A Critical Review: 2,3,7,8 –Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) Effects on Gonad Development in Bivalve Mollusks

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A Critical Review: 2,3,7,8 –Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) Effects on Gonad Development in Bivalve Mollusks

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Bivalve mollusks are equally sensitive to 2,3,7,8-tetrachlorodibenzo-p-dioxin's (2,3,7,8-TCDD) effect on gonad development, embryonic development, and epithelial lesion occurrence as higher vertebrates. 2,3,7,8-TCDD alters normal development of reproductive organs and early development in bivalve mollusks at 2 to 20 pg/g wet weight. In both *Crassostria virginica* and *Mya arenaria*, 2,3,7,8-TCDD preferentially accumulates into the gonads. The sensitivity of gonad maturation is likely due to disruption of cross-talk between highly conserved steroid, insulin, and metabolic pathways involved in gonad differentiation. The altered gonad development and decreased veliger larval survival can partially explain the lack of self-sustaining bivalve populations in 2,3,7,8-TCDD contaminated estuaries.

Key Words: 2,3,7,8-TCDD; bivalve; mollusks; reproductive toxicity

INTRODUCTION

In any ecological epidemiological study the ability to demonstrate a direct link between a contaminant and a specific disease state is complicated by exposure to multiple chemicals and other confounding factors (1). When chemicals
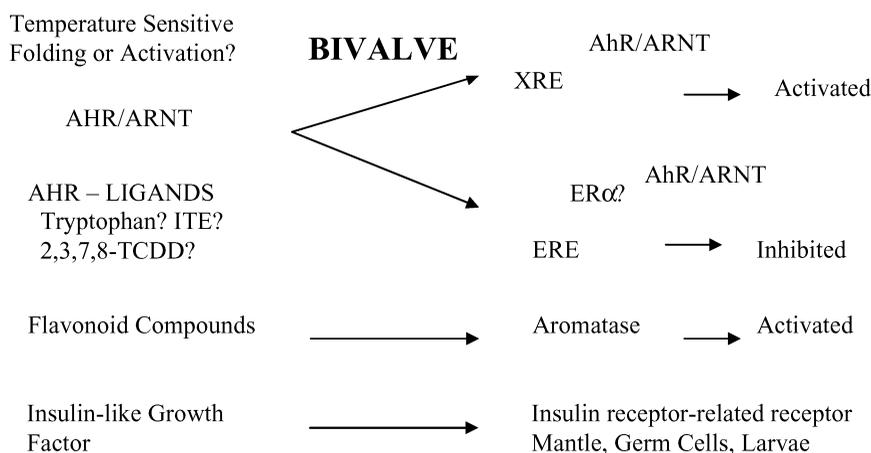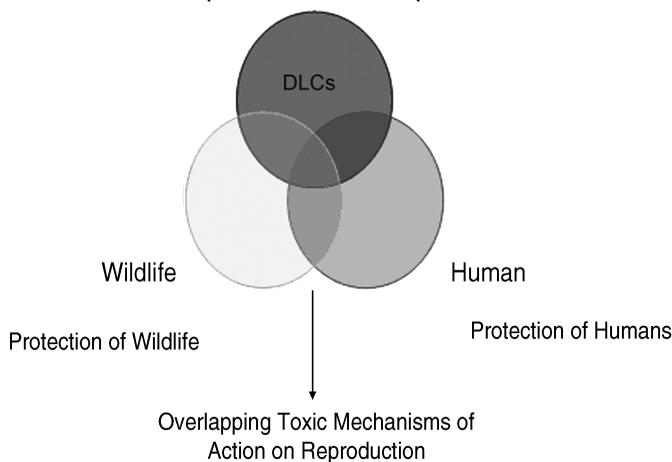
Figure 1: Conserved mechanisms of action on reproduction and early development between humans and wildlife and potential cross-talk. DLCs = dioxin like compounds, AhR= aryl hydrocarbon hydroxylase, ARNT = aryl hydrocarbon receptor translocