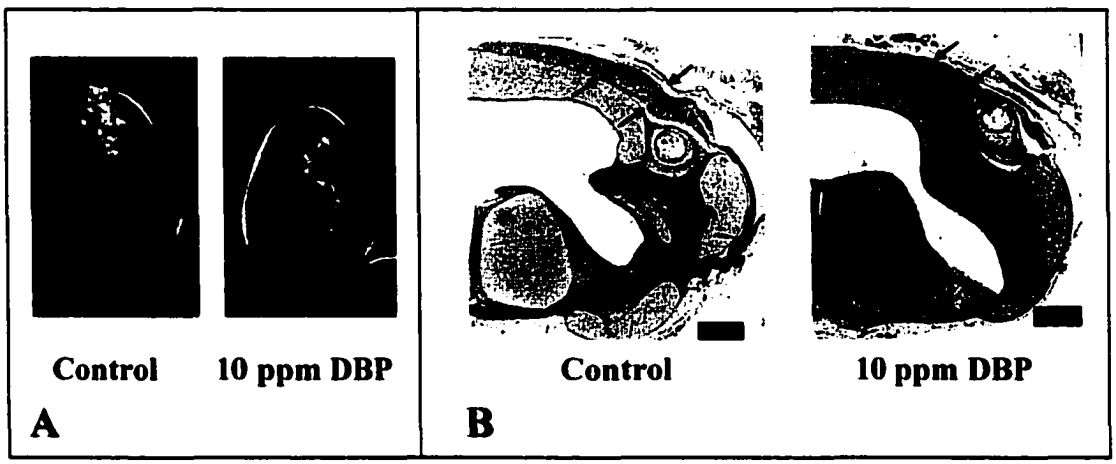


FIG. 4. Gross and histological appearance of male larynges at 36 wk of age of *Xenopus laevis* following treatment with 0 (Control) or 10 ppm DBP from 3 to 36 wk of age. Larynx weights did not differ in control and DBP-treated frogs. Although there were only 2 males surviving in DBP groups, the reduction in laryngeal size was obvious both at macroscopic ***(A)*** and microscopic ***(B)*** levels. Transverse sections through mid anterio-posterior axis of larynges ***(B)*** revealed a 3.5X decrease in dilator larynges muscle fibers ***(arrows)*** in DBP-treated frogs.

from breeding pairs (n = 4) injected with human chorionic gonadotropin (hCG) (Intervet Inc., Milsboro, DE) and exposed to 0 (n = 100), 1 (n = 50), 5 (n = 50), 7.5 (n = 50), 10 (n = 50), 15 (n = 25), 20 (n = 25), or 25ppm (n = 50) DBP and 2500 ppm (n = 50) 6-aminonicotinamide (6-AN) positive control; dissolved in FETAX solution from 6 hr post-fertilization (stage 8-11) to 96 hr (stage 46) in a static-24 hr-renewal-system. At 96 hr, when primary organogenesis is normally complete, the assay was terminated. Results from this assay are presented in Table 4. The percent mortality rates for 0, 1, 5, 7.5, 10, 15, 20, and 25 ppm DBP were 2, 4, 27, 48, 52, 100, 100, and 100%, respectively. Mortality for 6-AN was 100%. The incidences of developmental malformations were significantly increased ($p<0.05$) in 5 (34.7%), 7.5 (35.5%) and 10 (54.6%) ppm DBP groups compared to control (3.6%). The most frequent malformations encountered from exposure to DBP were axial, gut, cardiac, and optic. In general, the incidence of these malformations increased with increasing concentration (Figure 5). Body length as well as progression of normal development were significantly retarded ($p<0.05$) in the 5 (stage 45.3, 8.1 mm), 7.5 (stage 45.5, 8.2 mm) and 10 (stage 45.1, 7.9 mm) ppm DBP groups compared to controls (stage 46.1, 8.5 mm) (Table 4, Figure 5). The 96 hr-LC_{50} was estimated to be between 7.5 and 10 ppm DBP, while the 96 hr-EC_{50} (malformations) was 10 ppm DBP. The teratogenic index, calculated by dividing the LC_{50} by the EC_{50} was 0.75-1.0. The minimum concentration to inhibit growth was 5 ppm DBP. The no-observable-effects-level (NOEL) for mortality, malformations and body length was 1 ppm DBP. However the NOEL for stage of development was determined to be <1 ppm DBP since 1 ppm DBP significantly decreased the stage of development.

TABLE 4
Effects of DBP on *Xenopus* embryo development at 96 hr of age

Treatment	n	Mortality[a]	Malformations[b]	Developmental Stage[c]	Body Length (mm)[d]
FETAX Solution	400	2 (392)	3.6	46.1 ± 0.02	8.5 ± 0.03
2500ppm 6-AN	200	100 (0)	...	...	...
1ppm DBP	200	4 (193)	9.3	45.9 ± 0.03*	8.4 ± 0.05
5ppm DBP	200	27 (147)	34.7*	45.3 ± 0.06*	8.1 ± 0.05*
7.5ppm DBP	200	48 (104)	35.5*	45.5 ± 0.06*	8.2 ± 0.05*
10ppm DBP	200	52 (97)	54.6*	45.1 ± 0.05*	7.9 ± 0.05*
15ppm DBP	100	100 (0)	...	...	...
20ppm DBP	100	100 (0)	...	...	...
25ppm DBP	100	100 (0)	...	...	...

[a] Reported as percent of total embryos for each treatment (number of surviving tadpoles)

[b] Reported as percent of surviving embryos at 96 hr with at least one malformation

[c,d] Values represent mean ± SEM

* Significantly different ($p<0.05$) from control using ANOVA and Tukey/Kramer post-hoc test

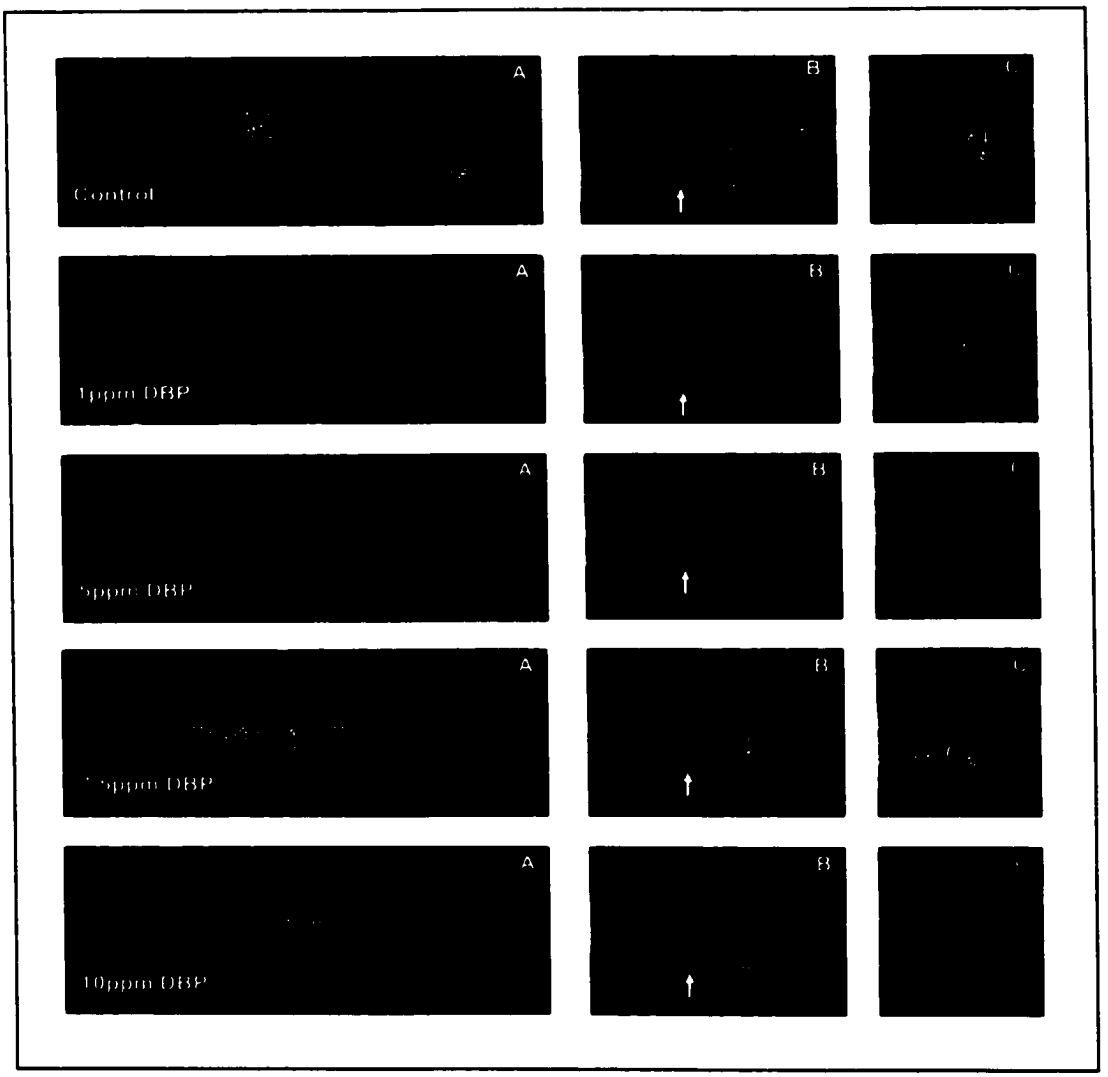


FIG. 5. Macroscopic differences in *Xenopus laevis* tadpoles exposed to 0, 1, 5, 7.5, and 10 ppm DBP at 96 hr of age. ***(A)*** Lateral view of entire tadpole. ***(B)*** Ventral aspect of cranial pole. ***(C)*** Lateral aspect of abdominal region. All images within each panel were captured at the same magnification ***A*****:** 7X ***B*****:** 9X ***C*****:** 12X. ***Large Arrows*** point to gut. ***Circles*** enclose the area where hindlimb-bud emerges and ***small arrows*** point to hindlimb-bud.

3.3.3 Determining the optimal rearing conditions for *Xenopus laevis*

At 96 hr of age (stage 46), the environment provided in FETAX (100 mm Pyrex petri dish at a population density of 2.5 tadpoles/ml) does not satisfy the environment required for maximal growth and development of tadpoles. Thus it becomes necessary to transfer the tadpoles to larger stock tanks and decrease the population density to approximately 2.5 tadpoles/L (Etheridge and Richter, 1978). Because the overall objective of the study was to determine the effects of DBP on tadpole development, which extends beyond 96 hr, it was necessary to determine the optimal environmental conditions for rearing *Xenopus laevis* tadpoles for toxicological studies to ensure that developmental defects unrelated to treatment were avoided. Accordingly, we compared rearing tadpoles at different temperatures (incubator at 24 ± 2°C (IT) or room at 19-21°C (RT)) in two different media (FETAX solution or Morton's Pickling Salt (MPS)) for the first 96 hr and in two different media (FETAX and MPS) at room temperature after 96 hr. MPS was compared since it is routinely used by numerous investigators (Wu and Gerhart, 1991), and is the current method used in the *Xenopus* colony at Colorado State University. Embryos were obtained from breeding pairs (n = 2) injected with hCG (*Section: 3.4.1.5: FETAX Procedures*) and randomly assigned to FETAX-IT (n = 150), FETAX-RT (n = 150), MPS-IT (n = 150), MPS-RT (n = 150) (media-temperature). Media were changed daily. At 96 hr of age (stage 46), (n = 200) tadpoles from each environmental condition were euthanized and evaluated according to ASTM (1991) standards. The remaining 96 hr tadpoles reared in FETAX-IT and FETAX-RT were transferred to 20-gallon glass aquariums at RT containing FETAX (n = 50) or MPS (n = 50) at a population density of 2.5 tadpoles/L. MPS-IT and MPS-RT 96 hr tadpoles were

not continued beyond 96 hr, because of the high incidence of malformations and retarded development observed (Table 5). On day 11 (~stage 48) all tadpoles were euthanized and evaluated according to ASTM (1991) standards. We found that tadpoles were most sensitive to differences in temperature or medium during the first 96 hr of life. Mortality rates were low (<8%) for all environmental conditions evaluated. However, the incidence of malformations in MPS-IT (26%) was significantly higher ($p<0.05$) compared to FETAX-IT (Table 5). The body length and overall development were significantly retarded ($p<0.05$) in FETAX-RT (stage 44, 7.4 mm), MPS-IT (stage 45.8, 7.0 mm) and MPS-RT (stage 43.9, 6.9 mm) compared to FETAX-IT (stage 45.9, 9.4 mm) (Table 5). Subsequent development of tadpoles following transfer to 20-gallon glass aquariums was not as sensitive to tested environmental conditions as during the first 96 hr of life (Table 6). Mortality was not a factor as survival rates were greater than 98% with no observed malformations in any group. Developmental stage and body length were retarded ($p<0.05$) in tadpoles that were raised at RT for the first 96 hr (Table 6). Based on these observations, rearing tadpoles in FETAX-IT for the first 96 hr, and in FETAX medium thereafter was optimal as it provided the maximal growth based on body length and stage of development with minimal mortality and malformations.

3.4 Effects of Developmental Exposure to DBP

A majority of the current research conducted on the effects of xenobiotics on amphibians utilizes short-term assays to determine immediate effects such as mortality and teratogenicity (Fort, 1995), induction of vitellogenin synthesis (Palmer and Palmer, 1985), sex ratios (Ohtani *et al.*, 2000) and gonadal differentiation (Hayes *et al.*, 2002). Unfortunately critical periods of development such as thyroid-dependent metamorphosis,

TABLE 5
Effects of environmental conditions on *Xenopus* embryo development at 96 hr of age

Environmental Conditions (medium-temperature)	n	Mortality[a]	Malformations[b]	Developmental Stage[c]	Body Length (mm)[d]
FETAX-IT	200	2 (197)	0.5	45.9 ± 0.02	9.4 ± 0.03
FETAX-RT	200	2 (197)	0	44.0 ± 0.03*	7.4 ± 0.02*
MPS-IT	200	7 (186)	26.3*	45.8 ± 0.03*	7.0 ± 0.06*
MPS-RT	200	1 (198)	4	43.9 ± 0.03*	6.9 ± 0.03*

FETAX = FETAX solution (ASTM 1991) MPS = 10 mM Morton's pickling salt solution

IT = Incubator temperature (24 ± 2°C) RT = Room temperature (19-21°C)

[a] Reported as percent of total embryos for each treatment (number of surviving tadpoles)

[b] Reported as percent of surviving embryos at 96 hr with at least one malformation

[c,d] Values represent mean ± SEM

* Significantly different ($p<0.05$) from FETAX-IT using ANOVA and Tukey/Kramer post-hoc test

TABLE 6
Effects of environmental conditions on *Xenopus* development at 11 days of age

96 hr Environmental Conditions (medium-temperature)	Post-96 hr Environmental Conditions (medium)	n	Mortality[a]	Malformations[b]	Developmental Stage[c]	Body Length (mm)[d]
FETAX-IT	FETAX	50	0 (50)	0	48.6 ± 0.07	14.2 ± 0.09
FETAX-IT	MPS	50	2 (48)	0	48.6 ± 0.08	14.0 ± 0.16
FETAX-RT	FETAX	50	0 (50)	0	46.8 ± 0.09*	13.4 ± 0.17*
FETAX-RT	MPS	50	0 (50)	0	47.2 ± 0.14*	12.3 ± 0.11*

FETAX = FETAX solution (ASTM 1991) MPS = 10 mM Morton's pickling salt solution

IT = Incubator temperature (24 ± 2°C) RT = Room temperature (19-21°C)

[a] Reported as percent of total embryos for each treatment (number of surviving tadpoles)

[b] Reported as percent of surviving embryos at 96 hr with at least one malformation

[c,d] Values represent mean ± SEM

* Significantly different ($p<0.05$) from FETAX-IT-FETAX using ANOVA and Tukey/Kramer post-hoc test

androgen-dependent laryngeal development, and long-term effects on reproductive competence are not evaluated. Therefore, we studied the long-term effects of chronic developmental exposure to an ubiquitous pollutant, DBP, at environmentally relevant doses using *Xenopus laevis*.

3.4.1 Materials and Methods

An overview of the experimental design is presented in Figure 6 and the procedures are described in detail in subsequent sections. Embryos were raised and dosed according to ASTM (1991) standards for the first 96 hr of life (FETAX conditions). At 96 hr a random sample of 100 tadpoles from each of 6 DBP treatment groups, 100 from Dimethyl sulfoxide (DMSO) (Fisher Scientific, Pittsburgh PA) control and 200 from FETAX-solution-only control were euthanized and evaluated according to ASTM (1991) standards. The remaining tadpoles were transferred to 20-gallon glass aquariums, maintained at a constant population density, and dosed until 12 wk (when 90% of controls metamorphosed). Starting at 3 wk (stage 52, premetmorphosis) metamorphic changes were documented by digitally photographing a random sample of at least 25% of surviving tadpoles in each treatment group and measuring the hindlimb:tail ratio. Triiodothyronine (T_3) was assayed in whole body homogenates of 20 tadpoles in each treatment at 7 wk (stage 59-62, metamorphic climax) and 12 wk (stage 66, when metamorphosis is complete). Male frogs (n = 5-15/treatment group) were evaluated at 38 wk (puberty) for laryngeal development, hypothalamic GnRH content and concentrations of testosterone. At 68 wk (sexual maturity), two groups of male frogs (n = 4-8/treatment group) were evaluated for sexual capacity and laryngeal development or structural integrity of testis and excurrent ducts, hypothalamic GnRH content, and

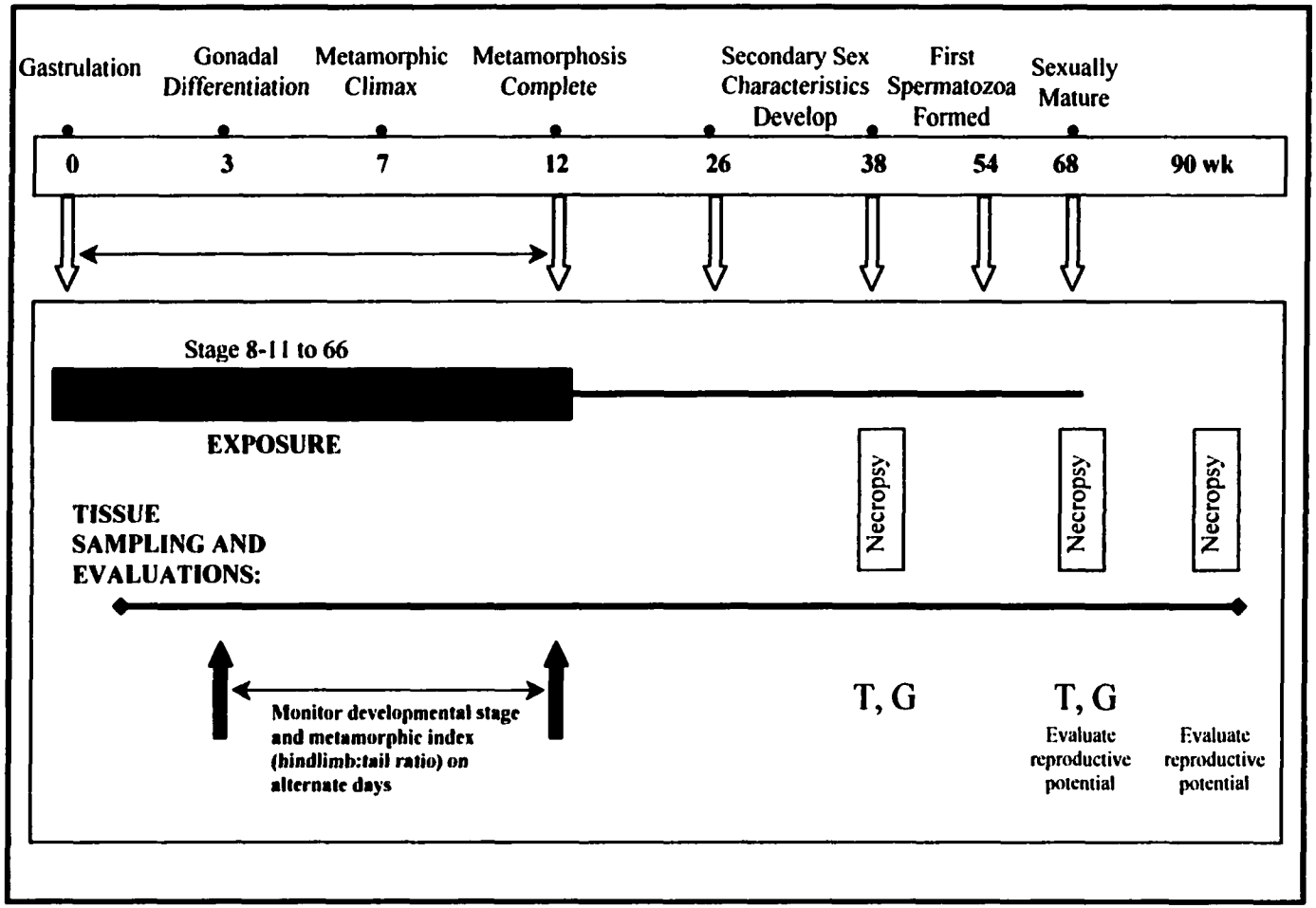


FIG. 6. Overview of study design to characterize the effects of DBP in *Xenopus laevis*. Open arrows indicate evaluation of developmental defects and progression of growth. Solid arrows indicate measurement of thyroid hormones. ***(T)*** Indicates measurement of testosterone in serum. ***(G)*** Indicates determination of hypothalamic gonadotropin-releasing hormone (GnRH) content.

concentration of testosterone. A subset of frogs from control and DBP groups were continued for extended observation of sexual capacity and laryngeal development at 90 wk.

3.4.1.1 Breeding Frogs

Proven breeder *Xenopus laevis* frogs were obtained from Xenopus One (Dexter, MI) and housed by sex (2 frogs/gallon) in opaque polypropylene tanks containing 10 mM NaCl (Morton's Pickling Salt) solution in aged-deionized water (Wu and Gerhart, 1991). Frogs were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility at Colorado State University. The room was maintained at 12 hr light-dark cycle and 19-21°C. Aquamax Grower 600 (PMI Nutrition International, Inc., Brentwood, MO) was provided three times a week; water was changed several hours later.

3.4.1.2 Breeding and Embryo Collection

Breeding was induced by fasting breeding pairs (n = 8) for 48-72 hr and injecting hCG (500 IU for males and 900 IU for females) into the dorsal lymph sac. Each breeding pair was placed into a 2.5-gallon glass aquarium fitted with a false bottom to facilitate egg collection, covered with a cardboard box, and isolated for 24 hr. Aquariums contained FETAX solution (pH = 7.6-7.9) (Appendix B). Twenty-four hr post-hCG injection (6 hr post-fertilization), breeding pairs were removed, and each viable clutch was individually washed in 2% w/v L-cysteine (pH= 8.1) (Sigma-Aldrich, St. Louis, MO) and rinsed several times with fresh FETAX solution. Good quality embryos were selected from 2 breeding pairs using a two-tier method based on stage of development

(stages 8-11; Nieuwkoop and Faber, 1967) and viability. Throughout the experiment embryos from the two clutches were maintained separately.

3.4.1.3 Dosing

DBP (99.8% pure) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Dosing solutions were made weekly by dissolving the appropriate amount of DBP into DMSO so that the final concentration of DMSO was 0.01%. DMSO was chosen because of its high LC_{50} (1.92% v/v) and EC_{50} (1.57%) in *Xenopus* embryos (Dresser *et al.*, 1992).

Each clutch was exposed to 0.1, 0.5, 1, 5, 10, or 15 ppm DBP in 0.01% DMSO (n = 200/dose group), vehicle alone (0.01% DMSO; n = 200), or FETAX solution only (n = 250) from 6 hr post-fertilization (stage 8-11) until metamorphosis at 12 wk (stage 66; when at least 90% of controls metamorphosed). The doses selected for DBP were based on the levels found in industrial ports (Penalver *et al.*, 2000), urinary metabolites found in a human reference population (high ends; Blount *et al.*, 2000), and our preliminary data on the effects of DBP on embryo development (*Section: 3.3.2: Dose Response*).

3.4.1.4 General Procedures

Throughout the experiment, mortality was recorded each time the water was changed. Body weights were recorded at 7, 12, 26, 38, 54 and 68 wk. At 36, 68, or 90 wk frogs were euthanized in a 0.2-0.5% w/v 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO) solution, and blood samples were collected by ventricular puncture. Each time 0.05 to 2 ml of blood was collected, plasma separated, and stored at -20°C for hormone assays.

3.4.1.5 FETAX Procedures

Embryos were placed in 100 mm Pyrex petri dishes at a population density of 2.5 tadpoles/ml and incubated at 24 ± 2°C. Media were changed every 24 hr. Temperature and pH of the media were recorded daily, and any dead embryos were removed and counted. At 96 hr of age (stage 46), when primary organogenesis is normally complete, a random sub-population of surviving tadpoles from each clutch (n = 50) for 0.1, 0.5, 1, 5, 10, 15ppm DBP, 0.01% DMSO (n = 50) and FETAX solution only (n = 100) were euthanized and fixed in 3% formalin for evaluation. The number of tadpoles evaluated (n = 100 per treatment and n = 200 from FETAX-solution-only control) represents the minimum number of tadpoles required for a valid FETAX assay (ASTM, 1991). Tadpoles were stained with 2% basic fuchsin and examined using a dissecting microscope (10-15X magnification) to determine developmental stage based on criteria defined by Nieuwkoop and Faber (1967) and identify any malformations (Bantle *et al.*, 1999b). The 96-hr EC_{50} was determined based on the number of surviving tadpoles with at least one malformation. Images of tadpoles were captured using a Sony DXC15A color video camera fitted with a Nikon AF Nikkor Lens and body length measured using Image Pro Plus (version 3.0). These values were used to determine the minimum concentration to inhibit growth as reflected by body length.

3.4.1.6 Post-FETAX Rearing Procedures

At 96 hr of age (stage 46), the remaining tadpoles were transferred to 20-gallon glass aquariums (24x12x12 inches) containing the appropriate dosing solution at a population density of 2.5 tadpoles/L. Because of the large volume of medium (~60 L) required, medium was prepared directly in the aquarium. A 60X concentrated FETAX

solution (Appendix B) and 120X bicarbonate solution were first added to the aquarium and then aged-deionized water was pumped into the tanks by a sump-pump while adding the corresponding dosing solution. Animals were transferred to the aquarium 30 minutes later. The room was maintained with a 12 hr light-dark cycle at 19-21°C. Starting on day 5 (~stage 47) tadpoles were fed daily ground trout chow (Xenopus One, Dexter, MI) blended in deionized water until 12 wk (stage 66). Beginning at 12 wk tadpoles were fed on alternate days Aquamax Grower 400 from 12-36 wk or a combination of Aquamax Grower 400 and 600 from 36 to 68 wk. Feeding and population density were adjusted to facilitate unrestricted growth. Clean aquaria and media were provided every other day.

3.4.1.7 Metamorphosis Assay

A. Metamorphic Index and Stage of Development

To assess attainment of metamorphosis, stage of development and metamorphic index (hindlimb:tail ratio) (Tata, 1993) were recorded. A random subpopulation of at least 25% of surviving tadpoles (n = 40 or 80/ treatment group) was photographed using a Nikon 990 digital camera on alternate days from 3-20 wk. The number of tadpoles observed was based on preliminary data (not presented), which was used to determine that 20 was the minimum number of tadpoles per treatment group for α=0.05 and β=0.05. Lengths of hindlimb and tail for metamorphic index were measured using Image Pro Plus 4.0 for Windows. Tadpoles were considered to have attained metamorphosis when they reached stage 66 (Nieuwkoop and Faber, 1967).

B. Radioimmunoassay of Triiodothyronine

Of the tadpoles used in metamorphosis assay, 10-20 tadpoles/treatment group were terminated at 7 wk (stages 59-62, metamorphic climax,) and 12 wk (stage 66, when

at least 90% of controls metamorphosed), frozen in liquid nitrogen and stored at -80°C (Gutleb *et al.*, 2000) for subsequent quantification of triiodothyronine (T_3) in whole body homogenates. Because of the small size of the tadpoles and difficulty obtaining sufficient blood samples at this time, whole body homogenates were utilized. T_3 was extracted according to procedures described by Bray and Sicard (1982) and Gutleb *et al.* (2000) with minor modifications. Briefly, tadpoles (n = 1-3) were homogenized in an Eberbach semi-micro stainless steel container using a Warning blender for three bursts of 10 seconds in 30 ml of 1:10 formic acid: methanol solution. The homogenate was transferred to 50 ml conical tube and the blender was rinsed with 10 ml formic acid: methanol solution. ^{125}I-labeled T_3 (~45000 CPM) (Diagnostic Systems Laboratories Inc., Webster, TX) was added to determine the extraction efficiency and recovery. The samples were vortexed and incubated at 4°C for 24 hr.

Samples were centrifuged for 30 min at 3000 rpm and 4°C in a Beckman J-6B, the supernatant poured into 15 ml conical tubes and stored for 24 hr at -20°C (Moss et al, 1980). This step was repeated two to three times. After the final centrifugation, 4 ml was pipetted into 12x75 mm glass tubes, dried under N_2 at ~30°C and counted for one minute using an Apex Automatic Gamma Counter (Micromedia Systems Inc.) to determine extraction efficiency. One ml of zero calibrator serum (Diagnostic System Laboratories Inc., Webster, TX) was added to each sample, vortexed, and stored at 4°C for 24 hr. Finally, samples were vortexed, centrifuged 30 min at 2000 rpm and 4°C, poured into 12x75 mm polypropylene tubes and counted to determine recovery. Extraction efficiency and recovery were 89% and 65%, respectively.

Amounts of T_3 (bound and free) were determined using a commercially available kit (Diagnostic System Laboratories Inc., Webster, TX) following the protocol outlined. Data from the manufacturer indicated that this assay was highly specific for T_3 (0.004% cross reactivity with reverse T_3, 0.003% cross reactivity with T_4 and <2.76% for other T_3 metabolites) and the sensitivity of the assay was 4.3 ng/dl (two standard deviations below maximal binding). Prior to measuring T_3 in tadpoles included in the study, an RIA procedure with sufficient specificity, accuracy and precision (Midgely *et al.*, 1969; Nett and Malvey, 1999) to quantify T_3 from whole body homogenates of tadpoles was validated (Appendix C). Specificity was defined as the lack of interference from substance other than the one to be measured (Nett and Malvey, 1999). Specificity of the assay was determined by comparing the parallelism of the T_3 standard curve with dilutions (0.25, 0.5, 0.75) of two different samples. There was no significant difference ($p>0.42$) between the slope of the T_3 standard curve and the serial dilutions. Accuracy was the extent to which the mean of an infinite number of measurements of a particular ligand agrees with the exact amount of ligand present (Nett and Malvey, 1999). Accuracy was determined by comparing the amount of T_3 added (25, 50, 100 and 200 ng/dl) to serum samples with the amount measured. The slope of the line was 0.961 and coefficient of correlation was 0.995. Precision was defined as the extent to which a given set of measurements for the same sample agrees with the mean (Nett and Malvey, 1999). Intra-assay coefficient of variation was determined from 5 or 13 replicates of two different extracts and inter-assay coefficient of variation was determined from 2 samples measured in 2 separate assays. These values were 4.4 and 15.6%, respectively.

3.4.1.8 Tissue Collection and Processing

Following euthanasia at 36 and 68 wk, visceral and reproductive organs were examined for any gross abnormalities and weighed. Larynx and kidneys-testes were fixed in a solution containing 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer (Robertson and Kelley, 1996) and processed for histological evaluation. Kidney and testes were removed as a unit to ensure that testicular excurrent ducts were not damaged.

Kidneys-testes unit was rinsed in 0.1 M phosphate buffer, cut mid-sagitally, immersed for 90 minutes in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, rinsed in buffer, dehydrated through graded ethanols, and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thick sections were cut and stained with Toluidine Blue or Laczko stain with Safranin O (Laczko and Levai, 1975) for light microscopy. Thin (60-80 nm) sections were cut, stained with Sato's stain (Sato, 1968) and examined using a JEOL-1200EX transmission electron microscope.

Larynges were bisected along the mid anterio-posterior axis (this is the site where number of muscle fibers are largest (Sasson and Kelley, 1986)), dehydrated through graded ethanols, embedded in paraffin, sectioned at 5μm, and stained with hematoxylin and eosin (H&E).

3.4.1.9 Morphometry of Testis, Excurrent Ducts and Larynx

Using toluidine blue-stained thick sections from the proximal and distal poles of the testis, stages of cell nests in the seminiferous epithelium were quantified in 3 groups (n = 4 frogs/treatment) of 68-wk-old frogs – FETAX-solution-only control; 0.5ppm and 5ppm DBP. Spermatogenic nests within all essentially round (<1.5X width) cross

sections of seminiferous tubules were classified into one of four stages of spermatogenesis based on Kanamadi *et al.* (1983) with minor modifications (Table 7).

Normalcy of the seminiferous epithelium and interstitium were evaluated with particular attention to changes in the germ cell population, interstitium, and vascular supply. A separate set of serial sections was cut from the same tissue blocks used in spermatogenic evaluation and stained with Laczko stain with Safranin O. Patency of testicular excurrent ducts and normalcy of epithelium were evaluated using Nikon Microphot FXA light microscope and JEOL-1200EX transmission electron microscope.

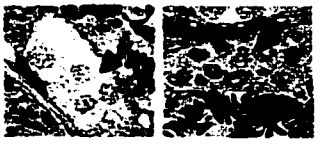
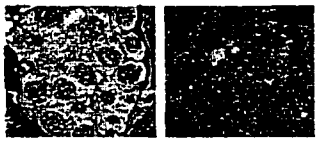
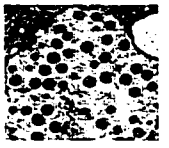
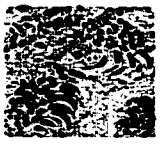
The number of dilator larynges muscle fibers, a measure of androgen-dependent development (Kelley, 1996; Sasson and Kelley; 1986; Tobias *et al.*, 1998) was determined in male frogs at 36 and 68 wk of age. Images of dilator larynges muscle fibers for one side of the larynx were captured using an MTI CCD camera attached to Nikon Microphot-FXA microscope interfaced with a Macintosh G3 computer having Image Pro Plus (version 3.0). Area of dilator larynges was measured at 50X magnification and individual muscle fibers were counted at 500X. Six rectangular reticles (5015.5 μm^2) were randomly placed on the images captured at 500X and the number of muscle fibers was counted within each rectangle. The total number of muscle fibers per larynx was calculated using the equation below:

$$\frac{\text{Average \# of muscle fibers}}{5015.5\ \mu m^2} * \text{area } (\mu m^2) * \frac{\text{2 sides}}{\text{larynx}} = \text{total number of muscle fibers in larynx}$$

3.4.1.10 Reproductive Hormones

Hypothalamic GnRH content (Sower *et al.*, 2000) and serum testosterone (Kang *et al.*, 1995) were assayed at 36 and 68 wk.

TABLE 7
Staging criteria for evaluation of *Xenopus laevis* spermatogenesis

Stage	Cell type	
1	Spermatogonia (primary and secondary)	
2a	Young primary spermatocytes (Leptotene to Zygotene)	
2b	Old primary spermatocytes (Pachytene to Diplotene)	
3	Secondary spermatocytes	
4a	Round spermatids	
4b	Condensing spermatids	
4c	Condensed spermatids	

Based on Kanamadi *et al*., 1983

A. Radioimmunoassay of Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) was extracted according to Moss *et al.* (1980). Briefly, hypothalami were homogenized in storage media using a Polytron PT10/35 with a standard generator with saw teeth (Kinematica Inc., Cincinnati, OH) at a setting of 7 for three bursts of 10 seconds. ^{125}I-GnRH (~2,500 CPM) was added to determine extraction efficiency and recovery. Samples were extracted as described above (*Section: 3.4.1.7.B: Radioimmunoassay of Triiodothyronine*) and 2 ml of 0.1 M phosphate-buffered saline containing 0.1% gelatin (PBS-gel) was added to each sample, vortexed and stored at 4°C for 24 hr. Finally, samples were vortexed, centrifuged from 30 min at 2000 rpm at 4°C, poured into 12x75 mm polypropylene tubes and counted to determine final recovery. Extraction efficiency and recovery were 91 and 87%, respectively.

RIA procedures were according to Nett and Adams (1977) and have been previously validated for amphibians (Sower *et al.*, 2000). The antisera used for this assay were R-42 (final dilution = 1:80,000) and A853 (final dilution = 1:30) and their specificity has been previously described (Nett *et al.*, 1973). Prior to measuring the hypothalamic GnRH content, the specificity and accuracy of the assay were verified in a preliminary experiment (Appendix D). Specificity of the assay was determined by comparing the parallelism of the GnRH standard curve with dilutions (0.025, 0.05, 0.1, 0.25, 0.5) of one sample. There was no difference ($p<0.1$) in the slopes of the standard curve and serial dilution. Accuracy of the assay was determined by adding 1, 1.25, 2.5 and 5 ng/ml GnRH to samples and plotting the amount of ligand added to the amount measured. The slope of the line was 1.09 and coefficient of correlation was 0.976.

B. Radioimmunoassay of Testosterone

Testosterone (bound and free) was measured using a commercially available kit (Diagnostic Products Corporation, Los Angeles, CA), which has been previously validated for *Xenopus laevis* (Kang *et al.*, 1995). Data from the manufacturer indicated that this assay was highly specific for testosterone (3.3% cross reactivity with dihydrotestosterone (DHT), 0.02% cross reactivity with estradiol and <2% for other steroids present in serum) and the sensitivity of this assay is 4 ng/dl (two standard deviations below maximal binding). Prior to measuring testosterone in tadpoles used in this study, specificity and accuracy of the testosterone assay were verified in a preliminary experiment (Appendix E). Specificity of the assay was determined by comparing the parallelism of the testosterone standard curve with dilutions (0.0625, 0.125, 0.25, 0.5) of one sample. There was no difference ($p<0.7$) in the slopes of the standard curve and serial dilution. Accuracy of the assay was determined by adding 20, 100, 400, and 800 ng/dl testosterone and plotting the amount of ligand added to the amount measured. The slope of the line was 0.998 and coefficient of correlation was 0.998. Due to the small volume of serum collected from some frogs, serum samples from frogs with similar body weights within a treatment were pooled.

3.4.1.11 Reproductive Ability: Male Advertising Call and Amplexus

Reproductive ability of male frogs was assessed at 68 wk (sexual maturity) and in a subset of frogs from FETAX solution control and 0.5 and 5 ppm DBP at 90 wk. Each time, 4-8 males/treatment were injected 500IU hCG and placed with primed adult females (750IU hCG) in 20-gallon glass aquariums filled with 45 L of FETAX medium. A hydrophone (High Tech Inc., model MTI-96-min) with an output sensitivity of -165 dB

at 1V/μPa and a frequency sensitivity of 0.002 to 10 kHz was placed in the center of the aquarium at a depth of 0.6 m. Beginning at 2 hr post-hCG, the sounds generated by frogs were recorded for 2 hr with a Marantz CDR-631 CD recorder.

The male advertisement call is a loud and distinct set of trills given only by sexually receptive males to attract females (Tobias *et al.*, 1998). To avoid any effects caused by exogenous noises, tanks were enclosed in 24x18x30 inch particleboard boxes lined with 1.5-inch open cell foam. This enclosure confined the sounds to the aquarium and minimized extraneous noises. Recorded male advertisement calls were filtered (to reduce 60-Hz noise) and the amount of time calling was determined using Goldwave (version 2.45).

After the sound recording, frogs were left in the enclosures and the ability to form successful amplexus was recorded at 24 hr. If no amplexus was formed, males were injected a second time with 500IU hCG and placed into the enclosure with a new primed female for a second set of recordings. Frogs were euthanized and tissues collected (*Section: 3.4.1.8: Tissue Collection and Processing*) at the end of observation period.

3.4.1.12 Statistical Analysis

For each point of observation, data were analyzed by analysis of variance (ANOVA) using Statview (version 5.0, SAS Institute Inc., Cary, NC). Treatment was fixed and all parameters were randomly measured. Difference between means were determined by Fisher's protected least significant differences and Tukey/Kramer post-hoc test. The level of significance was set at $p<0.05$. This model was used to evaluate body weights, hormone concentrations, and necropsy data. Percentage values were transformed using arcsine of the square root of the percentage/100 to account for any

inequalities in variance (Ott, 1993). Linear regression was performed on body weight, metamorphic index, and for determining accuracy of RIA. Logistic regression was performed on standard curves for RIA and to determine the specificity of the assay. Slopes of the lines from regressions were compared using analysis of covariance (ANCOVA).

3.5 Results

3.5.1 General Toxicology

The cumulative mortality rates for all treatment groups from 0 to 68 wk of life are presented in Figure 7. During the first 96 hr of life, mortality rates for FETAX solution, DMSO, 0.1, 0.5, 1, 5, 10, and 15 ppm DBP were 6, 5, 3, 5, 5, 7, 33, and 75%, respectively. Mortality reached 100% in 15 ppm DBP group by 1 wk. At this age, cumulative mortality rates for FETAX solution, DMSO, 0.1, 0.5, 1, 5, and 10 ppm DBP were 9, 6, 5, 6, 44, 30, and 59%; and by 68 wk these values were 35, 32, 29, 38, 60, 76, and 98%.

Progression of normal development as evidenced by the slope of the linear regression of body weights at 7, 12, 26, 38, 54 and 68 wk was significantly retarded ($p<0.05$) in the 5 (slope = 0.10) and 10 (slope = 0.15) ppm DBP groups compared to FETAX solution and DMSO controls (slope = 0.56 and 0.58) (Figure 8).

3.5.2 FETAX

Incidence of malformations, stage of development and body length in a random subpopulation of surviving tadpoles at 96 hr used to evaluate early sequelae are presented in Table 8. The incidences of developmental malformations were significantly increased ($p<0.05$) in 5 (19.3), 10 (39.2%) and 15 (90.6%) ppm DBP groups compared to FETAX

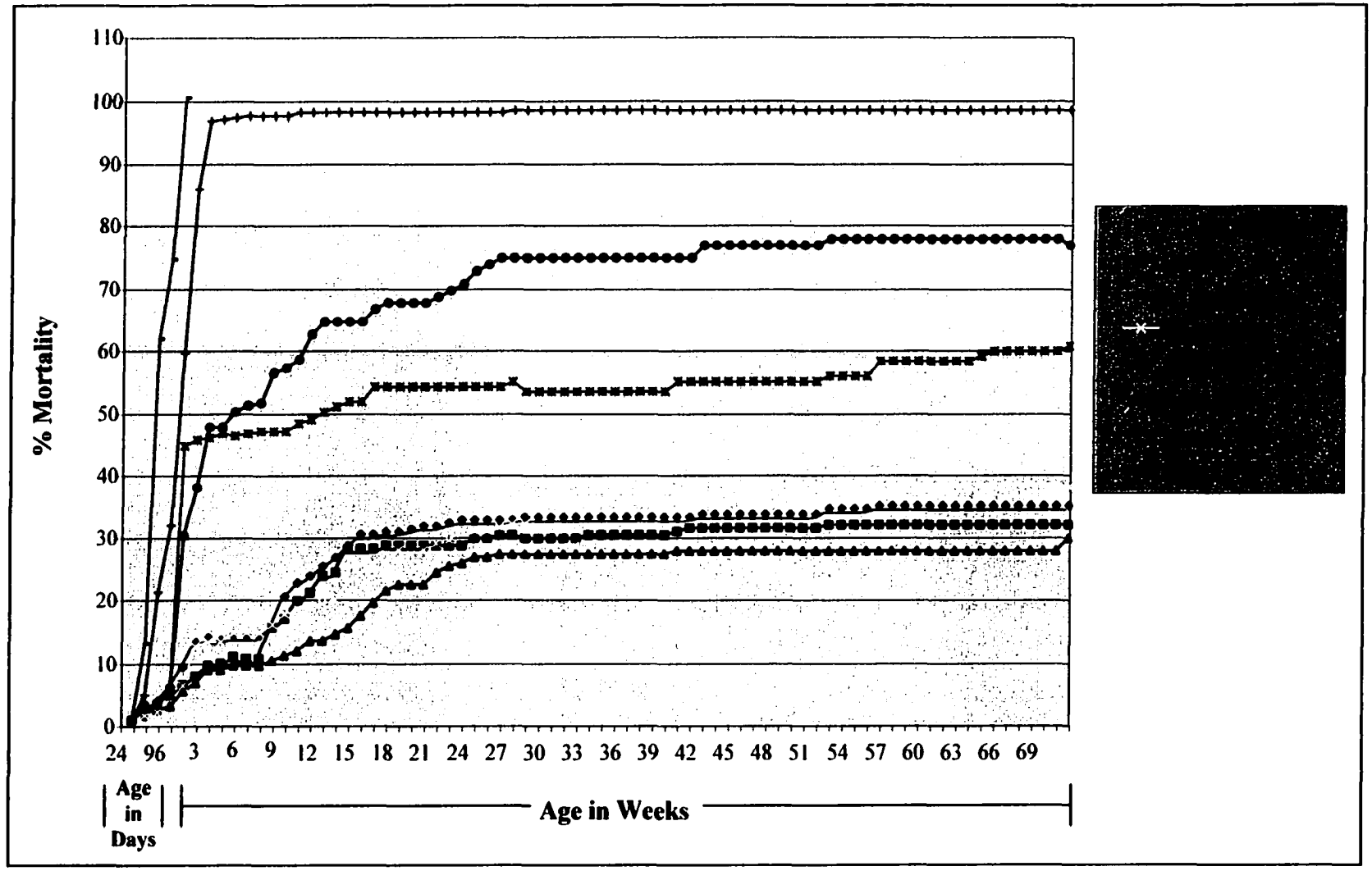


FIG. 7. Cumulative mortality rates of *Xenopus laevis* for FETAX solution or DMSO controls, and 0.1, 0.5, 1, 5, 10 or 15 ppm DBP.

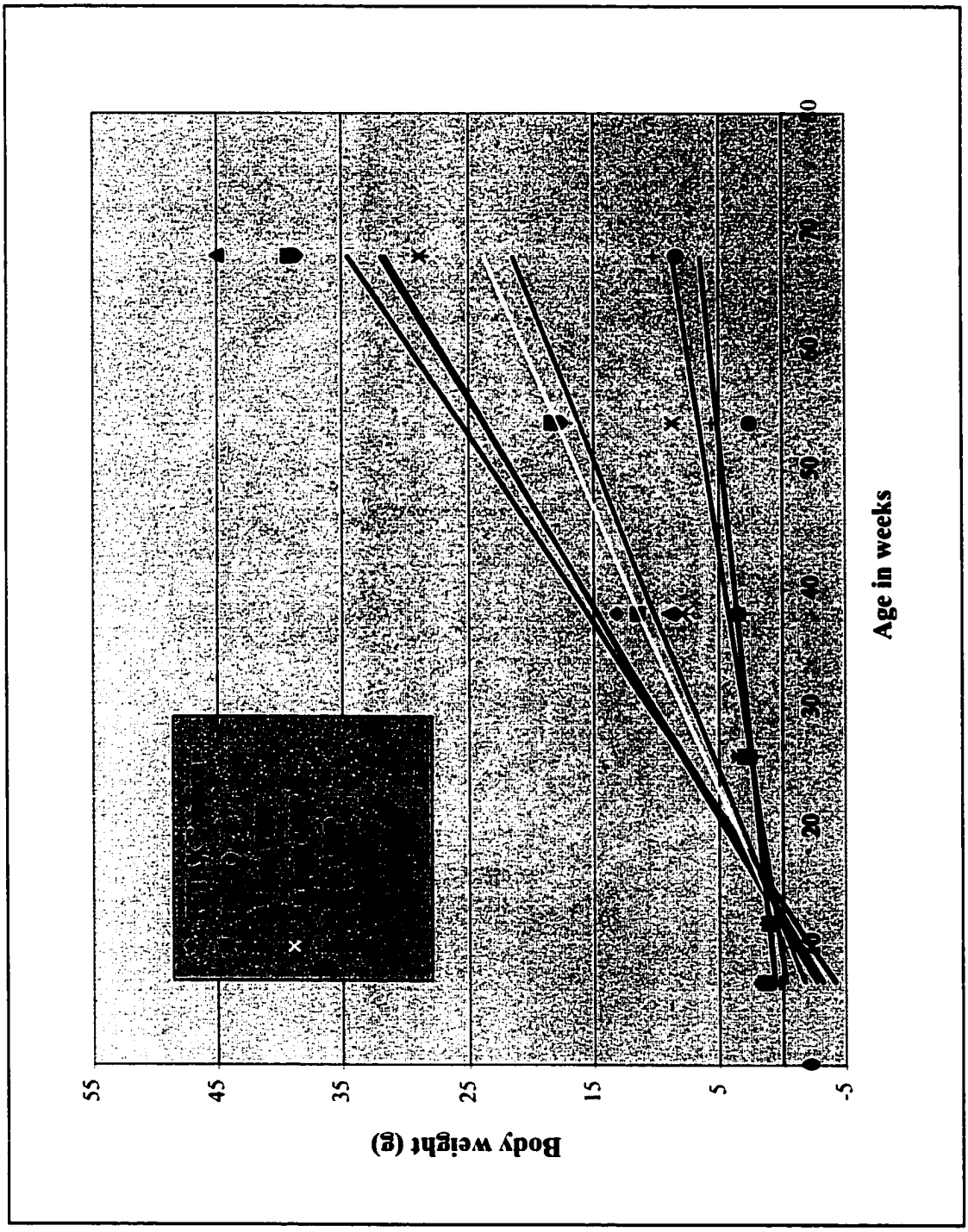


FIG. 8. Linear regression of body weights of *Xenopus laevis* at 7, 12, 26, 38, 54 and 68 wk of age.

TABLE 8
Effects of DBP on *Xenopus* embryo development at 96 hr of age

Treatment	n	Malformations[b]	Developmental Stage[c]	Body Length (mm)[d]
FETAX solution control	185	4.3	46.0 ± 0.04	9.7 ± 0.04
DMSO control	90	6.7	46.0 ± 0.07	9.7 ± 0.06
0.1 ppm DBP	97	8.2	45.9 ± 0.05	9.6 ± 0.04
0.5 ppm DBP	93	8.6	45.8 ± 0.04	9.6 ± 0.05
1 ppm DBP	92	3.4	45.7 ± 0.04	9.6 ± 0.05
5 ppm DBP	88	19.3*	45.8 ± 0.05	9.3 ± 0.05*
10 ppm DBP	51	39.2*	45.3 ± 0.11*	8.5 ± 0.05*
15 ppm DBP	32	90.6*	44.8 ± 0.15*	8.4 ± 0.05*

[a] Reported as percent of total embryos for each treatment (total number of surviving tadpoles)

[b] Reported as percent of surviving embryos evaluated for FETAX at 96 hr with at least one malformation

[c,d] Values represent mean ± SEM

* Significantly different ($p<0.05$) from control using ANOVA and Tukey/Kramer post-hoc test

solution and DMSO controls (4.3 and 6.7%) Similar to data for preliminary studies (*Section: 3.3.2: Dose Response*) the most frequent malformations were axial, gut, cardiac, and optic and their incidence increased with dose (Figure 9). Progression of normal development and mean body length were significantly retarded ($p<0.05$) in the 10 (stage 45.3, 8.5 mm) and 15 ppm (stage 44.8, 8.4 mm) dose groups compared to FETAX solution (stage 46.0, 9.7 mm) and DMSO (stage 46.0, 9.7 mm) controls. The 96 hr-LC_{50} was between 10 and 15 ppm DBP, while the 96 hr-EC_{50} (malformations) was between 10 and 15 ppm DBP. The teratogenic index (TI) was 0.67-1.5. The minimum concentration to inhibit growth and NOEL for stage of development was 5 ppm DBP. The NOEL for mortality, malformations and body length was 1 ppm DBP. These values are higher/lower than those observed in preliminary studies (*Section: 3.3.2: Dose Response*) and may be due to the use of DMSO as a solvent in the definitive experiment; since DMSO is an effective anti-inflammatory agent (reviewed by Murdoch, 1982) and may have ameliorated any treatment-related injuries.

3.5.3 Metamorphosis Assay

3.5.3.1 Metamorphic Index and Stage of Development

At 12 wk 76 (61/80), 80 (69/80), 68 (27/40), 35 (14/40) and 0% (0/8) of 0.1, 0.5, 1, 5 and 10 ppm DBP groups attained metamorphosis versus 91 (73/80) and 96% (77/80) in FETAX solution and DMSO controls (Figure 10) indicating a dose-dependent delay in metamorphosis. In those that completed metamorphosis after 12 wk, the delay ranged from 1 to 8 wk. Reflective of the delay in metamorphosis, there was a dose-dependent decrease in the slope of the linear regression of the metamorphic index (hindlimb:tail ratio) from prometamorphosis (5 wk, stage 55) to metamorphosis (12 wk, stage 66)

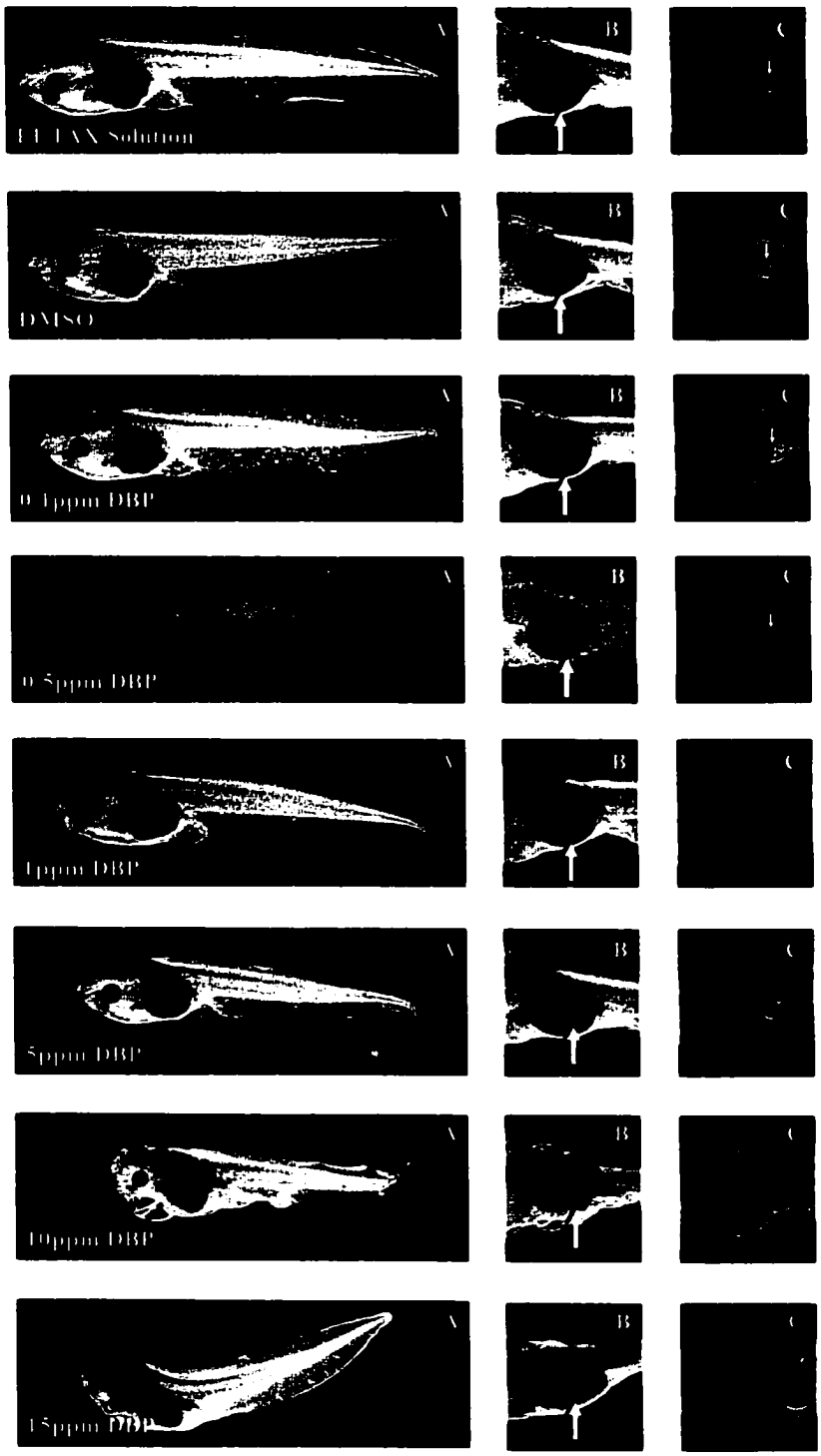


FIG. 9. Macroscopic differences of *Xenopus laevis* tadpoles exposed to FETAX solution, DMSO, 0.1, 0.5, 1, 5, 10 and 15 ppm DBP at 96 hr of age. ***A.*** Lateral view of entire tadpole. ***B.*** Ventral aspect of cranial pole. ***C.*** Lateral aspect of abdominal region. All images within each panel were captured at the same magnification ***A*****:** 6X ***B*****:** 8X ***C*****:** 8X. ***Large Arrows*** point to gut. ***Circles*** enclose the area where hindlimb-bud emerges and ***small arrows*** point to hindlimb-bud.

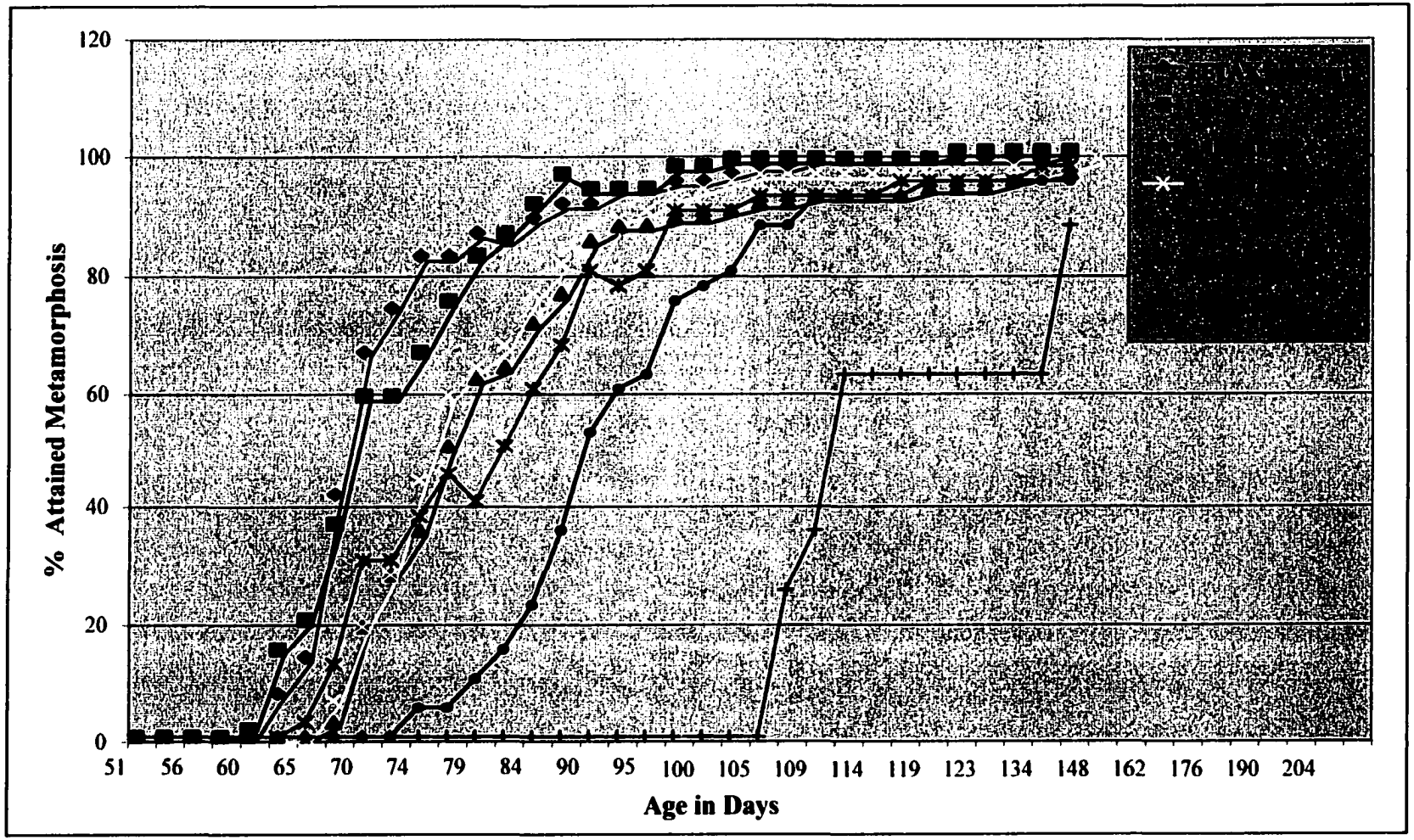


FIG. 10. Time to complete metamorphosis was monitored every other day in at least 25% of surviving animals from 21 to 140 days of age.

(Figure 11). This period encompasses the onset of metamorphosis until completion of metamorphosis in at least 90% of control animals. The slopes of the lines from prometamorphosis to metamorphosis were significantly less ($p<0.05$) in 1, 5 and 10 ppm DBP (0.219, 0.097 and 0.005) compared to FETAX solution and DMSO controls (0.343 and 0.341). The qualitative differences in stage of development at 5, 7, 9 and 12 wk are obvious in univariate scattergrams (Figure 12A) and upon macroscopic observation (Figure 12B and 12C).

Interestingly, one tadpole in the 0.5 ppm DBP group failed to complete metamorphosis even at 26 wk of age and had a malformed hindlimb (Figure 13). One froglet from the 0.1 ppm DBP group had an extra limb protruding from the axilla (Figure 13).

3.5.3.2 Concentrations of Triiodothyronine

Unfortunately problems were encountered measuring T_3 in the whole body homogenates of experimental tadpoles. When the assay was validated, concentrations of T_3 in whole body homogenates were 69.4 and 3.7 ng/g for stage 60-62 and 66, respectively. This difference in T_3 between stage 60-62 and 66 has been previously reported (Leloup and Buscaglia, 1977, Bray and Sicard, 1982). However, this difference was not observed between stage 59-62 and stage 66 animals from FETAX solution only, 2.43 and 4.93 ng/g, respectively. In order to identify the potential problem, all samples were re-extracted after adding ~2500 CPM ^{125}I-labeled T_3. The extraction efficiency and total recovery were 81.3 and 61.9%. In spite of extraction efficiency and total recovery values close to validating parameters, there was still no change in the difference in T_3 between stage 59-62 (3.69 ng/g) or stage 66 (5.58 ng/g). Adjusting for individual

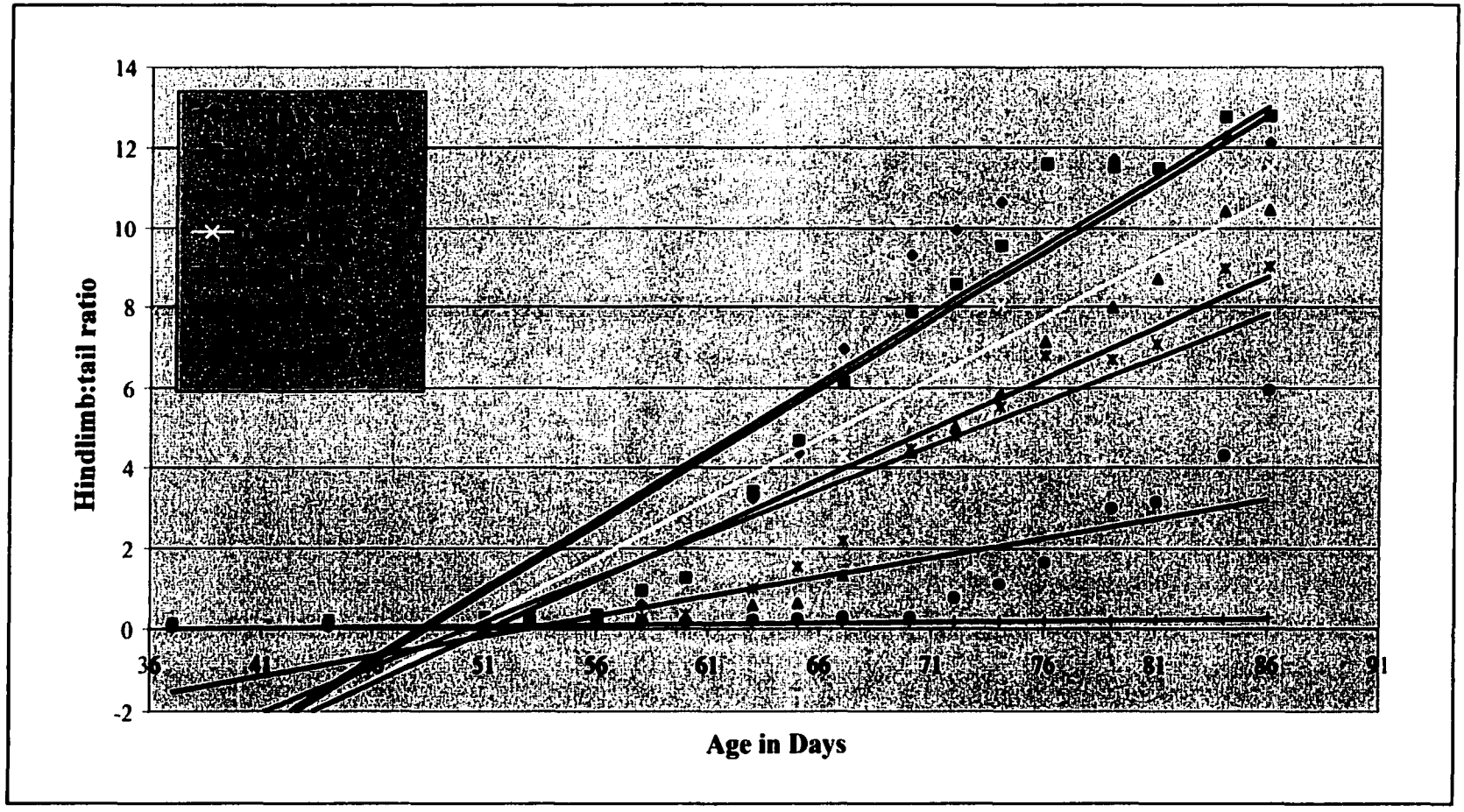


FIG. 11. Linear regression of the metamorphic index from prometamorphosis (5 wk, stage 55) until metamorphosis was complete in 90% of controls (12 wk, stage 66). This period encompasses the onset of thyroid-dependent metamorphosis until metamorphosis was complete in 90% of controls.

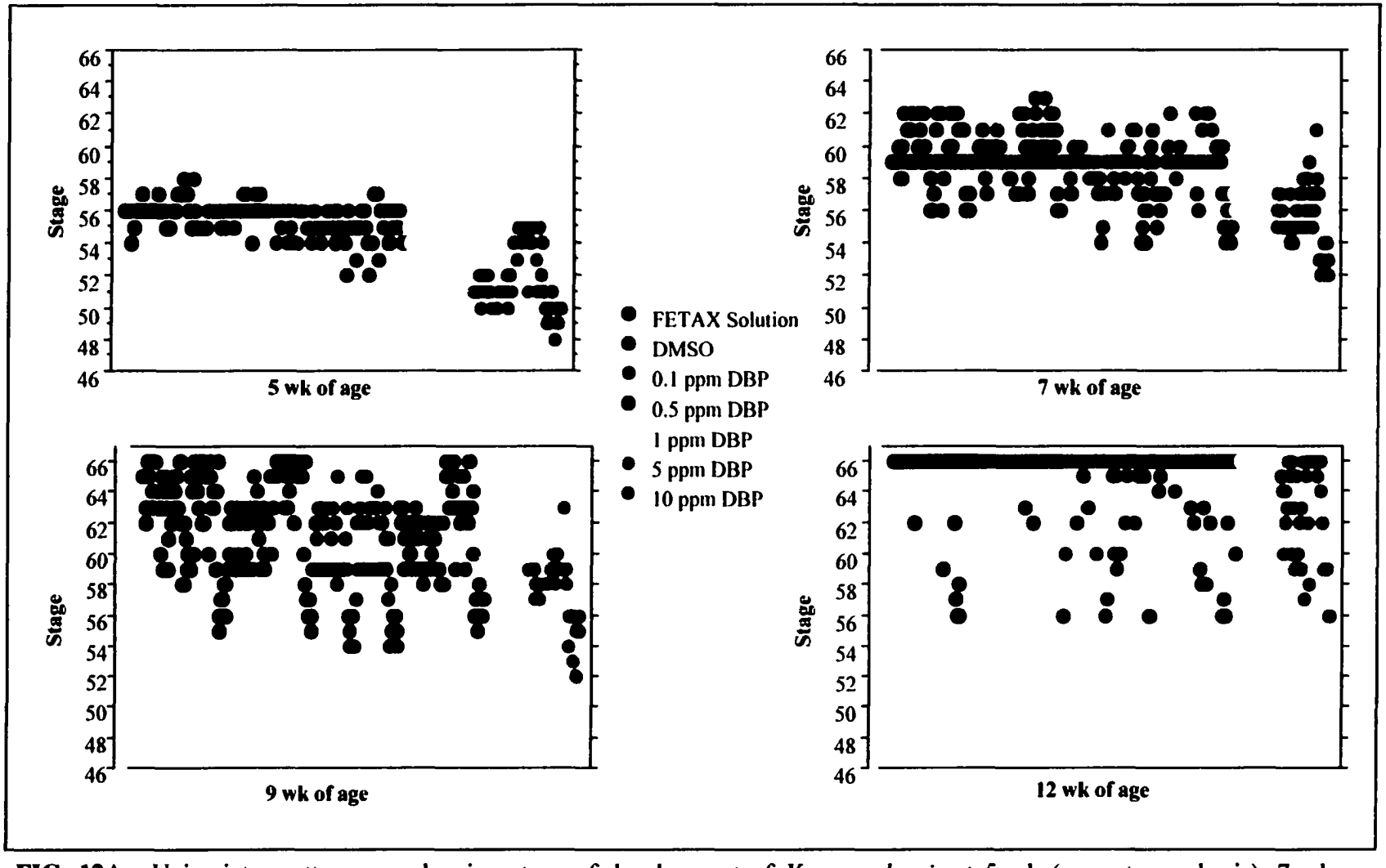


FIG. 12A. Univariate scattergrams showing stage of development of *Xenopus laevis* at 5 wk (prometamorphosis), 7 wk (metamorphic climax), 9 wk, and 12 wk (when 90% of controls attained metamorphosis).

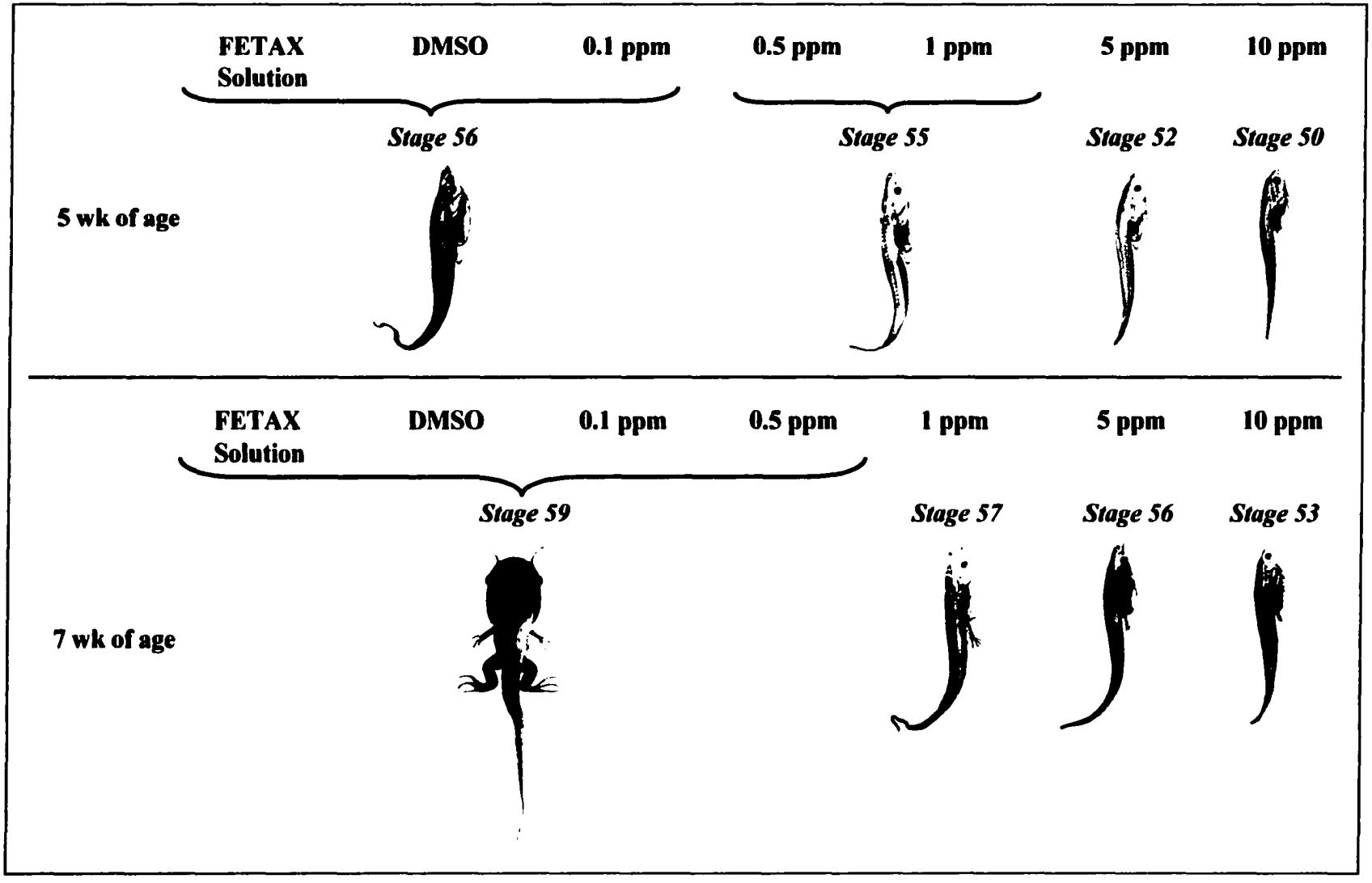


FIG. 12B. Median stage of development of *Xenopus laevis* at 5 wk (prometamorphosis) and 7 wk (metamorphic climax). Staging based on Nieuwkoop and Faber, 1967.

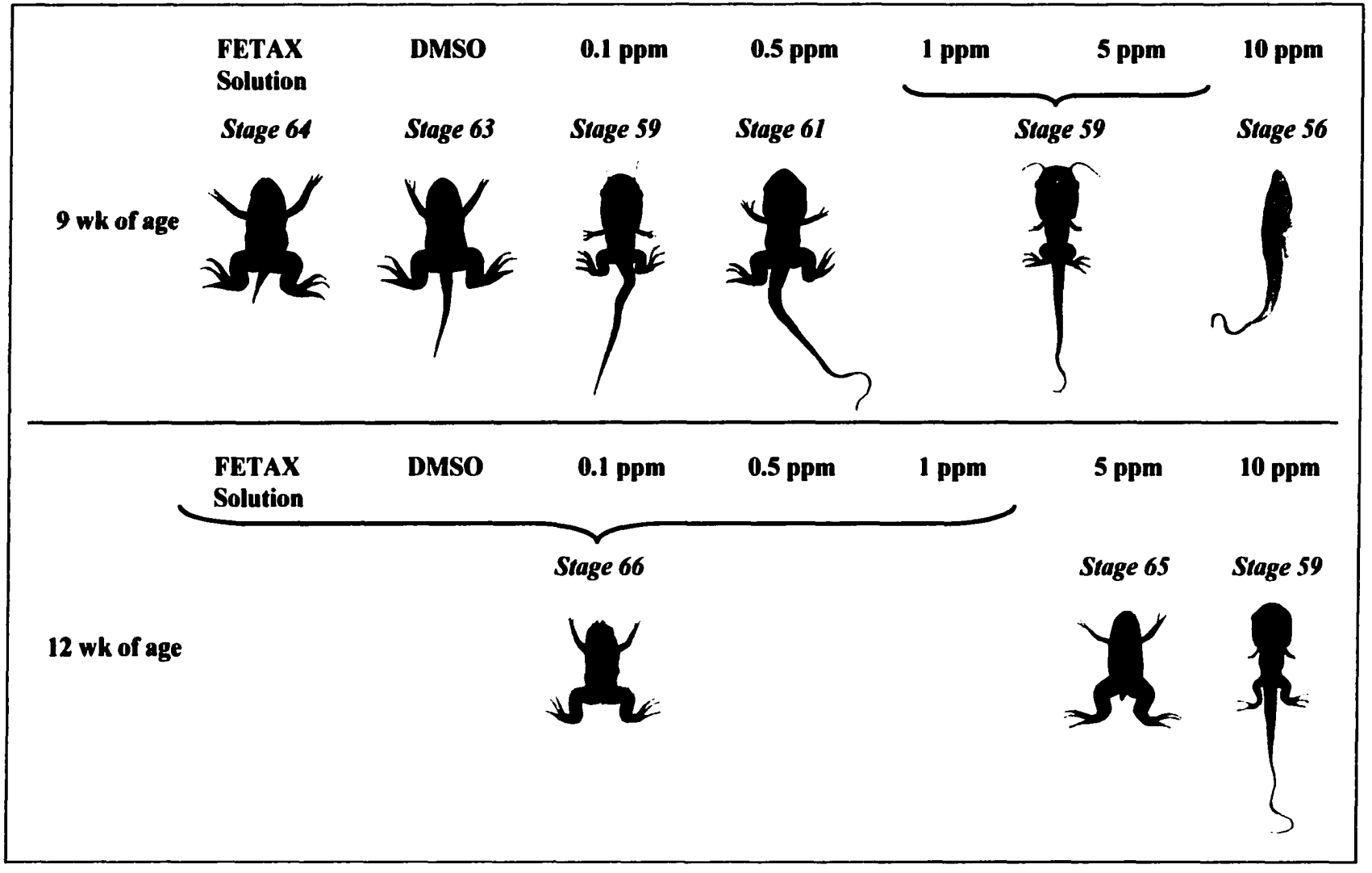


FIG. 12C. Median stage of development of *Xenopus laevis* at 9 wk (as metamorphosis continues) and 12 wk (when 90% of controls attained metamorphosis). Staging based on Nieuwkoop and Faber, 1967.

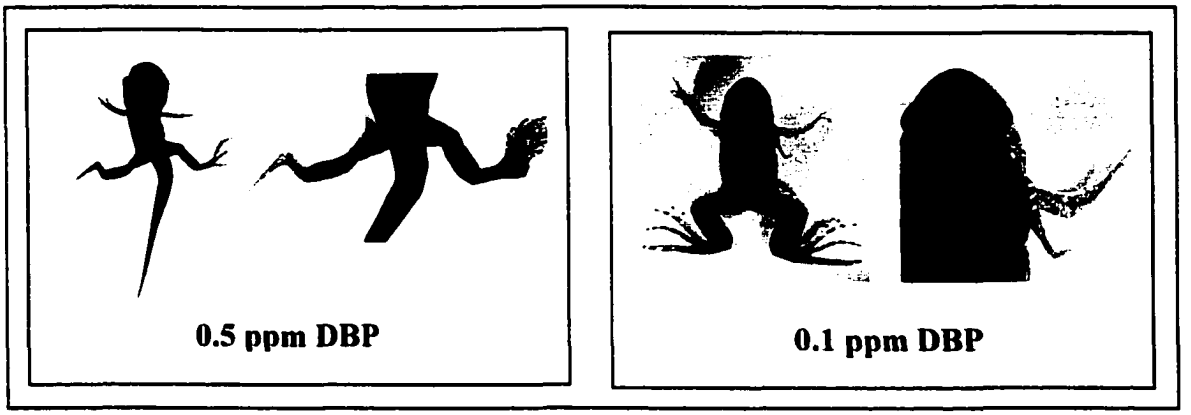


FIG. 13. Photographs of one tadpole from 0.5 ppm DBP group who failed to metamorphose even at 26 wk of age and had a malformed hindlimb and one froglet from 0.1 ppm DBP with an extra limb protruding from the axilla.

recoveries did not affect these parameters. During this time the measurement of the precision, accuracy and specificity did not change from validating parameters.

3.5.4 Organ Weights

At 38 wk of age body weights of male frogs were significantly lower ($p<0.05$) in 5 ppm DBP group compared to FETAX solution and DMSO controls (Table 9). At this age, weight of kidneys plus testes was also significantly lower ($p<0.05$) in 5 ppm DBP. At 68 wk body weights of male frogs were significantly lower ($p<0.05$) in 0.5, 1, and 5 ppm DBP groups compared to FETAX solution and DMSO controls (Table 9), while weight of liver was lower ($p<0.05$) only in 5 ppm DBP.

3.5.5 Morphometry of Testis, Excurrent Ducts and Larynx

A full complement of stages of spermatogenesis was observed in all treatment groups at 68 wk (Figure 14). However, the number of stage 2a nests (early spermatocytes) per seminiferous tubule in 5 ppm DBP and the number of stage 4c nests (condensed spermatids) in 0.5 and 5 ppm DBP were significantly lower ($p<0.05$) compared to FETAX solution control (Table 10). Interestingly, in 5 ppm DBP numerous

TABLE 9

Effects of exposure to DBP from 0 to 12 wk of age on organ weights in 38- and 68-wk-old-males

Treatment	38 wk			90 wk		
	Body (g)	Liver (mg)	Kidneys with testes (mg)	Body (g)	Liver (mg)	Kidneys with testes (mg)
FETAX solution control	11.3 ± 2.3 (10)	406.1 ± 91.9 (10)	78.9 ± 18.7 (10)	28.9 ± 2.1 (15)	817.9 ± 93.6 (15)	226.5 ± 28.8 (15)
DMSO control	11.8 ± 1.5 (5)	362.8 ± 61.4 (5)	87.0 ± 10.0 (5)	39.2 ± 4.1 (8)	1255.1 ± 203.1 (8)	271.5 ± 35.7 (8)
0.1 ppm DBP	8.9 ± 1.4 (13)	372.9 ± 67.2 (13)	74.8 ± 12.7 (13)	24.0 ± 3.0 (16)	760.8 ± 108.6 (16)	162.2 ± 16.3 (16)
0.5 ppm DBP	8.0 ± 0.8 (12)	383.8 ± 158.5 (12)	56.5 ± 4.8 (12)	17.5 ± 2.2* (17)	463.7 ± 78.6 (17)	207.8 ± 74.3 (17)
1 ppm DBP	6.7 ± 1.2 (10)	214.2 ± 43.2 (10)	62.6 ± 13.9 (10)	18.2 ± 2.2* (16)	443.6 ± 73.9 (16)	125.0 ± 15.5 (16)
5 ppm DBP	3.1 ± 0.5* (10)	75.4 ± 15.8 (10)	22.0 ± 3.3* (10)	7.8 ± 1.7* (10)	198.1 ± 82.1* (10)	66.1 ± 12.9 (10)

Values represent mean ± SEM

Numbers in parenthesis indicate number of animals

*Significantly different ($p<0.05$) from control group using ANOVA and Tukey/Kramer post-hoc test

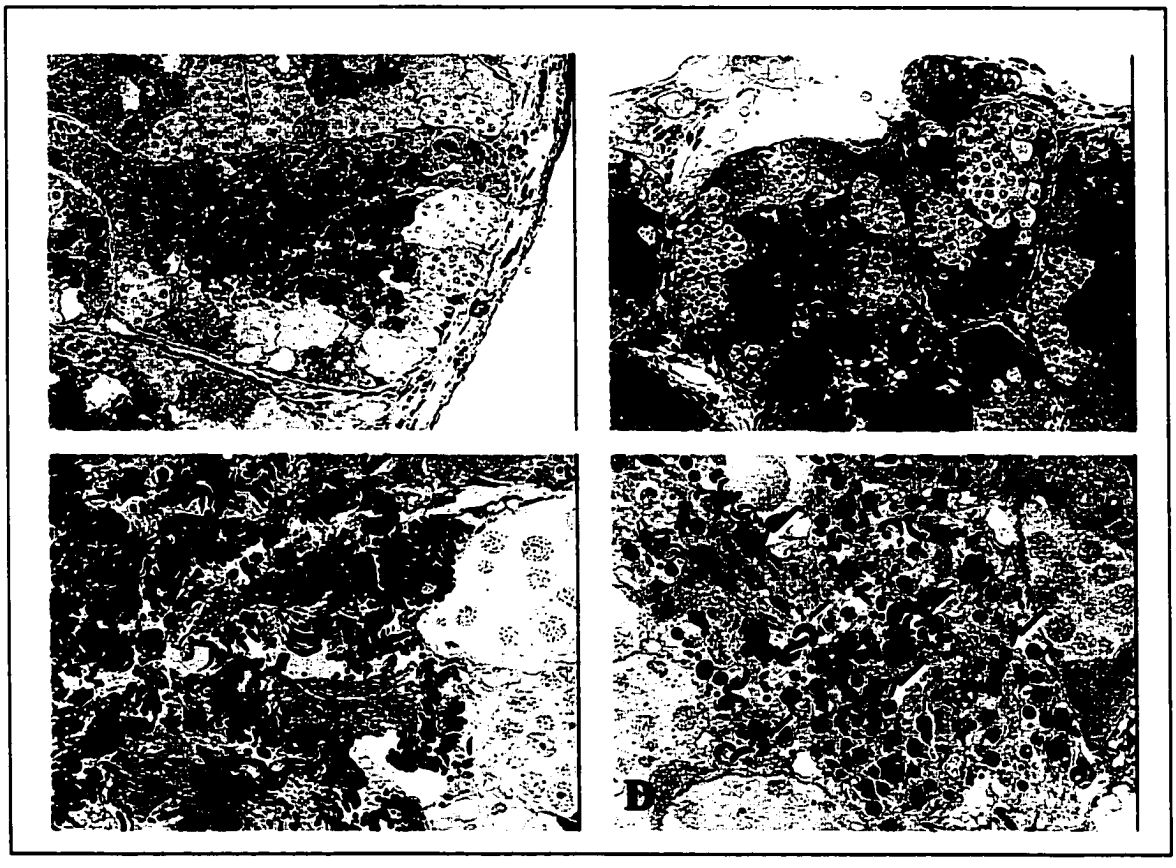


FIG. 14. Photomicrographs of seminiferous epithelium of *Xenopus laevis* at 68 wk of age. ***A,C.*** FETAX solution control. ***B,D.*** 5 ppm DBP. All stages of spermatogenesis were observed in seminiferous epithelium in control as well as DBP groups. In 5 ppm DBP numerous degenerating cells ***(large arrows)*** were identified in stage 4c nests (condensed spermatids), which contained abnormally condensed spermatids ***(small arrows) (D)***. Note changes compared to FETAX solution only stage 4c nests ***(C)***. Magnification. ***(A,B)*** 150X, ***(C,D)*** 380X. Toluidine Blue staining.

TABLE 10

Effects of exposure to DBP from 0 to 12 wk of age on spermatogenesis in 68-wk-old-males

Treatment	n	Number of cell nests per seminiferous tubule						
		Stage 1	Stage 2a	Stage 2b	Stage 3	Stage 4a	Stage 4b	Stage 4c
FETAX solution control	4	0.91	1.71	0.69	0.11	0.56	1.76	2.48
0.5 ppm DBP	4	0.81	1.44	0.56	0.09	0.48	1.32	1.43*
5 ppm DBP	4	0.77	1.00*	0.58	0.09	0.39	1.41	1.55*

*Significantly different ($p<0.05$) from control group using ANOVA and Tukey/Kramer post-hoc test

degenerating cells were observed in stage 4c (Figure 14). Electron microscopic evaluation revealed that these degenerating cells included condensing/condensed spermatids with nuclear vacuolation and inclusions, and acrosomal dysplasia (Figure 15). Some of these degenerate spermatids were located at the basal aspect of the nest undergoing phagocytosis (Figure 15B) while others on the apical aspect were being exfoliated into the lumen (Figure 15C). It is likely that these degenerative changes in late stages of spermiogenesis led to a reduction in stage 4c nests.

Normal testicular excurrent ducts lined with a squamous to cuboidal epithelium and a well-defined lumen were observed in the connective tissue joining the testis and kidney in control as well as DBP-treated frogs (Figure 16). However, several profiles of aberrant excurrent ducts that were characterized by lack of lumen and epithelial cells with irregular nuclear contours were observed in 0.5 and 5 ppm DBP groups. At ultrastructural level, normal excurrent ducts had a well-defined lumen lined with epithelial cells containing abundant filaments in the cytoplasm with few organelles and surrounded a well-defined lumen whereas aberrant ducts conspicuously lacked lumina and the epithelial cells were compacted giving the appearance of a solid cord (Figure 17).

At 38 wk of age weight of the larynx and number of muscle fibers per larynx were significantly lower ($p<0.05$) in 5 ppm DBP compared to controls (Table 11, Figure 18). The difference in number of muscle fibers per larynx between controls and 1 ppm DBP at 38 wk of age was not significant, possibly due to two outlier frogs (2 out of 10) whose values were ~1.5-fold greater. By 68 wk, weight of the larynx and number of muscle fibers per larynx remained lower ($p<0.05$) in 5 ppm DBP and the number of muscle fibers per larynx was significantly lower in 1 ppm DBP compared to FETAX solution and

FIG. 15 Transmission electron micrographs of stage 4c nests in seminiferous tubules from 5 ppm DBP group at 68 wk of age. ***A.*** Condensing/condensed spermatids with nuclear vacuolation and inclusions ***(arrows)***. ***B.*** A degenerate spermatid ***(arrow)*** at the basal aspect of the seminiferous tubule undergoing phagocytosis. ***C.*** An incompletely condensed spermatid ***(arrow)*** in the luminal aspect of the seminiferous tubule. Note normally condensed nuclei on the lower left. Magnification: 3000X

FIG. 16. Photomicrographs of testicular excurrent ducts of *Xenopus laevis* at 68 wk of age from FETAX solution control ***(A)***, 0.5 ppm DBP ***(B)*** and 5 ppm DBP ***(C)***. Excurrent ducts ***(arrows)*** with well defined lumina containing seminiferous elements were found adjacent to blood vessels ***(asterisks)*** in all treatment groups. Note the presence of aberrant excurrent ducts characterized by absence of lumen and compacted epithelial cells ***(arrowhead)*** only in 0.5 ppm DBP and 5 ppm DBP. All images are captured at 540X magnification. Laczko staining with Safranin O.

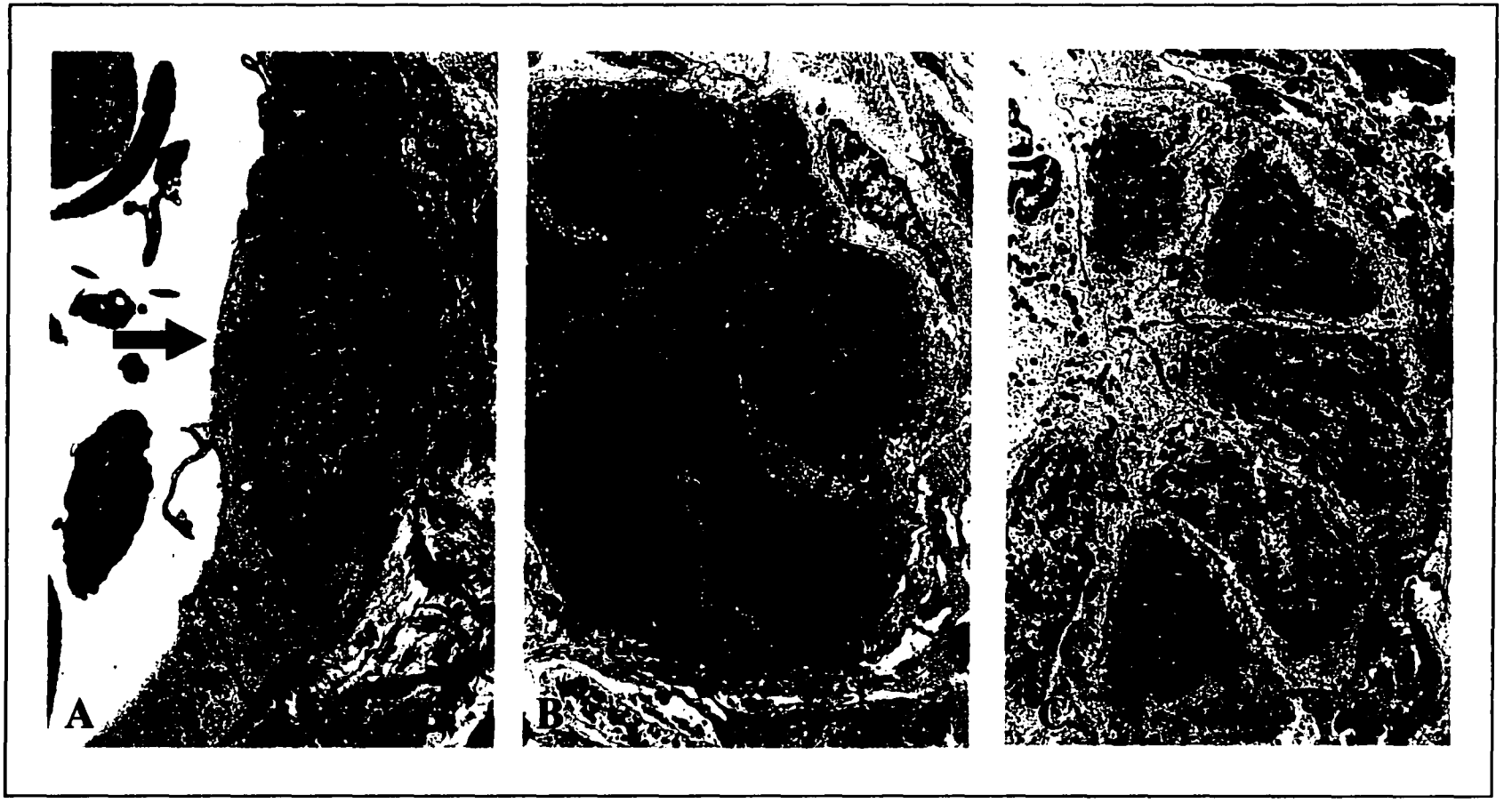


FIG. 17. Transmission electron micrographs of testicular excurrent ducts at 68 wk of age. ***A.*** Normal excurrent duct from FETAX solution control. Epithelial cells with an abundance of filaments ***(arrow)*** lining the duct with lumen containing sperm and remnants of seminiferous epithelial elements. ***B,C.*** Aberrant excurrent ducts from 0.5 ***(B)*** and 5 ppm DBP ***(C).*** Note compacted epithelial cells lining the ducts and conspicuous absence of lumina. Magnification: 5000X

TABLE 11

Effects of exposure to DBP from 0 to 12 wk of age on larynx structure in 38-, 68- and 90-wk-old-males

Treatment	38 wk		68 wk[a]		90 wk[a]
	Larynx (mg)	Muscle fibers (X 10^3/larynx)	Larynx (mg)	Muscle fibers (X 10^3/larynx)	Larynx (mg)
FETAX solution control	149.3 ± 34.6 (10)	22.6 ± 1.9 (8)	480.1± 45.2 (8)	32.9 ± 1.6 (8)	481.7 ± 14.4 (8)
DMSO control	161.8 ± 33.0 (5)	31.2 ± 1.9 (3)	435.8 ± 130.7 (4)	29.8 ± 2.2 (4)	...
0.1 ppm DBP	105.7 ± 18.3 (13)	25.2 ± 2.1 (11)	440.4 ± 41.1 (8)	30.1 ± 2.8 (8)	...
0.5 ppm DBP	128.4 ± 14.9 (12)	30.4 ± 2.5 (10)	350.8 ± 83.6 (7)	32.1 ± 2.7 (8)	444.8 ± 17.6 (8)
1 ppm DBP	95.8 ± 17.9 (10)	27.6 ± 2.1 (10)	395.2 ± 29.6 (8)	22.9 ± 0.8* (8)	...
5 ppm DBP	34.0 ± 9.0* (10)	13.2 ± 1.8* (9)	168.2 ± 56.2* (5)	22.8 ± 1.9* (5)	253.1 ± 29.7* (8)

[a]Determined from males randomly chosen for assessment of advertising call and ability to form amplexus

Values represent mean ± SEM

Numbers in parenthesis indicate number of animals

*Significantly different ($p<0.05$) from control group using ANOVA and Tukey/Kramer post-hoc test

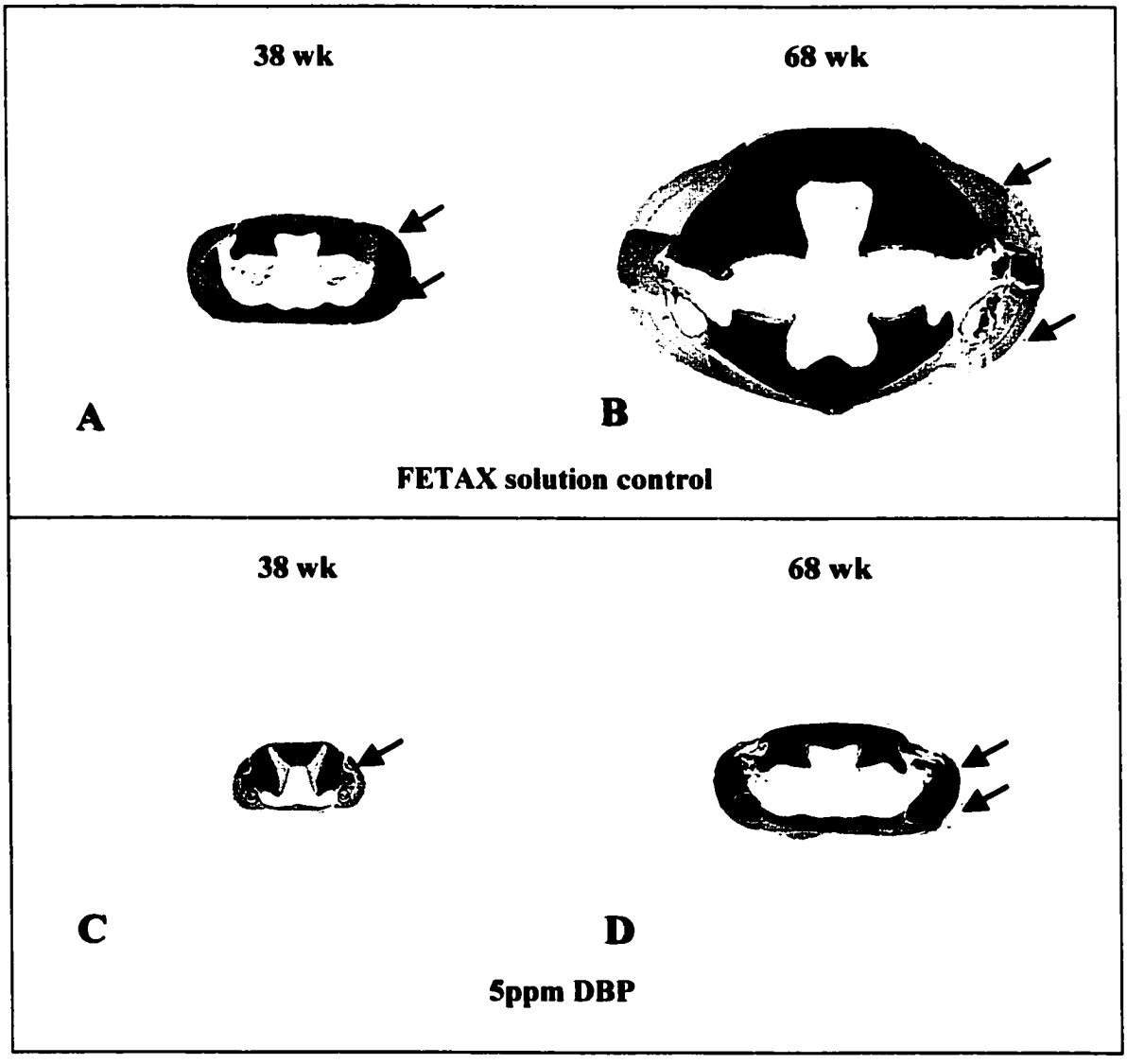


FIG. 18. Photomicrographs of laryngeal sections of males from FETAX solution control at 38 wk ***(A)*** and 68 wk of age ***(B)*** and from 5 ppm DBP at corresponding ages ***(C,D)***. The macroscopic differences in laryngeal structure are apparent at 38 wk as well as 68 wk of age between FETAX solution control and 5 ppm DBP. ***Arrows*** point to dilator larynges muscle. Magnification: 8X. H&E staining.

DMSO controls (Table 11, Figure 18). Larynx weights continued to be lower ($p<0.05$) in 5 ppm DBP at 90 wk of age.

3.5.6 Reproductive Hormones

At 38 wk of age, hypothalamic GnRH content was significantly lower ($p<0.05$) in 5 ppm DBP than in FETAX solution and DMSO controls (Table 12). Although this trend continued at 68 wk, the average value in 5 ppm being 30% lower than that of controls, the difference was not significant possibly due to 1 outlier frog whose hypothalamic GnRH content was ~3-fold greater. Concentrations of testosterone were unaffected at 30 or 68 wk of age (Table 12). Although not statistically significant, the average concentrations of testosterone at 68 wk in DBP groups were 40-80% lower than those in controls.

3.5.7 Reproductive Ability: Male Advertising Call and Amplexus

At 68 wk of age, the percentage of time spent producing the male advertising call during the 2 hr observation period was significantly decreased ($p<0.05$) in the 5 ppm DBP group (0.7%) compared to FETAX solution and DMSO controls (13.3 and 35.1%) (Table 13). Although no quantitative evaluations of the sound characteristics (frequency, amplitude modulation, etc.) were performed, the qualitative difference in the advertising call produced by a FETAX solution control male, that successfully formed amplexus was obvious compared to 1 and 5 ppm DBP males that failed to form amplexus (Figure 19). Reflective of the decrease in time spent producing the advertising call, the ability to form amplexus at 24 hr post-hCG was impaired (Table 13). Although 8 out of 8 FETAX solution and 4 out of 4 DMSO males formed amplexus with primed females at 24 hr post- hCG, 2 out of 8, 3 out of 8, 4 out of 8 and 3 out of 5 males in the 0.1, 0.5, 1

TABLE 12

Effects of exposure to DBP from 0 to 12 wk of age on reproductive hormones in 38- and 68-wk-old-males

Treatment	38 wk		68 wk	
	Hypothalamic GnRH (ng)	Testosterone (ng/ml) †	Hypothalamic GnRH (ng)	Testosterone (ng/ml) †
FETAX solution control	277.1 ± 53.2 (8)	2.1 ± 0.8 (2)	329.0 ± 70.7 (5)	3.6 ± 2.3 (6)
DMSO control	216.5 ± 37.2 (11)	1.4 ± 0.2 (2)	280.9 ± 43.2 (4)	3.4 ± 1.2 (4)
0.1 ppm DBP	181.4 ± 10.5 (13)	1.0 ± 0.3 (4)	330.5 ± 43.1 (9)	2.2 ± 0.4 (8)
0.5 ppm DBP	199.4 ± 26.0 (12)	0.8 ± 0.2 (5)	271.6 ± 28.2 (7)	0.8 ± 0.4 (5)
1 ppm DBP	167.3 ± 35.5 (11)	1.1 ± 0.2 (3)	312.7 ± 41.1 (7)	1.3 ± 0.3 (7)
5 ppm DBP	98.8 ± 18.6* (10)	1.6 ± 0.0 (1)	209.9 ± 70.4 (5)	0.7 ± 0.1 (3)

Values represent mean ± SEM

*Significantly different ($p<0.05$) from control group using ANOVA and Tukey/Kramer post-hoc test

†Samples pooled from 1-6 tadpoles

TABLE 13
Effects of exposure to DBP from 0 to 12 wk of age on sexual capacity and behavior in 68-wk-old males

Treatment	n	Percent of time spent producing male advertising call[a]		No. of males that failed to form amplexus with 1st primed female	No. of males that failed to form amplexus with 2nd primed female
		Average[a]	Range		
FETAX solution control	8	13.3	1.2 - 37.4	0	0
DMSO control	4	31.5	8.4 - 68.6	0	0
0.1 ppm DBP	8	10.1	0 - 42.8	2	2
0.5 ppm DBP	8	16	0.39 - 40.0	3	1
1 ppm DBP	8	10.1	0.4 - 33.7	4	2
5 ppm DBP	5	0.7*	0 - 2.1	3	3

[a] 2 hr-observation period: data exclude times when female was sexually unreceptive as evidenced by cloacal lips and body posture

*Significantly different ($p<0.05$) from control group using ANOVA and Tukey/Kramer post-hoc test

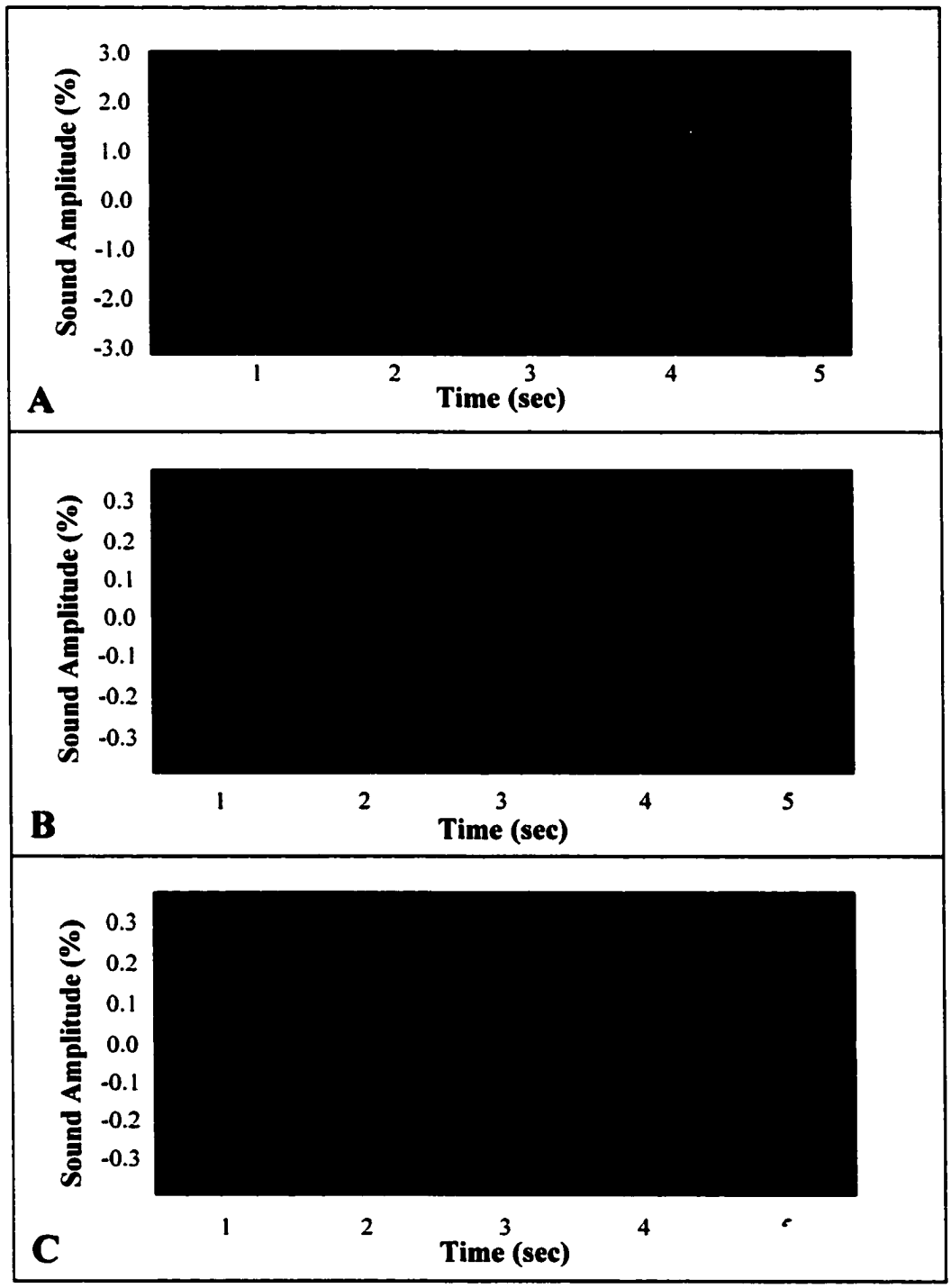


FIG. 19. Spectrographs of male advertising calls from FETAX solution control male that successfully formed amplexus ***(A)***, compared to 1 ppm DBP ***(B)***, and 5 ppm DBP ***(C)*** males that did not. Advertising call from FETAX solution male represents one continuous bout of calling that lasted a total of 98 seconds ***(A)***. Because the percentage of time spent calling was decreased in 1 and 5 ppm DBP males each call represents one bout ***(B,C)***.

and 5 ppm DBP groups failed to form amplexus. Of those frogs, 2 of the 8, 1 of the 8, 2 of the 8 and 3 of the 5 males completely failed to form amplexus during the observation period. Collectively, this constituted 41% (12/29) failure to form amplexus with the first primed female and 28% (8/29) completely failed to form amplexus. The average time spent producing the advertising call in males that completely failed to form amplexus was 0.2% compared to 3.4% in males that successfully formed amplexus with the second primed female. Interestingly, at 68 wk of age two males from the 1 ppm DBP group formed abnormal amplexus (Figure 20). In both cases, the male clasped the right hindlimb of the female instead of the abdomen.

Reproductive ability was still impaired at 90 wk of age in 0.5 and 5 ppm DBP groups (Table 14). Whereas 8 out of 8 FETAX solution controls formed amplexus at 24-hr post-hCG, 3 out of 8 and 3 out of 4 males in the 0.5 and 5 ppm DBP groups failed to form amplexus. Of those frogs, 2 of the 8 and 3 of the 3 males completely failed to form amplexus during the observation period. Collectively, this constituted 50% (6/12) failure to achieve amplexus with the first primed female and 42% (5/12) completely failed to form amplexus.

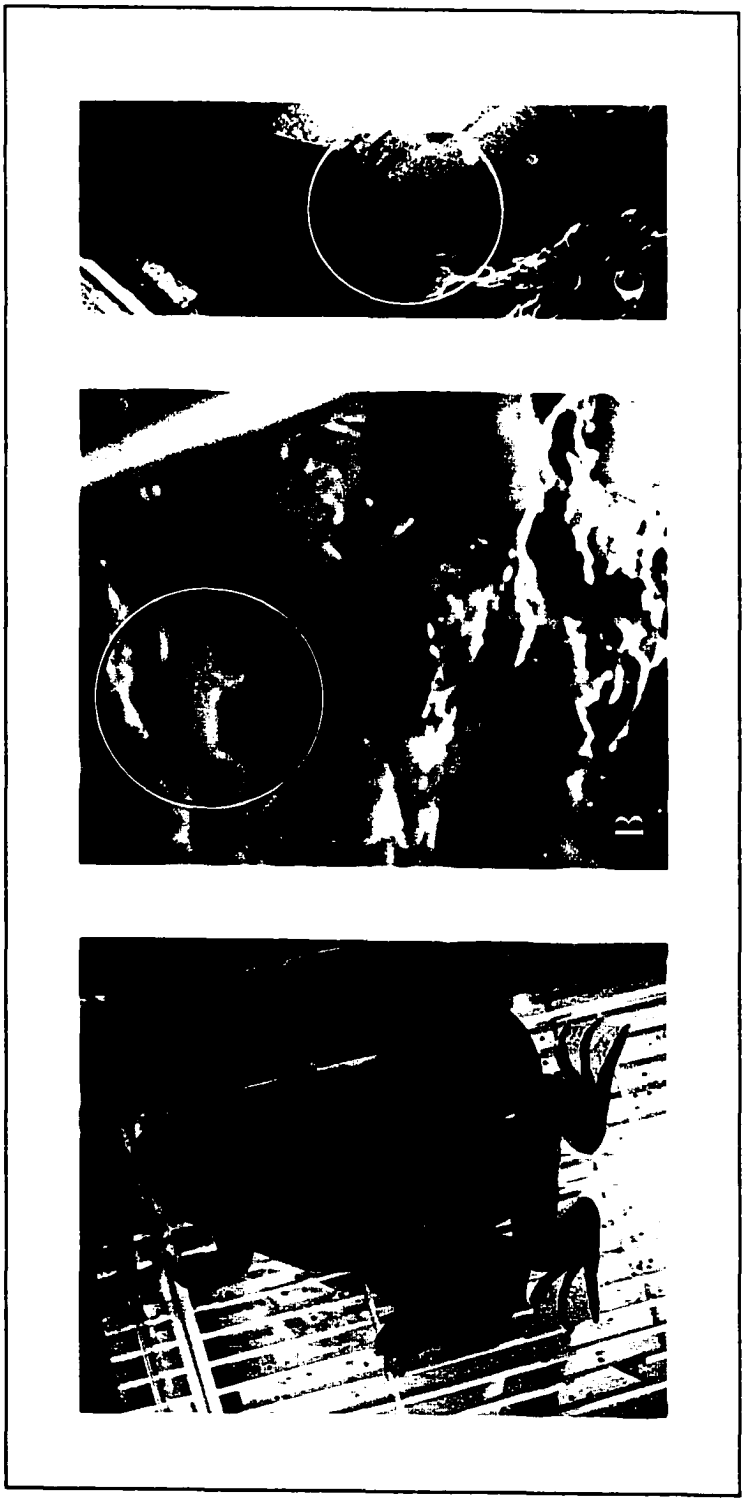


FIG. 20. Photographs of normal amplexus with male clasping around the female abdominal region *(A)* compared to abnormal amplexus observed in 1 ppm DBP frogs where males clasped the female hindlimb *(B,C)*. Circles point to males clasping the hindlimb of the females.

TABLE 14
Effects of exposure to DBP from 0 to 12 wk of age on sexual capacity and behavior in 90-wk-old males

Treatment	n	Percent of time spent producing male advertising call[a]		No. of males that failed to form amplexus with 1st primed female	No. of males that failed to form amplexus with 2nd primed female
		Average[a]	Range		
FETAX solution control	8	...	...	0	0
0.5 ppm DBP	8	...	...	3	2
5 ppm DBP	4	...	...	3	3

[a] 2 hr-observation period: data exclude times when female was sexually unreceptive as evidenced by cloacal lips and body posture

3.6 Discussion

Developmental exposure to relatively low concentrations of DBP from 0 to 12 wk of life adversely affected survival, growth, and development in *Xenopus laevis*. Furthermore, impaired male reproductive function and capacity in males at 56 and 78 wk after the cessation of treatment clearly demonstrates sustained long-term effects. This is the first documentation of long-term adverse effects of DBP on survival, growth, and male reproduction in an amphibian. It is possible that DBP can cause these adverse effects at lower concentrations than were tested since DMSO, the solvent used in this study to dissolve DBP, might have attenuated some of effects; DMSO is known to have anti-inflammatory properties (reviewed by Murdoch, 1982). In spite of this, the adverse effects of DBP on growth and reproduction occurred at doses ~34 times lower than the acceptable concentration in lakes and streams (EPA recommendation: 34 ppm; reviewed by ASTDR, 1990).

The LC_{50} of 10-15 ppm DBP determined for acute exposure to DBP during the first 96 hr of life in *Xenopus laevis* is similar to the values reported for other aquatic species (Table 2). In addition to increased mortality, DBP caused an increase in the incidence of malformations and retarded tadpole development. Similar developmental effects have been reported for rodents exposed to DBP *in utero* (Ema *et al.*, 1993; 1994; 1995; 1996; 1997; Hemm *et al.*, 1977; Saillenfait *et al.*, 2001; Shiota, 1980). The effects of DBP on survival and primary organogenesis in *Xenopus* are not due to a non-specific sensitivity of this animal to xenobiotic insult, as evidenced by a contemporaneous study in our lab that determined the LC_{50} in *Xenopus* for bromochloroacetic acid, a by-product of water disinfection was very high (between 10,000 to 12000 ppm; Weber *et al.*, 2000).

frogs raised from fertilization to metamorphosis in our *Xenopus* colony over the past 4 years.

As observed in rodents, DBP also affected differentiation of the testis and reproductive tract in developing frogs. The number of condensed spermatids (stage 4c) decreased in DBP groups even 56 wk after the cessation of treatment indicating permanent alterations to Sertoli and/or germ cells. This deterioration in spermiogenesis may be a result of delayed metamorphosis because blocking thyroid-dependent metamorphosis with the potent goitrogen, 6-*n*-propyl-2-thiouracil (PTU), leads to decreased germ cell density and altered testicular development that appears to recover with time (Robertson and Kelley, 1996). However, the stage in spermiogenesis that was affected coupled with ultrastructural observation of an increase in number of degenerating spermatids indicates direct effects on the germ cells and/or Sertoli cells. Regardless, deterioration in spermiogenesis may ultimately lead to a decrease in sperm output or an increase in the number of defective sperm.

It is unlikely that the observation of aberrant efferent ducts characterized by collapsed lumina in DBP groups was an artifact of tissue processing or plane of section since it was not observed in any control frogs. The histological features of normal (patent) excurrent ducts were similar to those described by Unsicker (1975). We did not characterize developmental events in the differentiating testis or excurrent ducts; therefore, a detailed explanation of the sequence of events leading to decreased incidence of stage 4c or aberrant excurrent ducts was not possible. However, it is clear that solid cord-like, aberrant excurrent ducts would compromise successful reproduction by preventing transport of any normal spermatozoa that were produced.

Xenopus inhabits murky ponds at high population densities with mating occurring at night so no visual cues are possible. Therefore, they must rely on auditory cues to broadcast receptivity and location (Tobias *et al.*, 1998). Only sexually active males accomplish this task by producing long bouts of a distinctive advertising call (Wetzel, 1983). Although only females are attracted to this call, sexually active males clasp onto the back of the abdomen of the nearest moving male or female releasing any unreceptive animal making a release call– "tick" (reviewed by Tobias *et al.*, 1998). Upon forming amplexus the pair moves around and eggs are fertilized upon ovulation (reviewed by Wu and Gehrhart, 1991). Production of male advertising calls and subsequent formation of amplexus requires intact androgen-dependent brain nuclei that control reproductive behavior, laryngeal function, and androgen secretion (reviewed by Kelley, 1996). Given that the number of laryngeal muscle fibers and the number of frogs accomplishing amplexus were decreased even 56 wk after the cessation of DBP exposure, we infer that DBP induces permanent alterations to the larynx and sexual capacity. Lack of changes in endocrine parameters at 38 and 68 wk of age further supports the inference that a permanent impairment occurred early on. Additional support comes from extended observations on a subset of frogs – from FETAX solution control, 0.5 and 5 ppm DBP groups that were continued until 90 wk of age. At 90 wk, there still was a significant decrease in larynx size and impaired reproductive behavior in 5 ppm DBP.

The abnormal amplexus observed in 1 ppm DBP male frogs – clasping of the hindlimb rather than the abdomen – may limit success of fertilization. Normally, to ensure that eggs are fertilized as they are released, males position their cloaca directly

above that of the females by clasping onto the abdomen (Figure 20). Without this positioning, release of sperm and egg are not coordinated limiting the physical access.

Changes in male reproductive behavior caused by DBP exposure may be due to altered androgen secretion during hormone-sensitive stages of development or lack of a functional androgen receptor. Earlier studies in rodents indicated that DBP causes anti-androgenic effects (Mylchreest *et al.*, 1998; 1999; 2000) without interacting with androgen receptor (Foster *et al.*, 2001; Paganetto *et al.*, 2000). In rodents DBP has adverse effects on developing Leydig cells leading to decreased testicular testosterone concentrations (Mylchreest *et al.*, 2002). In the present study concentrations of androgens during sexual differentiation or DBP exposure were not measured, therefore inferences about disruption of androgen secretion were not possible.

Thyroid hormones control numerous developmental processes in *Xenopus laevis*, including androgen-induced laryngeal development (Cohen and Kelley, 1996; Robertson and Kelley, 1996) and estrogen-induced vitellogenin synthesis (Huber *et al.*, 1979). Blocking thyroid hormone secretion with PTU inhibits laryngeal development (Cohen and Kelley, 1996), while treating with thyroid hormones causes precocious laryngeal development (Robertson and Kelley, 1996). Similar effects of thyroid hormones were observed in vitellogenin-producing hepatocytes (Kawahara *et al.*, 1989). Thyroid hormones do not affect steroid receptor expression, therefore it has been speculated that an additional thyroid-dependent transcription factor may be required during development for androgen- and estrogen-competency (reviewed by Kelley, 1996). In the present study a dose-dependent delay in metamorphosis, a thyroid-dependent phenomenon, with permanent alterations in male reproductive behavior and function in adult frogs was

observed Furthermore, no alterations in reproductive hormones were observed at puberty or adulthood. The timing of the manifestation of effects indicates that perturbations in thyroid-dependent development leads to compromised androgen receptor competency, which is not fully recovered 56 and 78 wk after the cessation of treatment. However, the ability of DBP to disrupt testosterone secretion during sexual differentiation and the role of thyroid hormones in DBP-mediated toxicity in *Xenopus laevis* need to be addressed in future studies.

Collectively, data obtained in this study provided evidence that frogs exposed to DBP during critical periods of development had delayed thyroid-dependent development and altered male reproductive function as adults. The impaired male reproductive function and sexual capacity observed in DBP-groups may ultimately lead to decreased fertility. Similar possibility has been suggested in a recent study of an indigenous population of malformed frogs in which it was observed that alterations to the male reproductive system early in development may lead to decreased reproductive efficiency in adult life (Sower *et al.*, 2000). Thus it is imperative that the long-term effects of xenobiotics on growth and reproduction be elucidated in order to identify the etiology of reported declines in amphibian populations and increased incidence of malformations.

CHAPTER 4

CHARACTERIZATION OF THE EFFECTS OF DBP IN MALE RABBITS

4.1 Introduction

As discussed in Chapter 2, the effects of DBP on the adult and developing reproductive system of rodents have been well characterized. However, no studies have been conducted using animal models with a relatively long infantile period that approximates human reproductive development. Similarly, studies evaluating the longitudinal effects of phthalates on sexual behavior, seminal parameters, the hypothalamic-pituitary-gonadal axis, or functional status of thyroid have not been performed. Furthermore sequelae induced by phthalates vary among species due to differences in metabolites formed (Foster *et al.*, 1983). Therefore, to determine if DBP affects a non-rodent mammalian species and if it causes immediate as well as long-term reproductive effects, we studied the effects of DBP using Dutch-Belted rabbits (*Orytylagus cunniculus*).

4.1.1 Declining Sperm Counts

Several reports indicate a decline in the quality of human semen. In 1974, Nelson and Bunge reported that 386 patients who were about to undergo elective vasectomies had an average ejaculate volume of 2.83 ml and a sperm count of 48 x 10^6 sperm/ml. This observation was in stark contrast to the established averages for ejaculate volume (3.4 ml) and sperm counts (>100 x 10^6 sperm/ml) (MacLeod and Gold, 1951). After

reevaluating their data and determining that no inherent errors had occurred, Nelson and Bunge (1974) speculated that an environmental factor had potentially altered semen quality. In another study (Leto and Frensilli, 1981), it was reported that potential semen donors had a decline in semen quality during 1973-1980 resulting in a decrease in acceptable applicants (from 77% in 1973 to 37% in 1980). This declining trend was observed in accepted as well as rejected donors. Collectively, these reports were viewed as an artifact due to changes in the policy of fertility clinics or selection bias and not a true biological phenomenon, since they were based on data collected from infertility clinics or a select group of fertile men (Toppari *et al.*, 1996).

In an attempt to gain a global perspective on the reports of declining semen quality, Carlsen *et al.* (1992) performed metaanalysis of data collected from 61 studies that included 14,947 men. Their results indicated that a significant decline (~50%) in sperm concentration (113 x 10^6 sperm/ml vs. 66 x 10^6 sperm/ml) and seminal volume (3.4 to 2.8 ml) had occurred from 1938 to 1991. This report, like the earlier reports on declining semen quality, has received scrutiny over statistical methodology and populations studied (Olsen *et al.*, 1995). Despite the criticism of the statistical model, Olsen *et al.* (1995) found a decline in sperm counts, yet the rate at which they occurred was decreased and the mean sperm counts were unchanged or slightly increased after 1970. Fisch and Goluboff (1996a) found substantial geographic variation in the sperm counts of 20 studies from 1938 to 1989 used in the original report by Carlsen *et al.* (1992). Therefore, the worldwide decline in sperm counts may only reflect clustering of significant geographic variations.

Following the initial report of Carlsen *et al.* (1992), several studies from around the world were undertaken to verify if a decline in semen quality had occurred. Using data from 1351 fertile men (fathered at least one child) in Paris, Auger *et al.* (1995) determined that sperm concentration had decreased from 89 x 10^6 sperm/ml in 1973 to 60 x 10^6 sperm/ml in 1992. There was also a decrease in the percentage of normal sperm and progressive motility by 0.5 and 0.6% per year. Another study of 1283 men from three different regions of the United States over a 25-year period found no decline in the quality of semen (Fisch *et al.*, 1996b). Mean sperm concentration for the three regions had significantly increased from 77 x 10^6 sperm/ml in 1977 to 89 x 10^6sperm/ml in 1994, yet the data from the three regions were significantly different from each other. Interestingly, a birth cohort study of 577 men over an 11 year period from Scotland revealed a significant decline in the median sperm concentration (78 x 10^6 sperm/ml), total sperm in the ejaculate (214 x 10^6) and number of motile sperm in the ejaculate (129 x 10^6) had occurred in men born after 1970 compared to men born before 1959 (98 x 10^6 sperm/ml, 301 x 10^6 sperm/ejaculate, and 168 x 10^6 motile sperm/ejaculate) (Irvine *et al.*, 1996). This was the first evidence that an exogenous factor may have been introduced after 1959, which influences semen quality. However, in another study of 5481 infertile Finnish men, no change in sperm counts was associated with year of birth (Vierula *et al.*, 1996).

There still is debate over the decline in semen quality (reviewed by Lipshutz, 1996). However, in an attempt to incorporate the critical issues raised to date into a reasonable model, Swan *et al.* (2001) analyzed 101 English-language studies published during 1936 to 1996 controlling for abstinence time, age, specimen collection method,

and history of proven fertility, while applying several statistical models. Similar to the original Carlsen *et al.* (1992) study, Swan *et al.* (2001) concluded that a decline in sperm counts has occurred in the United States (slope = -0.80) and Europe (slope = -2.35), while patterns in other countries remained unchanged (slope = -0.21).

If the decline in human sperm counts is a result of an environmental factor (Nelson and Bunge, 1974), then sperm counts from farm animals should follow a similar trend. Setchell (1997) found no decline in sperm counts from bulls, boars and rams during 1932-1995, and suggested that if the decline in human sperm counts is a result of an environmental factor it must not be affecting farm animals. However, as commonly practiced in animal husbandry, reproductively inefficient animals may have been culled from the test populations studied by Setchell (Veeramachaneni, 2000a).

4.1.2 Increased Incidence of Testicular Cancer

The incidence of testicular cancer, the most common malignancy in young men (25-34 years of age) has increased at a rate of 2-4% per annum from 1943 to 1989 (Adami *et al.*, 1994). This trend has considerable geographic (Adami *et al.*, 1994), socioeconomic and racial differences (Pearce *et al.*, 1987; Spitz *et al.*, 1986). In England and Wales the age-standardized incidence of testicular cancer has increased from 2.9 per 100,000 in 1971 to 5.4 in 1997 (Power *et al.*, 2001). This represents an 88% increase over the past 26 years. Similarly, the incidence of testicular cancer in Australia rose from 1.44 per 100,000 in the early 1950's to 4.16 in the mid 1980's (Stone *et al.*, 1991). The rates of testicular cancer in Scotland have increased almost 50% between 1959 and 1984 (Boyle *et al.*, 1987). Increased incidence of testicular cancer has also been documented in other Nordic and Baltic Countries, New Zealand, and United States (reviewed by

Toppari *et al.*, 1996). Caucasian men (Spitz *et al.*, 1986) and persons in the upper social class groupings (Pearce *et al.*, 1987) have a higher incidence of testicular cancer than African Americans and persons of lower social class groupings. Rates of testicular cancer in other countries have increased less drastically. In Finland the incidence of testicular cancer only rose from 0.9 per 100,000 to 1.6 from 1950 to 1982 (Halme *et al.*, 1989).

Similar to birth cohort studies for declining semen quality, the relative risk (RR) of developing testicular cancer follows the same trend. In six countries studied (Denmark, East Germany, Finland, Norway, Poland and Sweden), men born around 1965 had an average RR of developing testicular cancer of 7.7 compared to 1.3 for men born around 1920 (Bergstrom *et al.*, 1996). This increased RR varied by country; however, all countries showed a relative increase.

In 1972, Skakkebaek coined the term carcinoma *in situ* (CIS) to describe atypical germ cells in the seminiferous epithelium of 2 patients who developed testicular cancer 4.5 years after the original biopsy. Based on morphological and histochemical (rich in glycogen and alkaline phophatase) characteristics, CIS has been identified as malignant gonocytes (Skakkebaek *et al.*, 1987) and has been identified adjacent to the tumor in 75 to 99% of testicular cancers (Bringhurst and Amato, 1997). In one study progression of CIS to testicular cancer was estimated to be 50% in 5 years (Skakkebaek *et al.*, 1982). Based on these observations, CIS has been characterized as the uniform precursor to most forms of testicular cancer – seminoma and nonseminomas (Skakkebaek *et al.*, 1987) except for spermatocytic seminoma (Dekker *et al.*, 1992) and infantile germ-cell

neoplasm (Jorgensen *et al.*, 1995). This theory that most forms of testicular cancer arise from CIS is universally accepted (Dieckmann and Skakkebaek, 1999).

The prevalence of CIS in normal testis from patients without any underlying reproductive disorders (infertility, cryptorchidism, etc) is between 0.4 and 0.8% (Dieckmann and Skakkebaek, 1999). However, a number of reproductive conditions have been associated with an increased prevalence of CIS, and thus testicular cancer. Infertile men have a prevalence of ~1% and men with atrophic testis or sperm counts <3 x 10^6 sperm/ml constitute a higher risk. CIS occurs between 0 and 8% of adult men with a history of cryptorchidism, or maldescent of the testis (Dieckmann and Skakkebaek, 1999) and the relative risk of developing testicular cancer does not decrease with correction of the maldescent early in childhood (Prener *et al.*, 1996). Men with genital anomalies also have an increased prevalence of CIS. Muller and Skakkebaek (1984) found CIS in 25% of patients with testicular feminization, while patients with hypospadias have a relative risk of 4.2% (Prener *et al.*, 1996). Interestingly, the incidence of cryptorchidism and hypospadias has increased in certain populations, yet the inferences about a global phenomenon cannot be ascertained due to differences in screening techniques (reviewed by Toppari *et al.*, 1996).

4.1.3 Possible Etiological Factors

In spite of the enormous data indicating a decline in male reproductive health, few studies have given any regard to establish a causal link. It is well known that >5 million pregnant women were prescribed diethylstilbestrol (DES) between 1940 and 1970 (Palmlund *et al.*, 1993). DES is a potent synthetic estrogen used to maintain pregnancy in recurrently aborting women. However in clinical trials, DES caused the very effects

that it was intended to prevent – abortions, neonatal death, and pre-mature birth (Dieckmann *et al.*, 1953). DES was banned once clear cell carcinoma of the vagina, a very rare form of cancer was found in adolescent girls exposed *in utero* to DES (Herbst and Scully, 1970; Herbst *et al.*, 1971). A majority of the effects of DES on the development and functionality of the male reproductive tract have been generated from follow-up studies on the offspring of pregnant women testing the efficacy of DES. Severe reproductive tract anomalies have been described in males exposed *in utero* to DES. These anomalies included meatal stenosis, epididymal cysts, hypospadias, testicular defects (hypotrophic testis, cryptorchidism, capsular induration), and microphallus (Bibbo *et al.*, 1977; Gill *et al.*, 1977; 1979; Henderson *et al.*, 1976). Interestingly the frequency of genital anomalies correlated with the onset of exposure. Offspring of pregnant mothers exposed prior to gestation week 11 had twice as high a frequency as those exposed after gestation week 11 (Wilcox *et al.*, 1995). Seminal parameters were also adversely affected. Sperm concentration (83×10^6 vs. 123×10^6 sperm/ml), total sperm count, motility, normal sperm, and quality score were all significantly decreased in DES-exposed males versus placebo controls (Gill *et al.*, 1977; 1979). Men exposed to DES do not have an increased risk for developing cancer, however, it is still uncertain if DES exposure increases the risk for testicular cancer (Strohsnitter *et al.*, 2001). Similar results have been obtained for rodents exposed to DES (McLachlan *et al.*, 1975). Recently it was observed that the susceptibility for development of reproductive tumors in rodents is transmitted to subsequent generations (Newbold *et al.*, 2000). Female offspring (F_1) from pregnant mice (F_0) exposed *in utero* to DES when bred with untreated males resulted in male offspring (F_2) that had increased

incidence of proliferative lesions of the rete testis and tumors of the reproductive tract. Collectively these studies indicate that *in utero* exposure to a potent estrogen has profound effects on reproductive development with dire consequences later in life.

Exposure to known endocrine disrupting contaminants found at EPA superfund sites (Guillette *et al.*, 1994) or Kraft mill effluents (Howell *et al.*, 1980) had adverse effects on the reproductive system of several wildlife species. Alligators from Lake Apopka, which is directly adjacent to an EPA superfund site contaminated with dicofol, DDT, DDT metabolites, and agricultural contaminants, had abnormal reproductive parameters in female as well as males. Female alligators had high levels of estradiol and abnormal ovarian structure, while males had depressed testosterone, poorly organized testis and small phalli (Guillette *et al.*, 1994). Female mosquito fish (*Gambusia affinis holbrooki*) living downstream from the Kraft paper mills had masculinized anal fins, resembling the male gonopodium (Howell *et al.*, 1980).

Since the changes in human reproductive health have occurred over a relatively short period of time (~50 years) and based on the data from DES and wildlife at contaminated sites, it was hypothesized that many chemicals released in the environment are capable of disrupting the endocrine system and thus reproductive function of animals and humans (Colborn *et al.*, 1993; Jensen *et al.*, 1995; Sharpe and Skakkebaek, 1993). Specifically, the decline in human reproductive health may be the result of exposure during fetal life to estrogen-mimicking or other endocrine-disrupting chemicals. Support for this hypothesis comes from the numerous studies determining the effects of environmental estrogenic compounds on the developing reproductive system (reviewed by Toppari *et al.*, 1996). Furthermore, it was recently determined that chemicals may

block androgen signaling, leading to adverse effects on the developing reproductive system (reviewed by Gray *et al.*, 2001). Collectively these findings led to a United States Congressional mandate, under the Food Quality and Protection Act and Safe Drinking Water Act, to the EPA in 1996 to develop protocols to screen for endocrine effects of chemicals on reproductive development (reviewed in Gray *et al.*, 2001).

4.1.4 Rabbit as an Animal Model

One of the most critical aspects of research in reproductive toxicology is selecting an appropriate animal model (Amann, 1982). Currently, there are few research animal models available to delineate if a decline in male reproduction is a result of exposure to xenobiotics during critical periods of reproductive development or in adult life. Rodents have been commonly used in reproductive toxicology studies because of the ease of husbandry, cost effectiveness, and the size of the organs is large enough to easily determine and quantify any changes (Amann, 1982). However, this animal model has several limitations. Semen cannot be collected from rodents and evaluated in a longitudinal study (Amann, 1982). The period of reproductive development in rodents is extremely short. Whereas humans typically do not undergo pubertal changes until 12-13 years, rodents begin to initiate first spermatogonial division by the first week of age. This drastically decreases a critical window of exposure to gonocytes and pre-spermatogonia, and thus potential for toxicological insult. Finally, rodents typically develop teratomas, which are most likely a model for infantile germ-cell neoplasms (Looijenga and Oosterhuis, 1999). Therefore they may not be appropriate for identifying if exposure to xenobiotics is a causative factor in the increased incidence of testicular cancer.

An alternative animal model with numerous advantages over rodents is the rabbit. They are the smallest common species for which semen can be conveniently collected and analyzed in longitudinal studies (Amann, 1982) and parameters for critical evaluation of reproductive function are well characterized (Amann, 1970; 1982; 1986; Amann and Lambiase, 1967; 1969; Amann and Berndtson *et al.*, 1986; Swierstra and Foote, 1963). The chronology of reproductive development has been well characterized in male rabbits (Gondos *et al.*, 1973a; 1973b; 1976). Testicular differentiation occurs on GD 16 when germ cells become surrounded by supporting cells (precursors to Sertoli cells) and separated from each other by a distinct basal lamina (Gondos *et al.*, 1973a). Over the next few days differentiation occurs and by GD 22 germ cell proliferation begins. During the final days of gestation, the germ cells align in rows along the basal lamina. (Gondos *et al.*, 1973a). The infantile period in male rabbits is relatively long, which mimics human reproductive development. During late gestation to post-natal week (PNW) 6, the seminiferous epithelium contains only gonocytes, pre-spermatogonia, and Sertoli cells (Gondos *et al.*, 1973b). From PNW 5 to 7 Leydig and Sertoli cells undergo differentiation, and the onset of spermatogenesis begins around PNW 7-8 when the first adult spermatogonia (types A and B) are formed (Gondos *et al.*, 1973b; 1976). Spermatocytes are observed at PNW 8, spermatids appear by PNW 12 coinciding with the formation of the tubular lumen, and mature spermatozoa appear in the lumen by PNW 13-14 (Gondos *et al.*, 1973b). Efficiency of sperm production increases over the next few weeks attaining a maximum by 30 wk (Amann *et al.*, 1967).

Further support for the rabbit as an animal model in reproductive toxicology comes from numerous studies by Veeramachaneni *et al.* (1994; 1999a; 1999b; 2000a;

2000b; 2001a, 2001b). Male rabbits exposed to drinking water containing chemicals (arsenic, lead, chromium, benzene, chloroform, phenol and trichloroethylene) at concentrations typical of ground water near hazardous sites surveyed by EPA from GD 20 until PNW 15 had decreased mating desire/ability, sperm quality, and Leydig cell function even 45 wk after exposure (Veeramachaneni *et al.*, 2001a). Other studies revealed that exposure to xenobiotics (octylphenol, p,p' DDT, or zeranol) *in utero* or during infancy resulted in undescended testes, which manifested CIS-like atypical cells (Veeramachaneni *et al.*, 1994; 1999b; 2000a; 2001b). The incidence of these manifestations varied for each treatment. The determining factor in the cellular transformation of germ cells into CIS could result from the various actions of chemicals, which may include endocrine disruption, but not from the abdominal location of the testis per se (Veeramachaneni *et al.*, 2001b). Veeramachaneni and VandeWoude (1999a) identified atypical germ cells with morphological hallmarks of CIS directly adjacent to an intratubular seminoma-like tumor in a 3-year-old subfertile rabbit. This was the first documentation of occurrence of CIS-like atypical cells, preceding the formation of seminoma-like tumor in any laboratory species indicating the rabbit may be a good model for studying the pathogenesis of testicular cancer. Collectively, these studies indicate the rabbit may be the best model for determining the etiological factors of declining semen quality and increased incidence of testicular cancer.

4.2 Objective

The objective of this study was to evaluate the effects of DBP on development and reproductive function of male rabbits following gestational (GD 15-29), adolescent

(PNW 4-12) or post-pubertal (treatment for 12 wk) exposures. *Please see Figure 21 on the next page for an overview of the experiment.*

4.3 Materials and Methods

4.3.1 Rabbits

Adult Dutch-Belted rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN) and individually housed in standard stainless steel cages at the AAALAC-accredited facility of Colorado State University. The room was maintained with a 12 hr light-dark cycle at approximately 19-21°C and ~40% humidity. Animals were fed certified rabbit ration (#7009, Harlan, Teklad, Madison, WI), provided water *ad libitum*, and bedded with kiln-dried aspen shavings ~2 inches below the stainless-steel floor.

Two ejaculates of semen were collected from ten bucks using an artificial vagina and a teaser doe, evaluated for normalcy, and pooled. Female rabbits were inseminated with 20 x 10^6 spermatozoa. Ovulation was induced by an intramuscular injection of 10 μg GnRH followed by natural service by one of the ten bucks used for semen collection. Females were palpated for pregnancy 14 days after AI and pregnant does were randomly assigned to treatment groups. On GD 28 pregnant does were provided with nesting boxes, pups were allowed to nurse until weaning at PNW 6 and then individually housed.

4.3.2 Dosing

DBP (99.8% pure) was obtained from Aldrich Chemical Co. (Milwaukee, WI). In each treatment group, animals were dosed daily with 0 (vehicle alone) or 400 mg DBP/kg in a mixture of deionized water: Karo Light Corn Syrup (60:40 w/v) (Bestfoods, Englewood Cliffs, NJ). To account for changes in body weight, doses were computed daily during active periods of growth (PNW 4-6) and weekly for other age groups. The

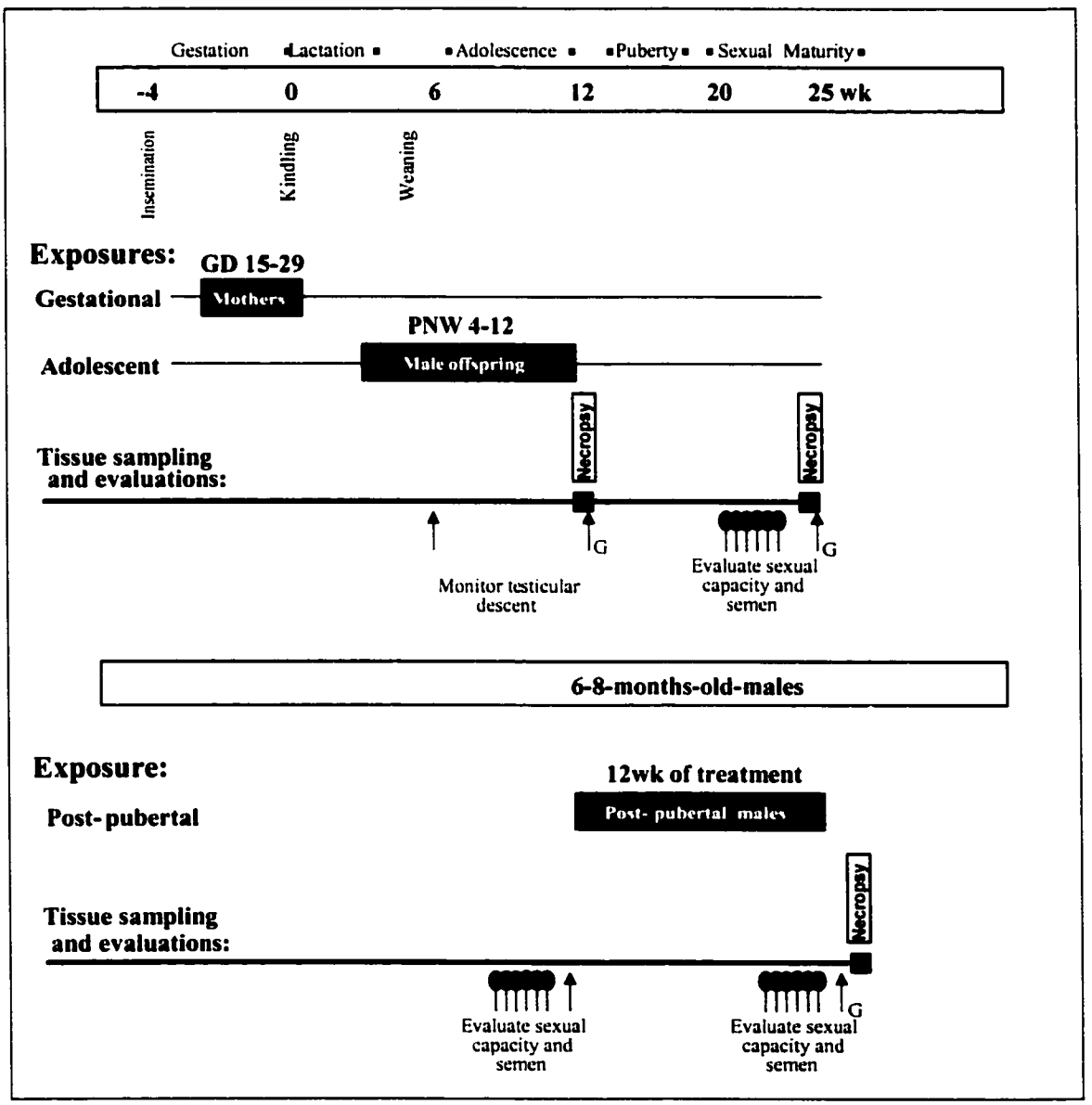


FIG. 21. Overview of study design to characterize the effects of DBP in Dutch-Belted rabbits. Arrows indicate measurement of hormones before and after a GnRH (arrows with a "G") challenge. ***GD:*** gestation day; ***PNW:*** post-natal week.

dose of DBP administered is based on previous reports that adverse effects on male reproductive development occurs between 100 and 500 mg DBP/kg/day without maternal or systemic toxicity (Foster *et al.*, 2001; Mylchreest *et al.*, 1998; 1999; 2000).

Three exposures were performed: gestational (GD 15-29), adolescent (PNW 4-12) and post-pubertal (12 wk of exposure to adult animals). For gestational exposure pregnant rabbit does were orally administered 0 (corn syrup only, n = 5) or 400 mg DBP/kg/day (n = 8) from GD 15 to 29. In adolescent exposure, rabbit pups (n = 11) were administered 400 mg DBP/kg/day from PNW 4 to 12. In order to minimize costs and use of animals, control animals from gestational exposure were utilized as controls for the adolescent exposure experiment. At 6 wk, at least one pup from each litter was randomly selected for the 12 and 25 wk groups and housed individually

For post-pubertal exposure, bucks (n = 12, 6-8 months of age) were obtained for post-pubertal exposure and initially trained to ejaculate into an artificial vagina. Three semen samples were collected on alternate days and analyzed prior to the onset of treatment (*Section: 4.3.5: Evaluation of Sexual Capacity and Semen Characteristics*). Animals were ranked based on body weight and before-treatment seminal parameters and alternately assigned to 0 (n = 6) or 400 mg DBP/kg/day (n = 6) ensuring representative distribution between the two groups. Treatment continued for 12 wk. This duration encompasses at least six cycles of the seminiferous epithelium, which is suggested to evaluate the effects of xenobiotics on gametogenesis in adult rabbits (Amann, 1982).

4.3.3 General Procedures

For gestational and adolescent exposure, body weights were recorded at 6, 12, and 25 wk of life. Testicular descent was monitored weekly from 6 wk of life by scrotal

palpation until descent was complete in all pups. Serum samples were collected at 6, 12, and 25 wk of life, by jugular puncture. Each time 4 to 5 ml of blood was collected, serum separated, and stored at -20°C for endocrine assays.

For post-pubertal exposure, body weights were recorded before treatment and weekly thereafter. Serum samples were collected before and at the conclusion of treatment and stored as described above.

In adolescent and post-pubertal exposure, animals were weighted weekly during the treatment period to determine any toxicity associated with DBP.

4.3.4 Tissue Collection and Processing

Rabbits were euthanized by carbon dioxide asphyxiation at 12 and 25 wk of age in gestational and adolescent exposures, and after 12 wk of post-pubertal exposure, one to three days after the evaluation of the hypothalamic-pituitary-gonadal axis (*Section: 4.3.8: Thyroid and Reproductive Hormones*). Visceral (thyroid, liver, kidney) and reproductive organs (testes, epididymides, accessory sex glands, hypothalamus) were evaluated for any gross abnormalities, removed, weighed and stored in the appropriate fixative. Testes and epididymides were weighed individually. Visceral organs were fixed in 10% formalin (for light microscopy). At 12 wk both testes and at 25 wk left testis were sliced into two pieces, one piece was fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate (for electron microscopy) and the other in Bouin's fixative (for light microscopy) along with the epididymis and accessory sex glands. Efficiency of sperm production and epididymal sperm reserves were determined at 25 wk in gestational and adolescent exposures and after 12 wk of post-pubertal treatment (*Section: 4.3.6 Efficiency of Sperm Production and Epididymal Sperm Reserves*). The right testis was decapsulated,

weighed, frozen and stored at -80°C to determine the daily sperm production (DSP). The right epididymis was dissected from the testis, cut into two pieces (caput-corpus and cauda), weighed, frozen and stored at -80°C to determine the epididymal sperm reserves (ESR).

The GnRH content was determined at 12 and 25 wk in gestational and adolescent exposures and after 12 wk of post-pubertal treatment. The hypothalamus was removed by incisions rostral to the preoptic area, caudal to the mamillary bodies, lateral to the hypothalamic sulci, and dorsal to the anterior commissure. The hypothalamus was bisected through the infundibulum and one half was weighed, placed in formic acid: methanol (1:9, v/v), and stored at -20°C until extraction and assay (Nett and Adams, 1977).

Tissues fixed for light and electron microscopy were processed as described in *Section: 3.4.1.8: Tissue Collection and Processing.* Thin sections for electron microscopy were stained with uranyl acetate (Polysciences Inc., Warrington, PA) and lead citrate (Polysciences Inc., Warrington, PA) and examined using a JEOL-1200EX transmission electron microscope (Veeramachaneni *et al.*, 1993).

4.3.5 Evaluation of Sexual Capacity and Semen Characteristics

The evaluation of sexual capacity and semen characteristics was performed according to Veeramachaneni *et al.*, (2001a). Male rabbits were trained to ejaculate into an artificial vagina using a female teaser, and ejaculates (n = 6 ejaculates/animal) were collected every third day from 22 to 24 wk for gestational and adolescent exposures. For post-pubertal exposure, 3 ejaculates were collected every other day before treatment (before-treatment) and at the end of treatment (after-treatment).

For each ejaculate collected, sexual behavior and capacity were subjectively evaluated by monitoring the outcome and recording: 1) sexual interest 2) status of penile erection 3) number of mounts to accomplish ejaculation; and 4) time to ejaculate after the introduction of a female teaser. Once the teaser was introduced, a maximum period of 180 seconds was allowed for evaluation.

For each sample, the ejaculate volume (after removing the gel) was recorded (to the nearest 0.05 ml) and an aliquant (50 µl) of semen was fixed in 950 µl phosphate-buffered formal saline and stored at 4°C until evaluation. Sperm concentration was determined by hemocytometer and sperm per ejaculate calculated by multiplying the sperm concentration by the corresponding ejaculate volume. To determine the sperm morphologic features, wet sperm smears were evaluated in a treatment-blinded manner, using a Nikon Microphot microscope equipped with DIC optics at 900X magnification. Two hundred spermatozoa/ejaculate were evaluated for abnormalities of the acrosome, head, mid- and principal pieces, retention of cytoplasmic droplets, and presence of residual cytoplasm using criteria previously established for rabbits (Veeramachaneni *et al.*, 2001a).

4.3.6 Efficiency of Sperm Production and Epididymal Sperm Reserves

The efficiency of sperm production (Amann, 1970; 1982; 1986; Amann and Berndtson, 1986) was measured by determining the number of homogenization-resistant spermatids per gram of testis according to Amann (1969) with minor modifications. Testicular parenchyma was thawed, minced on a watch glass, and homogenized for 1 minute in a semi-micro Warning blender using 50 ml buffer (0.145 M NaCl containing 4 mM NaN_3 and 0.05%(v/v) Triton X-100). The number of homogenization-resistant

elongated spermatids was counted without further dilution using a hemocytometer. DSP was calculated by using a time divisor of 5.35 days (Amann *et al.*, 1974) and expressed per weight of testicular parenchyma. Capita-corpora and caudae epididymidis were thawed, minced on a watch glass, homogenized for 3 minutes using 50 and 125 ml of buffer, and counted using a hemocytometer without further dilution. ESR was expressed per weight of epididymal segment.

4.3.7 Histopathology of the Testis

Normalcy of the seminiferous epithelium and interstitium were evaluated using H&E stained sections. One hundred randomly selected, essentially round (<1.5X width) seminiferous tubules from each animal were classified into one of eight different grades using criteria established for evaluating bulls (Veeramachaneni *et al.*, 1986) with minor modifications for rabbits (Veeramachaneni, unpublished results). Briefly, grade 0 – normal seminiferous epithelium; grade 1 – seminiferous epithelium with pynknotic cells and desquamation or focal vacuolation; grade 2 – seminiferous epithelium intermediate between grades 1 and 3; grade 3 – seminiferous epithelium with pre-meiotic and Sertoli cells; grade 4 – Sertoli cells only; grade 5 – no seminiferous epithelium leaving only the basement membrane; grade 6 – seminiferous tubule with sperm stasis, sperm granuloma, or mineralization; grade 7 – fibrotic seminferous tubule. A factor was assigned – 0, 1/4, 2/4, 3/4 to grades 0, 1, 2, 3, respectively, and 4/4 to grades 4 through 7 – and the relative degree of germinal epithelial loss (DGEL) was calculated by multiplying the number of tubules per grade by the respective assigned weight and summing products (Veeramachaneni *et al.*, 1986).

4.3.8 Thyroid and Reproductive Hormones

Based on the preliminary data (*3.3.1: Sensitivity of Xenopus to DBP and Selection of End Points/Parameters*) indicating exposure to DBP delays thyroid-dependent metamorphosis, the concentrations of triiodothyronine (T_3) were measured at 6, 12, and 25 wk in gestational and adolescent exposures and before and after treatment in post-pubertal exposure. To assess the normalcy of endocrine stimuli necessary to drive reproduction, hypothalamic GnRH content and serum concentrations of testosterone were determined by RIA.

4.3.8.1 Radioimmunoassay of Triiodothyronine

Serum samples were assayed using a commercially available kit described in *Section: 3.4.1.7.B: Radioimmunoassay of Triiodothyronine.* Prior to measuring the levels of T_3 in rabbit serum, an RIA procedure with sufficient specificity, accuracy and precision (Midgely *et al.*, 1963; Nett and Malvey, 1999) was validated. The specificity of the assay was determined by comparing the parallelism of the T_3 standard curve with dilutions (0.25, 0.5, 0.75) of two serum samples (Appendix F). There was no significant difference ($p>0.37$) between the slope of the T_3 standard curve and four replicates of the serial dilutions. Adding 12.5, 25, 50 and 100 ng/dl T_3 to two samples and plotting the amount of ligand added to the amount measured determined accuracy. The slope of the line was 0.994 and coefficient of correlation was 0.982. Precision was determined by measuring the intra-assay coefficient of variation from 16 replicates of two samples and the inter-assay coefficient of variation from two samples measured in 4 different assays. These values were 2.5 and 7.6%, respectively.

4.3.8.2 Radioimmunoassay of Gonadotropin-Releasing Hormone

Hypothalamic content of GnRH was determined at 12 and 25 wk in gestational and adolescent exposures and after 12 wk of post-pubertal exposure. This assay had been previously validated for rabbits and was performed as described in *Section: 3.4.1.10.A: Radioimmunoassay of Gonadotropin-Releasing Hormone.*

4.3.8.3 Radioimmunoassay of Testosterone

Concentrations of testosterone were determined at 6 wk in gestational and adolescent exposures. To assess the ability of the pituitary-gonadal axis to respond to GnRH input, GnRH challenge tests were conducted at 12 and 25 wk in gestational and adolescent exposures; animals in the 25 wk group were not subjected to GnRH challenge at 12 wk. In post-pubertal exposure concentrations of testosterone were determined before treatment and GnRH challenge tests were conducted after 12 wk of post-pubertal exposure. Each time, a baseline blood sample was taken, 10 μg GnRH was injected intra-muscularly, and two samples were collected at 30 and 120 minutes post-injection. Testosterone was assayed using a validated RIA (Berndtson *et al.*, 1974). For each sample 500 μl of serum was extracted twice in 5 ml Benzene:Hexanes (1:2 v/v), frozen in methanol cooled by dry-ice, and evaporated under nitrogen at 45°C. After the final evaporation, samples were reconstituted in 1.5 ml 0.1 M PBS-gel, vortexed and stored overnight at 4°C. Intra-assay and inter-assay coefficients of variation were 5.6 and 7.5%.

4.3.9 Statistical Analysis

For each parameter, data were analyzed using Statview (version 5.0, SAS Institute Inc., Cary, NC). Treatment was fixed for each experiment and all parameters were random. Difference between means was determined by t-test. The level of significance

was set at $p<0.05$. This model was used to evaluate body weights, hormone concentrations, and necropsy data. In post-pubertal exposure, data for repeated measures on the same animal (endocrine parameters, sexual behavior and semen characteristics) were analyzed by comparing before- and after-treatment values. Percentage values were transformed using arcsine of the square root of the percentage/100 to account for any inequalities in variance (Ott, 1993). Linear regression was performed on body weights during adolescent and post-pubertal exposures and also for determining accuracy of RIA. Logistic regression was performed on standard curves for RIA to determine the specificity of the assay. Slopes of the lines from regressions were compared using analysis of covariance (ANCOVA) with interaction.

4.4 Results

4.4.1 Organ Weights

Gestational exposure to DBP had no adverse effects on body weights at 6, 12, and 25 wk of age (Table 15). At 12 wk of age, there were no differences ($p>0.1$) in the weights of paired epididymides, but there was a significant decrease in the weights of paired testes ($p<0.05$) and accessory sex glands ($p<0.01$) in DBP pups compared to controls (Table 15). At 25 wk, there were no differences ($p>0.1$) in weights of paired epididymides or testes, however weight of accessory sex gland remained lower ($p<0.01$) in DBP-treated rabbits (Table 15). Testicular descent occurred between PNW 6-9 in 100% (12/12) of controls and 94% (16/17) of DBP rabbits. At 12 wk, one DBP rabbit was found to have severe malformations of the reproductive tract (Figure 22). These malformations included undescended testes, ambiguous genitalia, hypospadias, regressed prostate, and missing bulbourethral glands.

TABLE 15

Effects of gestational exposure to 400 mg DBP/kg/day from gestation days 15 to 29 on organ weights

Organ	6 wk		12 wk		25 wk	
	Control (n = 12)	DBP (n = 17)	Control (n = 6)	DBP (n = 11)	Control (n = 6)	DBP (n = 6)
Anogenital distance (mm)	4.90 ± 0.19	4.77 ± 0.24	4.73 ± 0.34	5.27 ± 0.42	7.03 ± 0.37	6.43 ± 0.32
Body (kg)	0.81 ± 0.05	0.79 ± 0.03	1.61 ± 0.06	1.54 ± 0.08	2.11 ± 0.05	2.04 ± 0.08
Liver (g)	...	...	...	...	64.45 ± 3.02	54.96 ± 4.24
Kidneys (g)	...	...	...	...	14.30 ± 0.61	13.52 ± 0.82
Thyroid (g)	...	...	...	...	0.16 ± 0.02	0.19 ± 0.03
Paired testes (g)[a]	...	...	2.28 ± 0.15	1.76 ± 0.17*	4.25 ± 0.37	4.32 ± 0.21
Epididymides (g)[a]	...	...	0.90 ± 0.05	0.79 ± 0.61	1.44 ± 0.04	1.36 ± 0.06
Accessory sex glands (g)[a]	...	...	1.81 ± 0.18	1.15 ± 0.12**	3.52 ± 0.17	2.54 ± 0.20**

Values represent mean ± SEM

[a]Data do not include undescended testes or malformed accessory sex glands

*$p<0.05$ and **$p<0.01$ using t-test

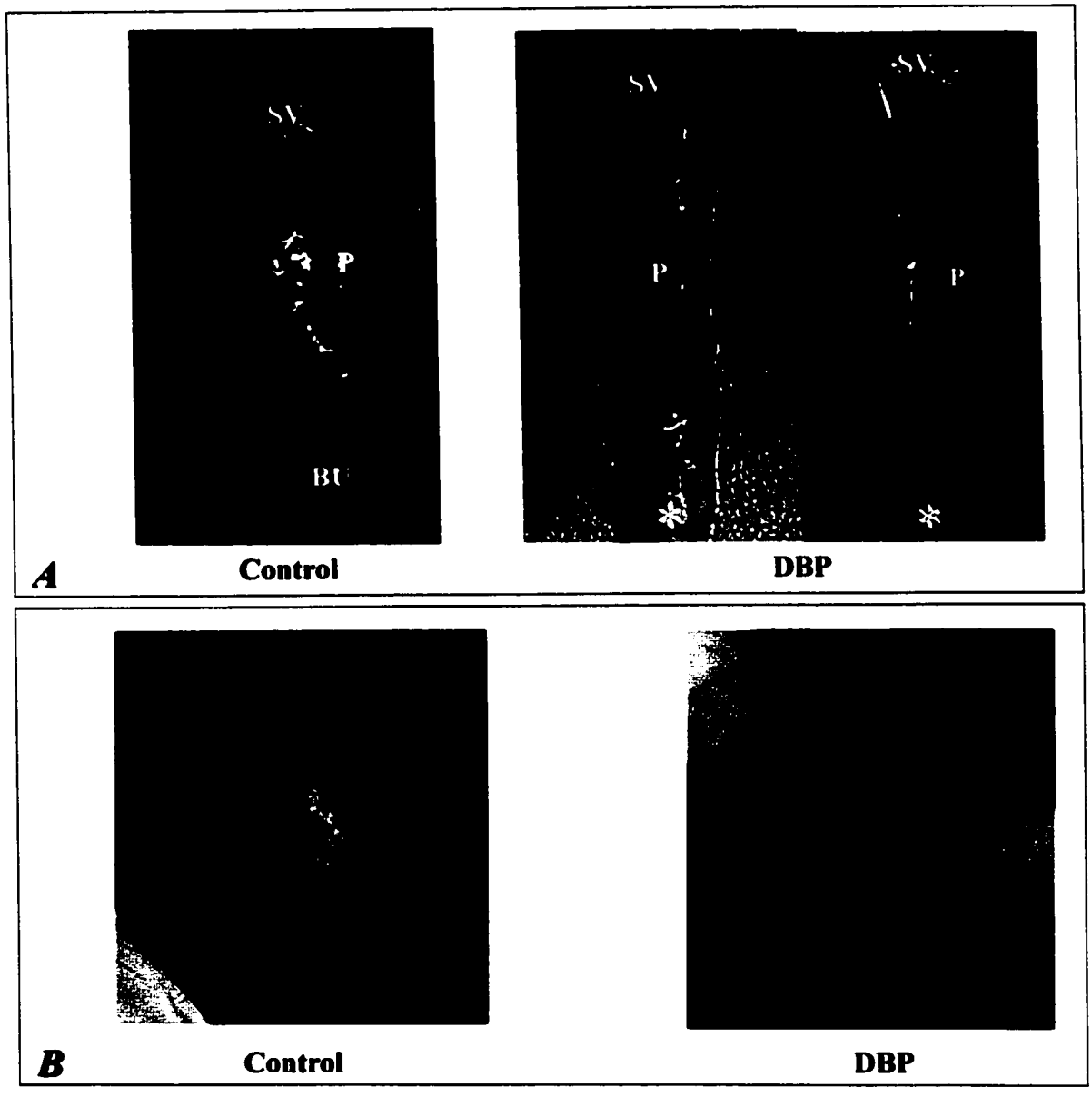


FIG. 22. Photographs of accessory sex glands and external genitalia of 12-wk-old rabbit exposed to 400 mg DBP/kg/day from GD 15 to 29. Gestational exposure to DBP reduced weights of paired testes and accessory sex glands, and at least in one animal (out of 12) caused severe regression or agenesis of individual components. ***A.***. Accessory sex glands: note regressed seminal vesicle ***(SV)*** and prostate ***(P)*** and missing bulbourethral gland ***(asterisks)*** in the DBP rabbit (*Dorsal (left) and Ventral (right) views*). ***B.*** In the same rabbit (*Right*), testes failed to descend to a scrotal position and the external genitalia were ambiguous.

Exposure to DBP during adolescence had no effect ($p>0.1$) on weight gain from PNW 4-12 (Figure 23) or body weight at 6, 12 and 25 wk of age (Table 16). Similar to gestational exposure, there were no significant differences ($p>0.1$) in weights of paired epididymides or testes, but a significant decrease ($p<0.05$) in the weight of accessory sex glands in DBP pups was observed at 12 wk (Table 16). This decrease in the weight of accessory sex glands in DBP-treated rabbits was not apparent at 25 wk (Table 16). Testicular descent occurred between PNW 6-9 in 100% (12/12) of controls and 91 % (10/11) of DBP rabbits. One DBP rabbit remained unilaterally cryptorchid at 25 wk with no gross malformations of the reproductive tract.

Post-pubertal exposure to DBP had no effect ($p>0.1$) on the maintenance of body weight throughout the dosing period (Figure 23). After 12 wk of exposure, weights of reproductive and visceral organs did not differ ($p>0.1$) in DBP and control rabbits (Table 17); there was a significant increase ($p<0.05$) in thyroid gland weight in DBP rabbits.

4.4.2 Sexual Capacity and Behavior

Gestational (Table 18) and adolescent exposure (Table 19) had no adverse effects on sexual behavior 25 and 13 wk after the cessation of treatment, respectively. Likewise, post-pubertal exposure caused no change in sexual behavior at the conclusion of treatment compared to before-treatment parameters (Table 20). Failure to ejaculate during 180 seconds of exposure to a teaser female only occurred once in gestational and once in post-pubertal exposure (Table 17 and 20). These incidences of failure only accounted for 3% (1/36) for gestational and 6% (1/18) for post-pubertal exposure of the total number of times sexual behavior was tested. In all of the failures, the rabbits expressed no sexual interest and achieved no erection.

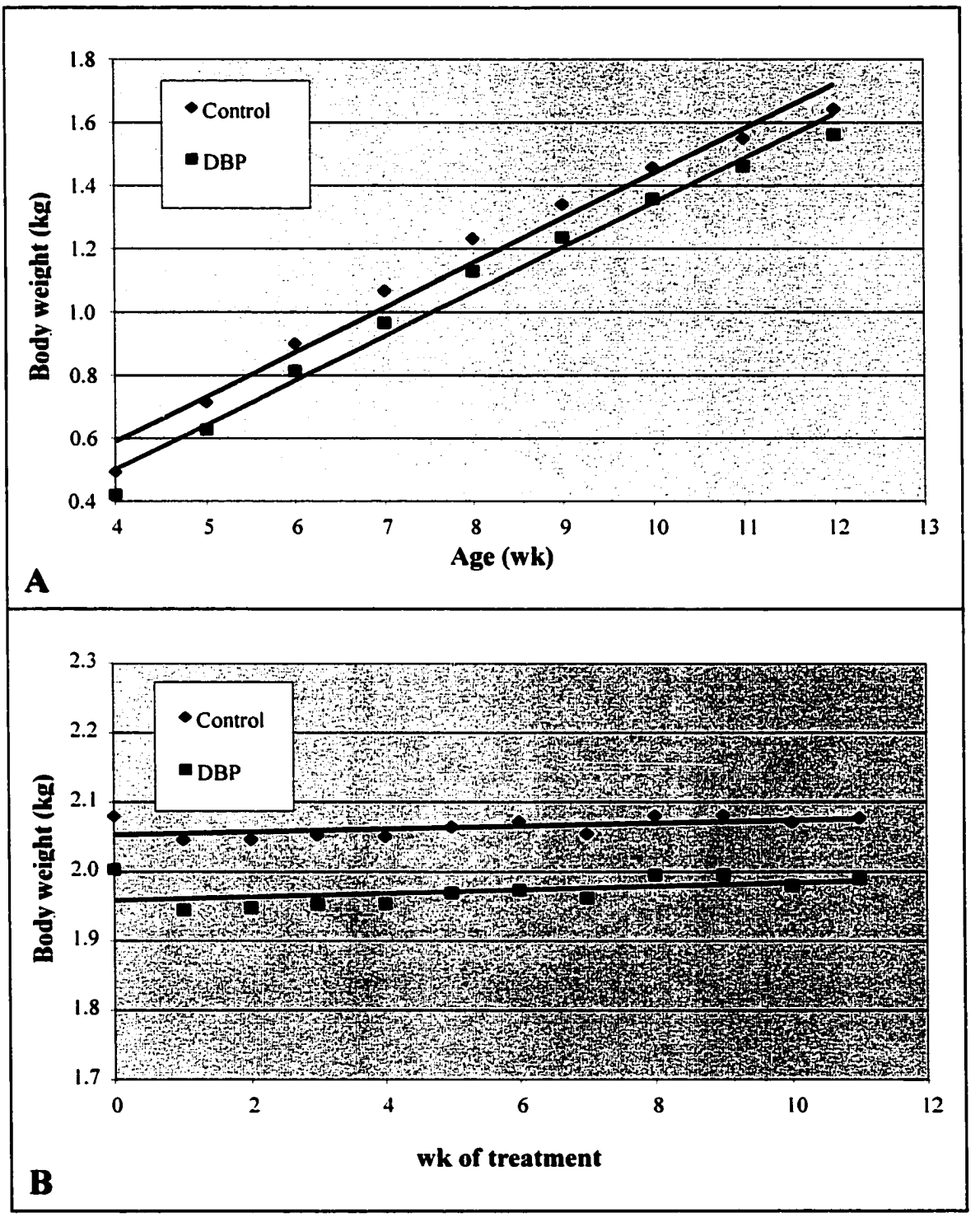


FIG. 23. Linear regression analysis of body weights during adolescent ***(A)*** or post-pubertal ***(B)*** exposure to 400 mg DBP/kg/day. In both exposures, there were no significant differences ($p>0.1$) in growth or maintenance between control and DBP-treated rabbits.

TABLE 16

Effects of adolescent exposure to 400 mg DBP/kg/day from post-natal week 4 to 12 on organ weights

Organ	6 wk		12 wk		25 wk	
	Control (n = 12)	DBP (n = 11)	Control (n = 6)	DBP (n = 5)	Control (n = 6)	DBP (n = 6)
Anogenital distance (mm)	...	...	4.73 ± 0.34	6.44 ± 0.16**	7.03 ± 0.37	7.28 ± 0.32
Body (kg)	0.81 ± 0.05	0.81 ± 0.03	1.61 ± 0.06	1.54 ± 0.06	2.11 ± 0.05	2.04 ± 0.08
Liver (g)	...	...	...	...	64.45 ± 3.02	62.67 ± 4.67
Kidneys (g)	...	...	...	...	14.30 ± 0.61	14.06 ± 0.82
Thyroid (g)	...	...	...	...	0.16 ± 0.02	0.15 ± 0.01
Paired testes (g)[a]	...	...	2.28 ± 0.15	1.82 ± 0.16	4.25 ± 0.37	4.74 ± 0.50
Epididymides (g)[a]	...	...	0.90 ± 0.05	0.86 ± 0.09	1.44 ± 0.04	1.65 ± 0.13
Accessory sex glands (g)[a]	...	...	1.81 ± 0.18	1.22 ± 0.18*	3.52 ± 0.17	3.85 ± 0.41

Values represent mean ± SEM

[a]Data do not include undescended testes

*$p<0.05$ and **$p<0.01$ using t-test

TABLE 17
Effects of post-pubertal exposure to 400 mg DBP/kg/day for 12 wk on organ weights

Organ	Treatment	
	Control (n = 6)	DBP (n = 6)
Anogenital distance (mm)	7.40 ± 0.35	6.92 ± 0.21
Body (kg)	2.08 ± 0.07	1.99 ± 0.05
Liver (g)	47.84 ± 2.59	45.90 ± 2.89
Kidneys (g)	12.78 ± 0.36	12.78 ± 0.63
Thyroid (g)	0.15 ± 0.01	0.17 ± 0.01*
Paired testes (g)	4.57 ± 0.26	4.52 ± 0.27
Epididymides (g)	1.72 ± 0.09	1.65 ± 0.10
Accessory sex glands (g)	3.31 ± 0.32	3.26 ± 0.42

Values represent mean ± SEM

*$p<0.05$ and **$p<0.01$ using t-test

TABLE 18
Effects of gestational exposure to 400 mg DBP/kg/day from gestation day 15 to 29 on sexual behavior and capacity at 25 wk of age

Treatment	n	Seconds to Ejaculate		# of Mounts to Ejaculate		No. of males that failed to ejaculate at least once
		Average[a]	Range	Average[a]	Range	
Control	6	18.1 ± 2.4	5 - 60	1.2 ± 0.1	1 - 3	0
DBP	6	15.4 ± 1.4	8 - 40	1.2 ± 0.1	1 - 2	1

Data for 6 occasions when collection of semen was attempted

[a]Excludes time for occasion when there was a failure to ejaculate (values represent mean ± SEM)

TABLE 19
Effects of adolescent exposure to 400 mg DBP/kg/day from post-natal week 4 to 12 on sexual behavior and capacity at 25 wk of age

Treatment	n	Seconds to Ejaculate		# of Mounts to Ejaculate		No. of males that failed to ejaculate at least once
		Average[a]	Range	Average[a]	Range	
Control	6	18.1 ± 2.4	5 - 60	1.2 ± 0.1	1 - 3	0
DBP	6	10.9 ± 1.1**	5 - 29	1.3 ± 0.1	1 - 2	0

Data for 6 occasions when collection of semen was attempted

[a]Excludes time for occasion when there was a failure to ejaculate (values represent mean ± SEM)

*p<0.05 and **p<0.01 using unpaired t-test

TABLE 20

Effects of post-pubertal exposure to 400 mg DBP/kg/day for 12 wk on sexual behavior and capacity

		Before-treatment					After-treatment				
		Seconds to Ejaculate		# of Mounts to Ejaculate		No. of males that failed to ejaculate at least once	Seconds to Ejaculate		# of Mounts to Ejaculate		No. of males that failed to ejaculate at least once
Treatment	n	Average[a]	Range	Average[a]	Range		Average[a]	Range	Average[a]	Range	
Control	6	19.8 ± 2.9	4 - 50	1.5 ± 0.1	1 - 3	0	15.4 ± 3.2	2 - 61	1.3 ± 0.1	1 - 3	0
DBP	6	36.8 ± 7.1	7 - 120	1.8 ± 0.2	1 - 3	0	30.0 ± 5.1	7 - 90	1.9 ± 0.3	1 - 4	1

Data for 3 occasions for before- and after-treatment when collection of semen was attempted

[a]Excludes time for occasion when there was a failure to ejaculate (values represent mean ± SEM)

4.4.3 Production of Spermatozoa

Gestational, adolescent and post-pubertal exposure to 400 mg DBP/kg/day did not have adverse effects on spermatogenesis as evidenced by no differences ($p>0.1$) in daily sperm production per gram of testis, and the number of spermatozoa in the caput-corpus or cauda epididymidi between control and DBP rabbits (Tables 21, 22, 23). However, gestational exposure significantly decreased the ejaculate volume ($p<0.01$), sperm concentration ($p<0.05$) and total sperm per ejaculate ($p<0.01$) compared to controls (Table 21). Although not statistically significant ($p=0.06$), there was a ~20% decrease in the sperm concentration in DBP-treated rabbits from adolescent exposure (Table 22). No decrease in semen parameters was observed in post-pubertal exposure during post-treatment evaluation compared to before-treatment values (Table 23).

Gestational and adolescent exposure decreased ($p<0.01$) the quality of sperm when evaluated by light microscopy. For gestational and adolescent exposure, DBP-treated rabbits had almost twice as many abnormal sperm (30.1% and 25.7%) than controls (16.6% and 16.6%) (Table 21 and 22). A majority of the abnormal sperm (16 and 18%) had acrosomal and nuclear defects that originated from the testis (Figure 24). These included acrosomal dysplasia and vesiculation and malformed nuclei. In post-pubertal exposure, there also was a significant reduction (5%; $p<0.01$) in the number of normal sperm in the after-treatment ejaculates from DBP-treated rabbits. A majority of the abnormal sperm that contributed to a reduction in normal sperm had acrosomal defects (4.5% increase). In comparison, control rabbits had a 2% increase in the number of normal sperm in the after-treatment ejaculates (Table 23).

TABLE 21

Effects of gestational exposure to 400 mg DBP/kg/day from gestation day 15 to 29 on sperm production and semen characteristics at 25 wk of age

	Treatment	
	Control (n = 6)	DBP (n = 6)
Organ Weights		
Right testis (g)	2.12 ± 0.17	2.21 ± 0.10
Right epididymis (g)	0.69 ± 0.02	0.68 ± 0.03
Tissue Homogenates		
Daily sperm production (x 10^6/g testis)	14.65 ± 2.46	16.98 ± 1.17
Caput epididymal sperm reserve (x 10^6)	139.87 ± 25.21	128.86 ± 13.26
Cauda epididymal sperm reserve (x 10^6)	407.72 ± 65.31	469.86 ± 39.40
Semen Characteristics		
Ejaculate volume (ml)	0.40 ± 0.03	0.28 ± 0.02**
Sperm concentration (x 10^6/ml)	563.59 ± 50.33	414.60 ± 28.90*
Total sperm/ejaculate (x 10^6)	214.74 ± 22.23	121.59 ± 12.08**
Morphologically normal sperm (%)	83.44 ± 0.76	69.26 ± 1.49**

For semen parameters, data from 6 ejaculates were pooled

Values represent mean ± SEM

*$p<0.05$ and **$p<0.01$ using t-test

TABLE 22

Effects of adolescent exposure to 400 mg DBP/kg/day from post-natal week 4 to 12 on sperm production and semen characteristics at 25 wk of age

	Treatment	
	Control (n = 6)	DBP (n = 6)
Organ Weights		
Right testis (g)	2.12 ± 0.17	2.36 ± 0.22
Right epididymis (g)	0.69 ± 0.02	0.79 ± 0.06
Tissue Homogenates		
Daily sperm production (x 10^6/g testis)	14.65 ± 2.46	21.98 ± 2.29
Caput epididymal sperm reserve (x 10^6)	139.87 ± 25.21	116.53 ± 17.14
Cauda epididymal sperm reserve (x 10^6)	407.72 ± 65.31	499.15 ± 37.91
Semen Characteristics		
Ejaculate volume (ml)	0.40 ± 0.03	0.45 ± 0.02
Sperm concentration (x 10^6/ml)	563.59 ± 50.33	444.09 ± 36.49
Total sperm/ejaculate (x 10^6)	214.75 ± 22.23	203.20 ± 18.18
Morphologically normal sperm (%)	83.44 ± 0.8	74.18 ± 0.90**

For semen parameters, data from 6 ejaculates were pooled

Values represent mean ± SEM

*p<0.05 and **p<0.01 using t-test

TABLE 23

Sperm production and semen characteristics before and after post-pubertal exposure to 400 mg DBP/kg/day for 12 wk

	Treatment			
	Control (n = 6)		DBP (n = 6)	
	Before-treatment	After-treatment	Before-treatment	After-treatment
Organ Weights				
Right testis (g)	...	2.24 ± 0.13	...	2.26 ± 0.15
Right epididymis (g)	...	0.85 ± 0.05	...	0.83 ± 0.05
Tissue Homogenates				
Daily sperm production (x 10^6/g testis)	...	23.89 ± 0.99	...	22.85 ± 0.74
Caput epididymal sperm reserve (x 10^6)	...	117.36 ± 11.50	...	116.94 ± 9.21
Cauda epididymal sperm reserve (x 10^6)	...	469.50 ± 51.35	...	449.33 ± 72.33
Semen Characteristics				
Ejaculate volume (ml)	0.56 ± 0.04	0.61 ± 0.04	0.50 ± 0.03	0.51 ± 0.05
Sperm concentration (x 10^6/ml)	442.71 ± 64.20	523.68 ± 67.09	552.99 ± 63.70	497.89 ± 45.11
Total sperm/ejaculate (x 10^6)	252.87 ± 40.14	327.33 ± 59.23	282.81 ± 37.24	250.75 ± 27.05
Morphologically normal sperm (%)	69.44 ± 3.69	71.06 ± 3.98	80.03 ± 1.2	75.25 ± 1.68*

For semen parameters, data from 3 ejaculates were pooled

Values represent mean ± SEM

*p<0.05 and **p<0.01 using t-test

FIG. 24. Photomicrographs of sperm showing acrosomal and nuclear defects. Differential interference contrast ***(A,B)***, and electron microscopy ***(C)*** revealed that these defects included acrosomal dysplasia and vesiculation and incomplete nuclear condensation. That these defects originated during spermiogenesis, and not during epididymal transit, is evident from histological examination of testicular sections ***(D)***. ***Arrows:*** Acrosomal defects, ***Arrowheads:*** Incomplete nuclear condensation. Images captured at ***(A)*** 930X, ***(B,D)*** 1030X. Magnification. ***(C)*** 5000X.

4.4.4 Histopathology of the Testis

Normalcy of the seminiferous epithelium was evaluated by determining DGEL at 25 wk of age for gestational and adolescent exposure, and after 12 wk of post-pubertal exposure. Pathological changes representative of grades 1 and 2 were observed in all rabbits from each treatment group (Table 24, Figure 25). The frequency of these changes was higher in DBP rabbits compared to controls and the incidence of tubules with total germinal epithelial loss (grade 4) was low, only occurring in a few isolated rabbits from each DBP group (Figure 25). Grades 5-7 tubules were not observed. Reflective of the increased incidence of grades 1 and 2 in DBP rabbits, the DGEL was significantly higher ($p<0.05$) in all DBP groups than in controls (Table 24). Furthermore desquamated cells were frequently observed in the lumen, where they may ultimately block a tubule leading to swelling of seminiferous tubules (Figure 25).

Electron microscopic evaluation of the testes from one 12-wk-old bilaterally cryptorchid rabbit from gestational exposure and one 25-wk-old unilaterally cryptorchid rabbit from adolescent exposure revealed atypical germ cells resembling gonocytes (Figures 26 and 27) with morphological features indicative of carcinoma *in situ* (CIS). These included unusual membranous profiles, swollen mitochondria, glycogen deposits, irregular nuclear contours, and chromatin clumps. Swollen mitochondria have been noted as a characteristic change associated with seminoma in humans (Ghadially, 1997). Gonocytes should not be present beyond the perinatal period of testicular development and their presence at 12 and 25 wk indicates an abnormality (Veeramachaneni, 2000a). The cellular atypia ranged from early alterations in the nucleolonema to more progressive changes characterized by unusual membranous profiles, irregular nuclear contours,

TABLE 24

Histopathological changes in the seminiferous epithelium after gestational, adolescent and post-pubertal exposure to 400 mg DBP/kg/day

		Percentage of seminferous tubules graded as:								Degree of germinal epithelial loss[a]
	n	0	1	2	3	4	5	6	7	
Gestational exposure										
Control	6	88.0	9.2	2.7	...	...	...	...	...	3.6 ± 0.2
DBP	6	79.7	14.8	5.0	0.2	0.3	...	...	...	6.7 ± 1.0*
Adolescent exposure										
Control	6	88.0	9.2	2.7	...	...	...	...	...	3.6 ± 0.2
DBP	6	81.0	15.7	4.2	...	0.2	...	...	...	6.2 ± 1.0*
Post-Pubertal exposure										
Control	6	86.2	9.8	2.2	...	...	...	...	...	3.5 ± 0.3
DBP	6	81.2	16.5	2.2	0.2	...	...	...	...	5.3 ± 0.7*

[a]Values represent mean ± SEM

*$p<0.05$ and **$p<0.01$ using t-test

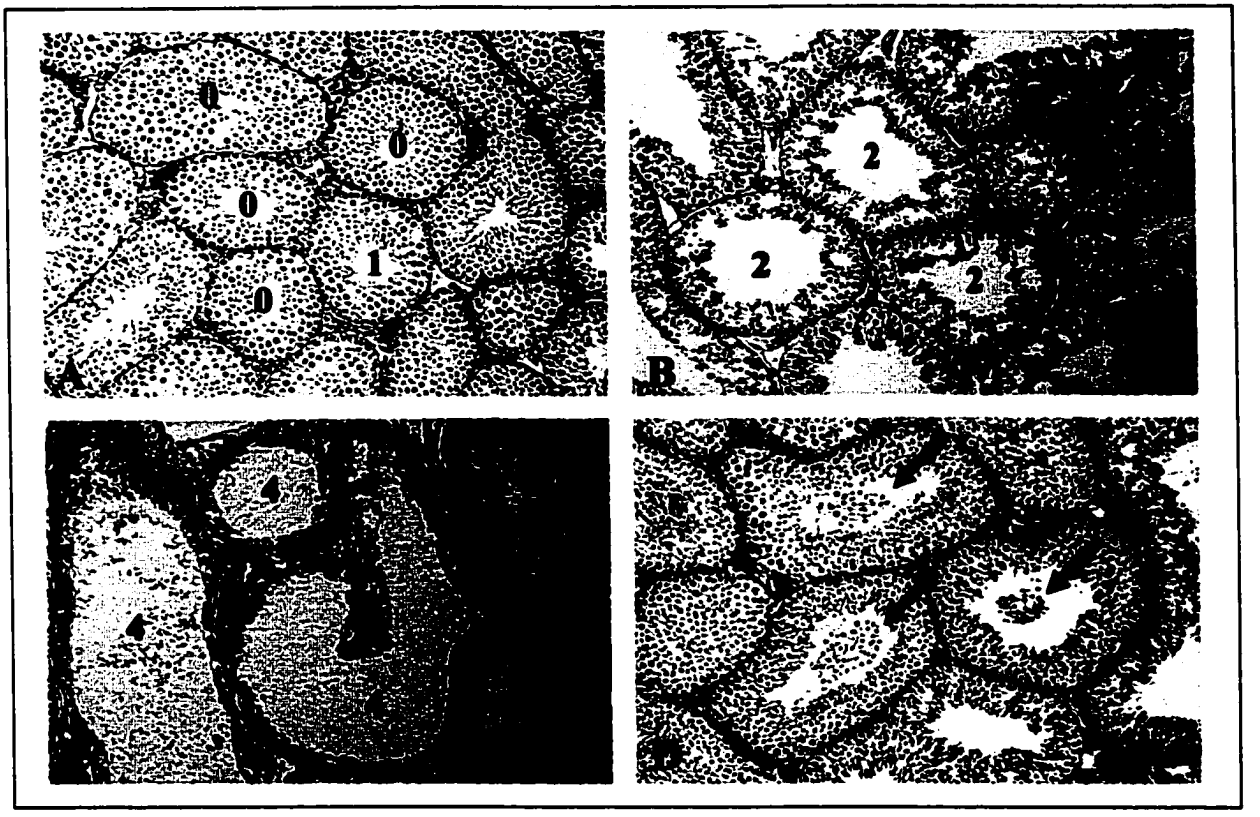


FIG. 25. Pathological changes of the seminiferous epithelium after gestational, adolescent, and post-pubertal exposure to 400mg DBP/kg/day. In control rabbits grades ≥1 were observed infrequently ***(A)*** compared to DBP rabbits ***(B)***. Grade 4 tubules containing predominantly Sertoli cells and a few germ cells were observed in gestational and adolescent exposure to DBP ***(C)***. Reflective of the increased ($p<0.05$) DGEL in DBP-treated rabbits, desquamated cells ***(black arrows)*** were frequently seen in the lumen ***(D)***. This cellular debris may ultimately block a tubule leading to swelling of seminferous tubules. Note the swollen grade 4 tubules in panel ***(C)***. ***Numbers*** indicate grade of pathological change. Magnification: 100X.

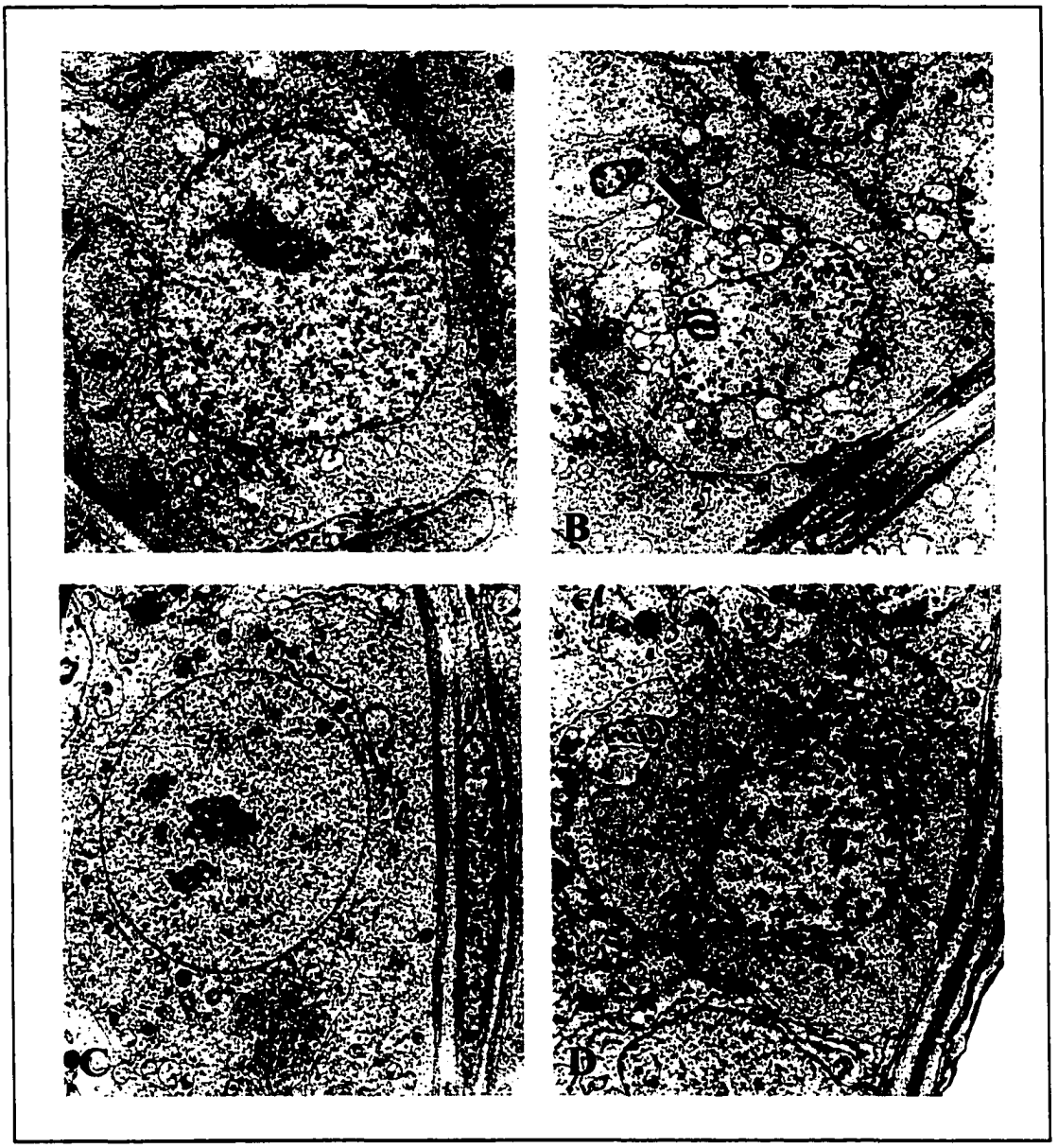


FIG. 26. Transmission electron micrographs of atypical gonocytes from a 12-wk-old bilaterally cryptorchid rabbit exposed to 400 mg DBP/kg/day from GD 15 to 29 ***(A,B)*** and a 25-wk-old rabbit exposed to 400 mg DBP/kg/day DBP from PNW 4 to 12 ***(C,D)***. Gonocytes are not usually present beyond perinatal period of testicular differentiation. Cellular atypia ranged from early alterations in the nucleolonema ***(A,C)*** to more progressive changes characterized by unusual membranous profiles, swollen mitochondria ***(black arrow)***, irregular nuclear contours, and chromatin clumps ***(B,D)***. The presence of these ultrastructural lesions is indicative of testicular carcinoma *in situ* (Skakkebaek, 1972) and have been associated with seminoma in humans (Ghadially, 1997). Magnification. ***(A,C,D)*** 3500X and ***(B)*** 3000X.

chromatin clumps, and swollen mitochondria (Figure 26). Atypical spermatogonia with similar morphological hallmarks of CIS were also identified in the seminiferous epithelium from the 25-wk-old unilaterally cryptorchid rabbit (Figure 27).

In addition to the adverse effects on differentiating stem cells, DBP induced unique vascular lesions in the interstitium. At post-natal day 5, extravascular erythrocytes undergoing phagocytosis were identified in the interstitium (Figure 28). This vascular lesion persisted long after the cessation of treatment (~25 wk) and was caused by widened fenestrae in the capillary endothelial wall (Figure 28).

Interestingly, atypical germ cells also were identified in the seminiferous epithelium of a rabbit in post-pubertal exposure group (Figure 29). These cells had perinuclear cytoplasmic inclusions, irregular nuclear contours and pale staining of the cytoplasm, suggesting a paucity of organelles. Electron microscopic evaluation confirmed light microscopic observations and further revealed pars amorpha in nucleolus, chromatin clumping and swollen mitochondria. It is assumed that these atypical cells were derived from a mature phenotype rather than a gonocyte since exposure began well beyond infantile and pubertal development.

4.4.5 Thyroid and Reproductive Hormones

Concentrations of thyroid and reproductive hormones for gestational, adolescent and post-pubertal exposures to DBP are presented in Tables 25, 26 and 27. Although not statistically different ($p>0.1$), the concentration of T_3 in rabbits exposed to DBP during gestation was increased by 38 and 13% at 6 and 12 wk compared to controls. However in adolescent exposure, serum T_3 increased significantly ($p<0.01$) at 6 and 12 wk in DBP pups compared to controls. Serum T_3 was not affected ($p>0.1$) in post-pubertal exposures

FIG. 27. Transmission electron micrographs of an unaffected spermatogonium ***(A)*** and atypical spermatogonia from a 25-wk-old rabbit exposed to 400 mg DBP/kg/day from PNW 4 to 12 ***(B)***. Note presence of meandering nucleolus ***(white arrow)***, unusual membranous profiles, swollen mitochondria ***(black arrows)***, glycogen deposits, irregular nuclear contours, and chromatin clumps ***(B)***. The presence of these ultrastructural features has been associated with seminomatous lesions in humans (Ghadially, 1997). Magnification: ***(A)*** 4000X and ***(B)*** 3500.

FIG. 28. Transmission electron micrographs of interstitial tissue ***(IT)*** of rabbit testis at post-natal day 5 ***(A)*** and 25 wk of age ***(B)*** showing lesions to the vascular endothelium. ***A.*** Extravascular erythrocytes undergoing phagocytosis ***(white arrow)***. ***B.*** Widened fenestrae in the capillary endothelial wall ***(black arrow)***. ***ST:*** Seminiferous tubule, ***LC:*** Leydig cell, ***RBC:*** Red blood cell. Magnification: 2500X.

FIG. 29. Photomicrographs of atypical germ cells from a male rabbit exposed post-pubertally to 400 mg DBP/kg/day for 12 wk. Light microscopic examination of H&E stained sections ***(A)*** revealed that these atypical germ cells ***(white arrows)*** had perinuclear cytoplasmic inclusions, abnormal nuclear contours, and pale cytoplasm compared to a normal germ cell ***(arrowhead)***. In addition, abnormal nucleoli, irregular chromatin clumping and mitochondrial swelling ***(black arrow)*** were observed in electron micrographs ***(B)***. ***Asterisks*** indicate Sertoli cell nuclei. Magnification: ***(A)*** 1125X ***(B)*** 3500X.

TABLE 25
Effects of gestational exposure to 400 mg DBP/kg/day from gestation day 15 to 29 on thyroid and reproductive hormones

	6 wk		12 wk		25 wk	
	Control	DBP	Control	DBP	Control	DBP
Triiodothyronine (ng/ml)	1.36 ± 0.04 (12)	2.14 ± 0.55 (12)	1.55 ± 0.04 (11)	1.77 ± 0.28 (11)	1.80 ± 0.08 (5)	1.82 ± 0.04 (6)
Hypothalamic GnRH (ng)	...		9.15 ± 2.20 (6)	11.08 ± 2.13 (6)	11.82 ± 1.28 (6)	10.07 ± 0.93 (6)
Baseline serum testosterone (ng/ml)	8.31 ± 0.84 (12)	5.62 ± 0.87*(12)	6.21 ± 2.29 (6)	2.05 ± 0.76 (6)	6.10 ± 2.31 (6)	3.07 ± 1.01 (6)
30min post-GnRH serum testosterone (ng/ml)	...	...	18.23 ± 1.30 (6)	22.02 ± 3.71 (5)	14.36 ± 0.70 (6)	16.49 ± 1.60 (6)
Change in serum testosterone (ng/ml)[a]	...	...	12.02 ± 2.35 (6)	20.63 ± 4.01 (5)	8.27 ± 2.02 (6)	13.42 ± 2.30 (6)
120min post-GnRH serum testosterone (ng/ml)	...	...	16.52 ± 0.98 (6)	21.82 ± 3.34 (5)	13.74 ± 2.19 (4)	17.11 ± 3.65 (6)

[a] Change from baseline serum testosterone concentration

Values represent mean ± SEM

Numbers in parentheses indicate number of animals

*$p<0.05$ and **$p<0.01$ using t-test

TABLE 26

Effects of adolescent exposure to 400 mg DBP/kg/day from post-natal week 4 to 12 on thyroid and reproductive hormones

	6 wk		12 wk		25 wk	
	Control	DBP	Control	DBP	Control	DBP
Triiodothyronine (ng/ml)	1.36 ± 0.04 (12)	1.80 ± 0.05** (11)	1.55 ± 0.04 (11)	1.89 ± 0.05**(11)	1.80 ± 0.08 (5)	1.87 ± 0.08 (6)
Hypothalamic GnRH (ng)	...	...	9.15 ± 2.20 (6)	17.62 ± 1.58*(5)	11.82 ± 1.28 (6)	11.23 ± 2.24 (6)
Baseline serum testosterone (ng/ml)	8.31 ± 0.84 (12)	5.03 ± 0.61**(11)	6.21 ± 2.29 (6)	8.06 ± 3.21 (5)	6.10 ± 2.31 (6)	1.35 ± 0.38 (6)
30min post-GnRH serum testosterone (ng/ml)	...	...	18.23 ± 1.30 (6)	7.80 ± 0.95**(5)	14.36 ± 0.70 (6)	10.40 ± 2.74 (6)
Change in serum testosterone (ng/ml)[a]	...	...	12.02 ± 2.35 (6)	-0.27 ± 2.43**(5)	8.27 ± 2.02 (6)	9.05 ± 2.54 (6)
120min post-GnRH serum testosterone (ng/ml)	...	...	16.52 ± 0.98 (6)	10.57 ± 2.08**(5)	13.74 ± 2.19 (4)	8.25 ± 2.53 (6)

[a] Change from baseline serum testosterone concentration

Values represent mean ± SEM

Numbers in parentheses indicate number of animals

*p<0.05 and **p<0.01 using t-test

TABLE 27
Effects of post-pubertal exposure to 400 mg DBP/kg/day for 12 wk on thyroid and reproductive hormones

	Control		DBP	
	Before-treatment	After-treatment	Before-treatment	After-treatment
Triiodothyronine (ng/ml)	1.83 ± 0.25 (6)	1.66 ± 0.06 (6)	1.63 ± 0.07 (6)	1.87 ± 0.10 (6)
Hypothalamic GnRH (ng)	...	13.97 ± 2.57 (6)	...	13.25 ± 1.25 (6)
Baseline serum testosterone (ng/ml)	2.44 ± 0.72 (6)	0.64 ± 0.09* (6)	2.35 ± 1.44 (6)	2.65 ± 0.83 (6)
30min post-GnRH serum testosterone (ng/ml)	...	13.85 ± 4.77 (6)	...	10.17 ± 0.80 (6)
Change in serum testosterone (ng/ml)[a]	...	13.21 ± 4.80 (6)	...	7.52 ± 1.39 (6)
120min post-GnRH serum testosterone (ng/ml)	...	8.90 ± 2.66 (6)	...	9.60 ± 0.84 (6)

[a] Change from baseline serum testosterone concentration

Values represent mean ± SEM

Numbers in parentheses indicate number of animals

at any time point measured. Clearly the effect of DBP on the concentration of serum T_3 is directly related to the period when exposure occurred.

Gestational and post-pubertal exposures to DBP did not affect ($p>0.1$) the hypothalamic content of GnRH (Table 25 and 27), but adolescent exposure significantly increased ($p<0.01$) the GnRH content at 12 wk in DBP pups. However, this increase in GnRH content was not observed at 25 wk of age. The mean baseline level of testosterone decreased significantly in DBP rabbits at 6 wk in gestational ($p<0.05$) and adolescent ($p<0.01$) exposures. This decrease in mean baseline concentration was not apparent in subsequent measurements at 12 and 25 wk. At the conclusion of post-pubertal exposure, there was no change ($p>0.1$) in baseline concentration of testosterone compared to before-treatment values.

Regardless of baseline testosterone concentrations, injection of GnRH increased serum testosterone concentration 30 minutes later in gestational and post-pubertal exposures (Table 25 and 27). Likewise, the magnitude of this change was not affected. But, at 12 wk in adolescent exposure, the concentration of testosterone and the magnitude of the change in testosterone at 30 minutes post-GnRH were significantly lower ($p<0.01$) in DBP pups (Table 26). At 120 minutes post-GnRH the concentration of testosterone was still lower ($p<0.05$) in DBP pups from adolescent exposure. This impaired ability of the pituitary-gonadal axis to respond to exogenous GnRH was not apparent at 25 wk. The concentration of testosterone was not affected ($p>0.1$) in gestational and post-pubertal exposures at 120 minutes post-GnRH.

4.5 Discussion

This study found that DBP causes reproductive toxicity in male Dutch-Belted rabbits when exposed *in utero*, during adolescence, or post-pubertally. The fact that most pronounced effects were observed in rabbits exposed during gestation or adolescence lends support to findings in rodents that the male reproductive system is more susceptible to DBP in developing animals than in post-pubertal animals. No changes in external genitalia or sexual behavior were seen when rabbits were exposed after puberty but subtle alterations in testicular function were observed.

Rats exposed to >500 mg DBP/kg/day from GD 12 to 21 had a high incidence of impaired differentiation of the derivatives of Wolffian duct (epididymis and seminal vesicle) and urogenital sinus/genital tubercle (prostate and external genitalia), and testicular maldescent. Reflective of these changes in androgen-dependent phenomena, the weights of the paired testes, epididymides, seminal vesicles and prostate were reduced at PND 100 (Mylchreest *et al.*, 1999). Similarly, we also found that rabbits exposed to DBP from GD 15 to 29 had decreased weights of the paired testes (at 12 wk) and accessory glands (at 12 and 25 wk), and reproductive tract malformations – regressed prostate, missing bulbourethral glands, undescended testes, ambiguous genitalia, and hypospadias at least in one rabbit. Likewise, rabbits exposed during adolescence had decreased weights of accessory sex glands at 12 wk and one rabbit was unilaterally cryptorchid. The low incidence of the reproductive tract malformations in observed exposures compared to high incidence of rodent studies may be a result of decreased dose (400 mg DBP/kg/day in rabbit vs. >500 mg DBP/kg/day in rodents) or reflect differences in metabolism, since some species have the propensity to detoxify DBP and its

metabolites more efficiently (Foster *et al.*, 1983). Despite the low incidence, these findings confirm the previously reported adverse effects of DBP on the developing reproductive system.

In rodents, gestational exposure to DBP also caused testicular atrophy with wide-spread germ cell loss, multinuclear gonocytes and Leydig cell hyperplasia (Mylchreest *et al.*, 1998; 2002; Wine *et al.*, 1997). Similarly, rodents treated with 1000 mg DEHP/kg/day from PND 6 to 11 had decreased proliferation of Sertoli cells immediately after the final dose, which eventually reaches normal levels by PNW 6. In spite of the recovery in number of Sertoli cells a decrease in the number of testicular spermatids was observed at PNW 19 (Dostal *et al.*, 1988). Our observations of altered semen parameters in rabbits exposed to DBP during gestation and adolescence is similar to data reported for rodents, yet there were differences between the two exposures. Gestational exposure significantly decreased ejaculate volume, sperm concentration, sperm per ejaculate, and percentage of normal sperm. Adolescent exposure decreased sperm concentration and percentage of normal sperm but not ejaculate volume. The differences in these observations may be a result of the window of exposure. Despite the differences in observed toxicity, DBP causes permanent alterations to the male reproductive system.

In spite of the differences in semen parameters both gestational and adolescent exposure caused histological lesions in the testis. Both exposures increased the DGEL 13 or 25 wk after cessation of treatment. These observations are consistent with the reports of increased germ cell loss in rodents without recovery as adults (Mylchreest *et al.*, 1998; 1999). In addition, we observed atypical germ cells resembling carcinoma *in situ* (CIS) in the cryptorchid testis of rabbits exposed to 400 mg DBP/kg/day during gestation and

adolescence. Since cellular transformation of germ cells in the rabbit testis to CIS is dependent on the nature of the chemical insult and not the abdominal location of the testis per se (Veeramachaneni *et al.*, 2001b), it is logical to conclude that DBP induces unique lesions in the rabbit testis which were not documented in rodents. Whether these cells ultimately transform into testicular cancer needs to be addressed in long-term studies since the development of CIS into testicular cancer in humans is estimated to be 50% in five years (Skakkebaek *et al.*, 1987). However, the ability of the rabbit model to detect these subtle alterations to germ cells will be important in elucidating the etiology and pathogenesis of human disease.

Phthalate-mediated male reproductive toxicity in the developing testis is thought to occur by a complex interaction between Sertoli, Leydig, and germ cells (Mylchreest *et al.*, 2002). Phthalates inhibit FSH-mediated processes in Sertoli cells, and increase the exfoliation of germ cells and incidence of multinuclear gonocytes (Li *et al.*, 1998, 2000). Furthermore DBP decreases the testicular testosterone concentrations by inhibiting key enzymes of the steroidogenic pathway (Mylchreest *et al.*, 2002). Gestational and adolescent exposure to DBP decreased concentrations of testosterone at 6wk. Gestational exposure did not alter the basal or GnRH-stimulated concentrations of testosterone or the hypothalamic GnRH content at 12 or 25 wk. However, at 12 wk rabbits exposed to DBP during adolescence had basal levels of testosterone similar to controls, yet GnRH-stimulated testosterone production was significantly decreased 30 min post-stimulation. Furthermore, the hypothalamic GnRH content was increased in DBP-treated animals at 12 wk. Without measuring the concentrations of gonadotropins we were unable to ascertain whether this effect was mediated by lowered pituitary responsiveness to GnRH

or the inability of the testis to respond to gonadotropin. This effect was not permanent, and the ability to respond to GnRH-stimulation returned to normal at 25 wk. Therefore it appears DBP directly affected the endocrine environment of the developing rabbit testis, probably by altering differentiating Leydig cells, since perturbations occurred 6 wk after gestational and during adolescent treatments.

Rabbits exposed *in utero* had induced unique vascular lesion in the testis – widened fenstrae in the endothelium resulting in leakage of blood into interstitium. This lesion persisted long after the cessation of treatment. Whether this vascular lesion, which can result in a local inflammatory response, has any association with altered Leydig cell function leading to decreased testosterone secretion at 6 wk is not known. Hemorrhagic testis resulting from similar vascular lesion has been reported to occur in rats exposed to 750 mg/kg/day DEHP from GD 14 to PND 3 (Gray *et al.*, 2000).

Because our preliminary studies in the frogs indicated that DBP altered thyroid-dependent metamorphosis, we determined the concentrations of T_3. Although not statistically different the concentration of T_3 was increased 38 and 13% at 6 and 12 wk in animals exposed during gestation, while concentration of T_3 was significantly increased during adolescent exposure at 6 and 12 wk. Therefore similar to the effects of DBP on the endocrine environment of the developing rabbit testes, it appears that DBP also directly affects concentrations of T_3. The potential role of thyroid hormones contributing to DBP-induced reproductive toxicity should not be overlooked. Other studies determined that thyroid hormones are vital for the proliferation and maturation of prepubertal Sertoli cells and perturbations led to altered development (Ulisse *et al.*, 1994; Arambepola *et al.*; 1998b). Inhibiting thyroid hormone-dependent processes in 21-day-

old rats led to a reduction in weights of testis, epididymis and prostate (Marty *et al.*, 2001). Furthermore, acute exposure to thyroid hormones stimulates steroid biosynthesis, StAR expression, and increased LH receptor gene expression and ligand binding *in vitro* (Manna *et al.*, 2001a; 2001b). Future studies characterizing the mechanism of phthalate-mediated toxicity need to address the etiology of perturbations in T_3-dependent processes.

In rodent studies, exposure to 80 or 385 mg DBP/kg from GD 0 to PND 21 produced no changes in the ability of F_1 males to mate with control females (Wine *et al.*, 1997). Similarly, in the current study, neither gestational nor adolescent exposure to DBP produced marked changes in mating desire/ability at 25 wk. These findings confirm that DBP is a reproductive toxicant, which does not interfere with sexual behavior and capacity. Furthermore they support the rabbit as an animal model to detect subtle changes in reproductive parameters, since in other studies male rabbits exposed via drinking water to common chemical contaminants (Veeramachaneni *et al.*, 2001a) or disinfection by-products (Veeramachaneni *et al.*, 2000b) had marked decrease in mating desire/ability.

Acute exposure of post-pubertal rodents to high doses of DBP (2000-2400 mg DBP/kg/day) caused an immediate reduction in testis weight and severe testicular atrophy (Cater *et al.*, 1976; 1977; Gray *et al.*, 1982; Foster *et al.*, 1980). However, post-pubertal rodents fed a diet containing 1% DBP for 14 wk had no changes in sperm parameters (Wine *et al.*, 1997), possibly because DBP-induced reproductive effects were partially reversible over time (Tanino *et al.*, 1987). This latter observation is somewhat similar to our observations that chronic administration of 400 mg DBP/kg/day for 12 wk in post-pubertal rabbits increased DGEL, but had no effect on testicular weights, sperm

production or semen parameters. However, utilizing the rabbit as a model, we demonstrated that post-pubertal exposure to DBP significantly reduced the percentage of normal sperm after 12 wk of DBP treatment compared to before-treatment values.

It is uncertain if the phthalate-induced testicular toxicity in post-pubertal rodents was a result of changes in reproductive hormones or direct effect on the testis. Majority of the studies indicates testicular toxicity is a result of compromised Sertoli cell function since metabolic and morphological changes are observed immediately after treatment (Creasy *et al.*, 1983, 1987; Foster *et al.*, 1980; Gray and Gangolli, 1986; Heindel and Chapin, 1989; Heindel and Powell, 1992). Because hypothalamic GnRH content and concentrations of testosterone and T_3 were not affected following DBP treatment, we assume DBP-induced changes in rabbit testis occurred without altering the hormones necessary to drive reproductive or metabolic processes, supporting the hypothesis of a direct effect on Sertoli or germ cells.

We observed atypical germ cells in post-pubertal rabbits exposed to DBP for 12 wk. Spermatocytic seminoma is a form of testicular cancer seen in elderly men, which develops from altered B spermatogonia, not from CIS (Dekker *et al.*, 1992). CIS is thought to arise from transformation of gonocytes or pre-spermatogonia, which are not expected to be present in post-pubertal animals. It is possible that these alterations to B spermatogonia may result from exposure to xenobiotics in adult life. Based on our observation of atypical germ cells in the post-pubertal testis following administration of DBP for 12 wk, we hypothesize that exposure to xenobiotics during adult life induces changes to germ cells that may manifest into spermatocytic seminoma later in life. This possibility needs to be pursued in detail with a larger number of animals and exposure to

different xenobiotics. Collectively, the similarities and differences between adverse effects observed in rodents compared to rabbits may be a result of the 5-6-fold lower dose administered to rabbits, lack of critical evaluation of the rodent testis following acute exposure, and advantages of the rabbit over the rodent model in longitudinal evaluation of seminal parameters.

Irrespective of the window of exposure, DBP treatment lowered the number of normal sperm in the ejaculates. Majority of the abnormal sperm, regardless of window of exposure, included acrosomal and nuclear defects. These defects were observed in the testis indicating that these malformations originated during spermiogenesis and not epididymal transport. Furthermore, the fact that these defects continued to occur 13 and 25 wk after the cessation of treatment (viz., gestational and adolescent exposure), indicated that these effects are lasting. Similar observations were made in studies testing exposure in drinking water to common chemical contaminants (Veeramachaneni *et al.*, 2001a) or disinfection by-products (Veeramachaneni *et al.*, 2000b).

The observation that gestational, adolescent, and post-pubertal exposure to DBP decreased the percentage of normal sperm in the ejaculate, increased the DGEL, and that exposure to DBP during gestation and adolescence decreased the sperm concentration and number of sperm in the ejaculate by 23 and 20% should not be overlooked. Human males have low reproductive efficiency, only ejaculating twice the number of sperm where fertility might be expected to decline (Amann, 1982). Therefore, the human testis would be more vulnerable to toxicological insult, assuming similar responses occur.

Extrapolating these data from rabbits to humans, it is likely that exposure to high levels of DBP may contribute to the decline in human reproduction. Several

epidemiological studies have attempted to correlate exposure to phthalate containing materials with decline in semen quality and increased risk of testicular cancer (Murature *et al.*, 1987; Hardell *et al.*, 1997; Hansen, 1999). A weak negative correlation between sperm density and phthalate concentration was found in a random population of college students (Murature *et al.*, 1997), while a 7-fold increase in risk for developing testicular cancer was determined in workers occupationally exposed to phthalate-containing materials (Hardell *et al.*, 1997). However another study using a larger population found no increased risk for testicular cancer in these workers (Hansen, 1999). Based on these observations it is likely that declines in male reproductive health are associated with exposure to ubiquitous chemicals such as phthalates, pesticides and estrogenic chemical pollutants.

Collectively, these observations support the hypothesis that males exposed to xenobiotics during late gestation or adolescence are more sensitive to toxicological insults to the reproductive system than post-pubertal animals. Furthermore, the alterations in reproductive development induced by late gestational or adolescent exposure remain undetected until after sexual maturity (Veeramachaneni *et al.*, 2001a). Without the rabbit model, many of these subtle changes viz., atypical germ cells and morphologically defective sperm would have gone unnoticed.

CHAPTER 5

CONCLUSIONS AND INFERENCES

This is the first report that DBP causes deleterious effects on development and male reproduction in frogs and rabbits. It also gives insight into the role of an ubiquitous pollutant, such as DBP, potentially contributing to the worldwide decline in amphibian populations and increased incidence of malformations, and the decline in human male reproductive health. Comparing the results obtained from these studies using two different animal models also gives insight into the mechanism of phthalate-mediated toxicity.

Thyroid hormone-mediated effects occur through binding of thyroid hormone to their receptors (TR), which are members of the proto-oncogene *c-erbA*-related supergene family of ligand activated transcription factors that includes retinoic acid receptor (RAR), 9-cis retinoic acid receptor (RXR), vitamin D_3 receptor (VDR), and peroxisome proliferator-activated receptor (PPAR). Upon binding to the receptor, the thyroid hormone-TR complex forms a heterodimer with RXR and activates transcription of their target genes by interacting with thyroid response elements. Although liganded TR monomers and homodimers interact with thyroid response elements, it is the liganded TR-RXR complex that is physiologically active (reviewed by Shi *et al.*, 1996; Tata, 1998). Because DBP delayed thyroid-dependent metamorphosis in frogs and increased concentration of T_3 in developing rabbits (indicating unavailability of TR) we propose DBP may alter thyroid homeostasis by acting at the level of the receptor.

Further support for this hypothesis comes from numerous studies characterizing the effects of phthalates in rodents. Phthalates are known to cause peroxisome proliferation by binding PPARα (Hasmall *et al.*, 1999) leading to stimulation of mitosis, suppression of apoptiosis and eventually hepatocarcinogenesis in rodents (reviewed by Roberts *et al.*, 1997). Similar to TR, the physiologically active form of liganded PPAR is a heterodimer with RXR. In vitro, PPAR-RXR heterodimers down-regulate the transcriptional activity of TR by competing for RXR (Juge-Aubry *et al.*, 1995). Therefore, phthalates may indirectly disrupt thyroid-dependent processes by binding preferentially with PPAR, which consumes most of the available RXR.

Inhibiting thyroid hormone action by itself also could result in testicular toxicity. Thyroid hormones are vital for the proliferation and maturation of prepubertal Sertoli cells and perturbations lead to altered development (Ulisse *et al.*, 1994; Arambepola *et al.*, 1998b). Inhibiting thyroid hormone-dependent processes in 21-day-old rats led to a reduction in the weights of testis, epididymis, and prostate (Marty *et al.*, 2001). Thyroid hormones have also been identified as a major regulator of MIS production in rat Sertoli cells (Arambepola *et al.*, 1998a). Acute exposure to thyroid hormones stimulates steroid biosynthesis, StAR expression, and increased LH receptor gene expression and ligand binding in vitro (Manna *et al.*, 2001a; 2001b). Furthermore, in frogs, thyroid hormones set the stage for androgen competency in the larynx (Cohen and Kelley, 1996; Robertson and Kelley, 1996).

By limiting available RXR, other RXR-dependent processes will be altered. The observation that retinoic acid-induced nuclear localization of RARα with concomitant

decreases in the retinoic acid-induced transcriptional activity of RARα (Vo *et al.*, 2001) supports this mechanism, since physiologically active RAR forms heterodimers with RXR (reviewed by Dufour and Kim, 1999).

Peroxisome proliferator-mediated effects may also contribute to phthalate-mediated toxicity. Peroxisome proliferators are hypolipidemic agents that inhibit 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase (reviewed by Johnson *et al.*, 2001) therefore the decreases in testosterone synthesis may also be a result of decreased cholesterol available for steroid biosynthesis. Furthermore, peroxisome proliferators inhibit Leydig cell steroidogenesis *in vivo* (Bookstaff *et al.*, 1990) and *in vitro* (Boujrad *et al.*, 2000). However PPAR independent pathways in phthalate-induced reproductive toxicity may exist, since PPARα-null (-/-) mice have testicular lesions when fed a diet containing 1200mg DEHP/kg (Ward *et al*, 1998). Collectively, disrupting thyroid-, retinoic acid-, and peroxisome proliferator-dependent pathways may constitute a complex biochemical mechanism of phthalate-mediated toxicity that accounts for some of the reproductive effects of DBP.

The utility of the frog and rabbit models for developmental and reproductive toxicology is apparent in the number of studies using these animals (Bantle *et al.*, 1994a; 1994b; 1995; 1996; 1999a, Dawson *et al.*, 1989, Fort *et al.*, 1998; 2000b; Hayes *et al.*, 2002; Ohtani et al, 2000; Palmer *et al.*, 2000; Veeramachaneni *et al.* 1994; 1999a; 1999b; 2000a; 2000b; 2001b; 2001a, 2001b). In addition, usefulness of these amphibian and non-rodent models is evident in their comparability and complementarity. Future studies should take advantage of these animal models.

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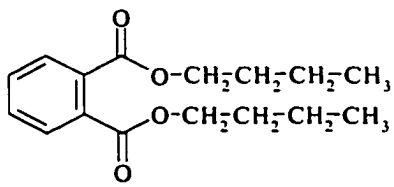
APPENDIX

APPENDIX A

CHEMICAL AND PHYSICAL PROPERTIES OF DIBUTYL PHTHALATE

Property	Value
Chemical	
Name	Dibutyl Phthalate (DBP)
CAS #	84-74-2
Synonyms	1,2-Benzenedicarboxylic Acid Dibutyl Ester; o-Benzenedicarboxylic Acid; Dibutyl Ester Benzene-o-Dicarboxylic Acid Di-n-Butyl Ester; Butylphthalate; Dibutyl 1,2-Benzene dicarboxylate; Di-*n*-butyl phthalate, Dibutyl-o-Phthalate, N-Butylphthalate; Phthalic acid dibutyl ester
Trade Names	Caswell NO. 292; Celluflex DBP; Elaol; Ergoplast FDB; Genoplast B; Hexaplast M/B; Palatinol C; Polycizer DBP; PX104; Satflex DBP; RC Plasticizer DBP; Uniflex DBP
Formula	$C_{16}H_{22}O_4$
Structure	
Density (g/ml)	1.047
Molecular Weight	278.35
Melting Point	-35°C
Boiling Point	340°C
Solubility	
Water (mg/L)	4.9
Organic Solvents	acetone, alchohol, ether, benzene
Log octanol water coefficeint	4.63

Adapted from ASTDR, 1990; Ellington and Floyd, 1996; IRIS, 2001; WHO, 1990

APPENDIX B

FETAX SOLUTION[a]

Solute	g/10 L	
	1X	60X
Sodium chloride	6.25	375.00
Potassium chloride	0.30	18.00
Calcium chloride	0.15	9.00
Calcium sulfate	0.60	36.00
Magnesium sulfate	0.70	42.00
Sodium bicarbonate	0.96	115.20[b]

[a] According to ASTM, 1991

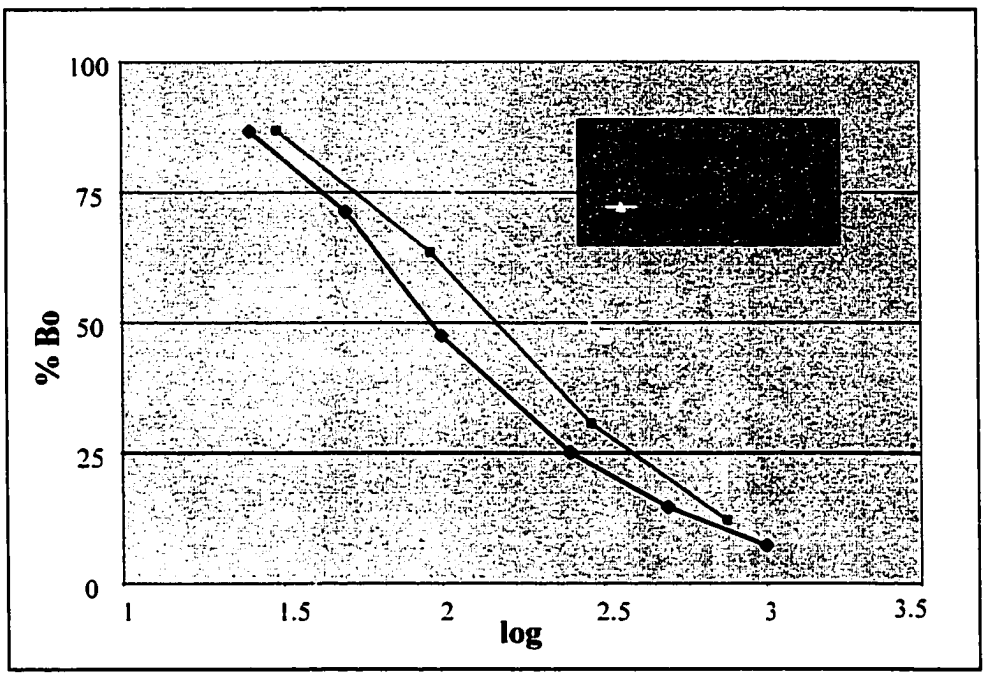
[b] A separate 120X Sodium Bicarbonate solution was made to avoid calcium bicarbonate formation

Solutions were allowed to stir a minimum of 6 hr prior to use

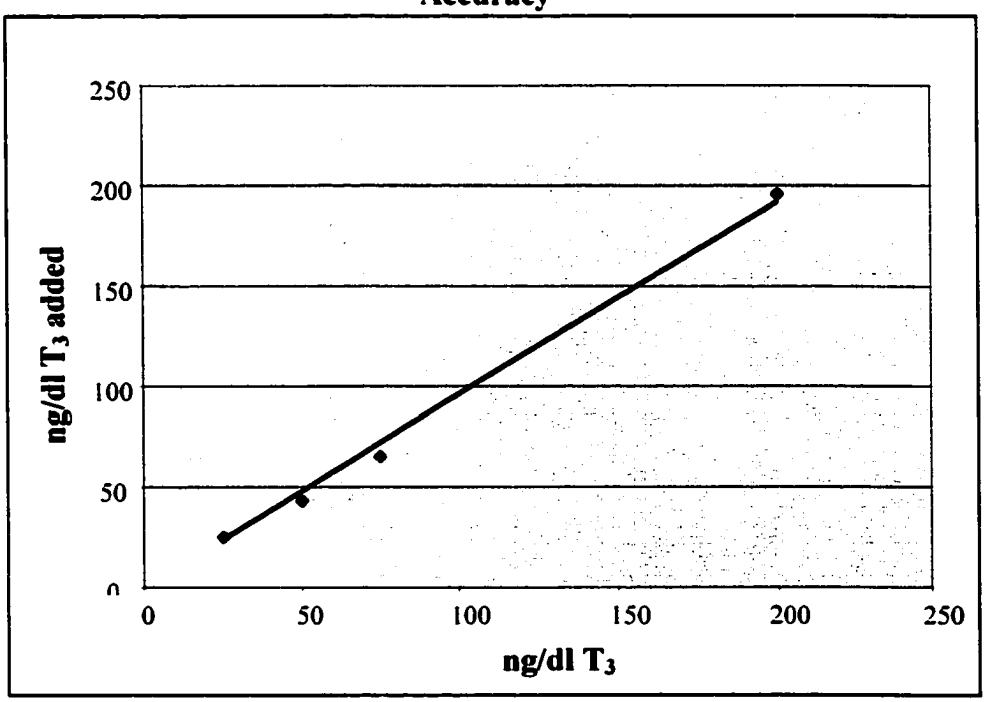
APPENDIX C

SPECIFITY AND ACCURACY OF A RADIOIMMUNOASSAY FOR TRIIODOTHYRONINE IN WHOLE BODY HOMOGENATES OF *XENOPUS LAEVIS*

Specificity



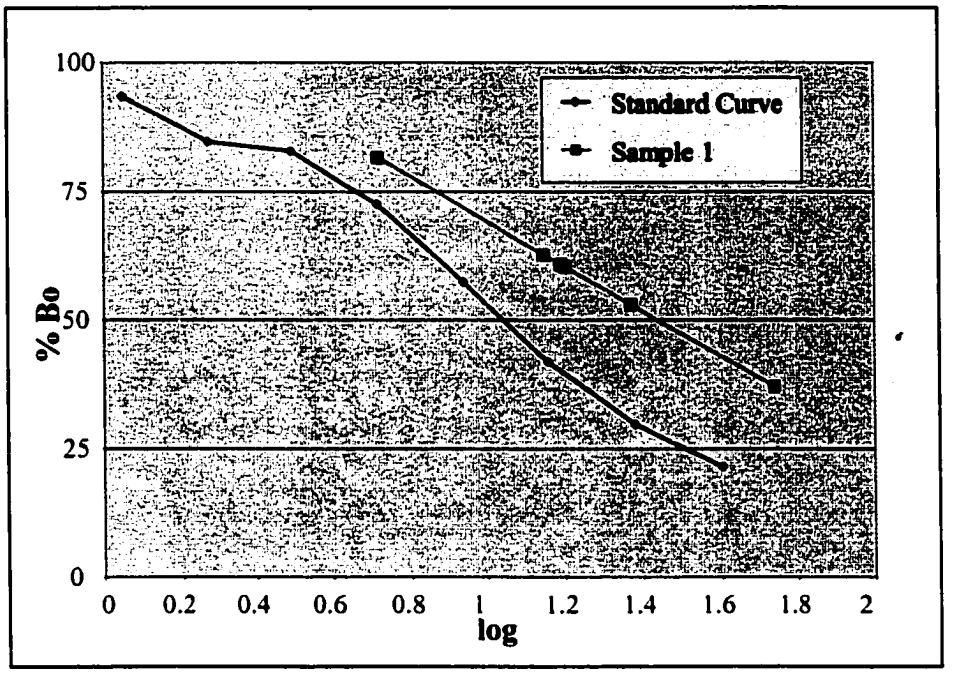
Accuracy



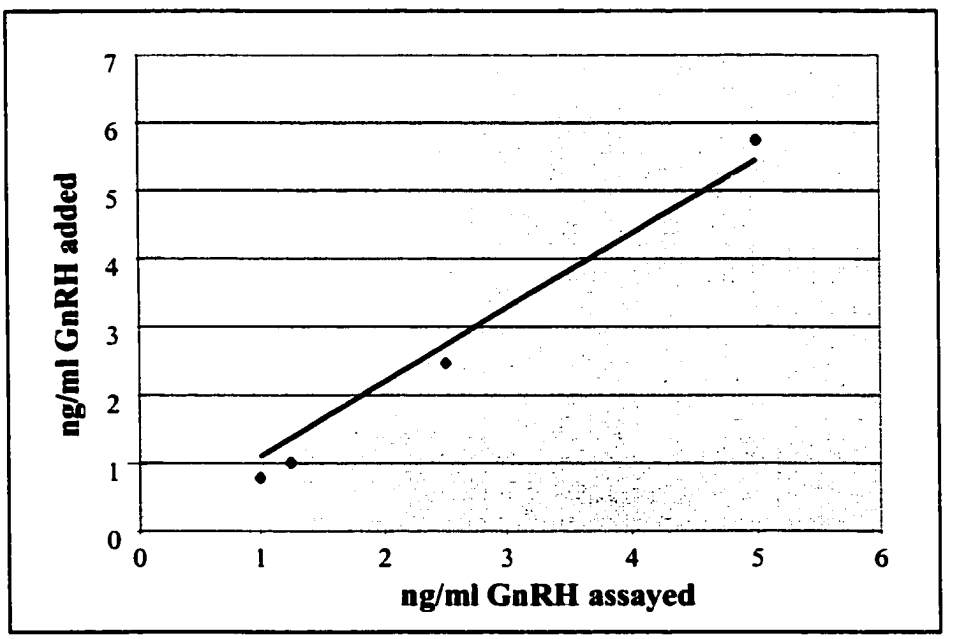
APPENDIX D

SPECIFITY AND ACCURACY OF A RADIOIMMUNOASSAY FOR HYPOTHALAMIC GONADOTROPIN-RELEASING HORMONE IN *XENOPUS LAEVIS*

Specificity



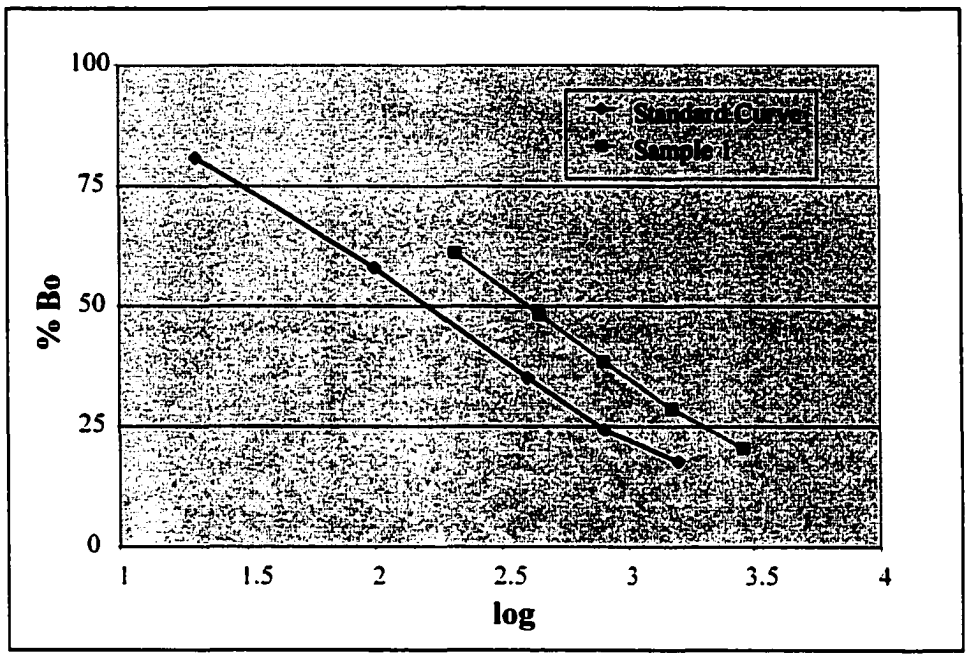
Accuracy



APPENDIX E

SPECIFITY AND ACCURACY OF A RADIOIMMUNOASSAY FOR TESTOSTERONE IN *XENOPUS LAEVIS* SERUM

Specificity



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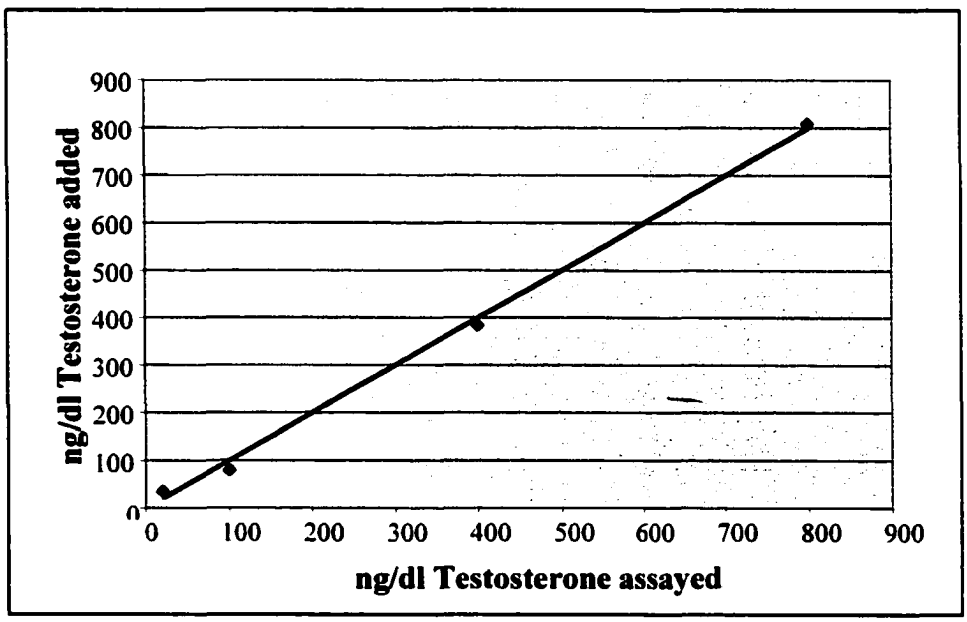
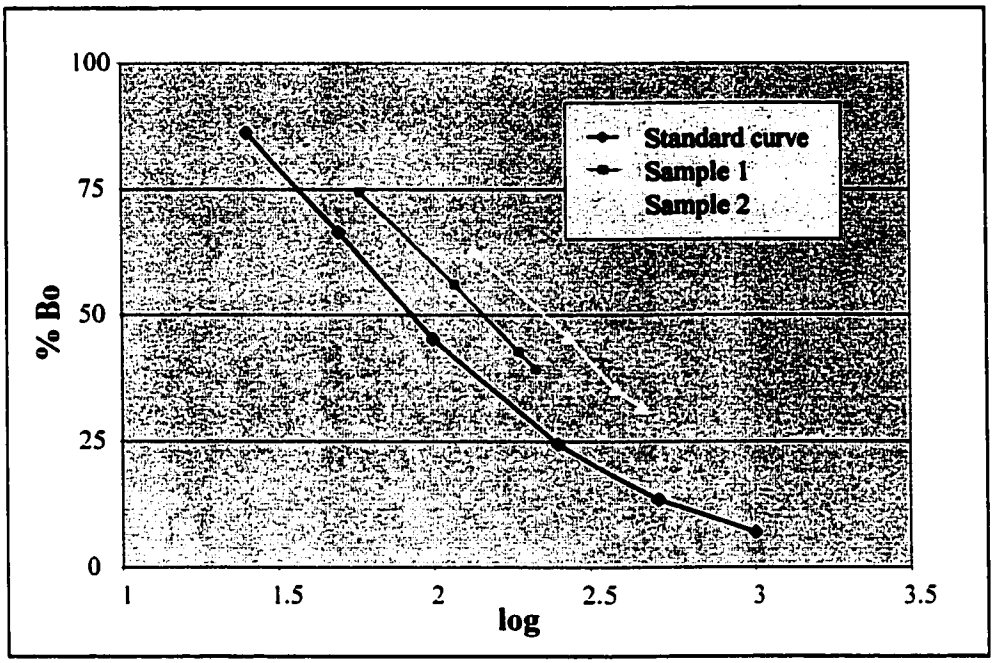


APPENDIX F

SPECIFITY AND ACCURACY OF A RADIOIMMUNOASSAY FOR TRIIODOTHYRONINE IN RABBIT SERUM

Specificity



Accuracy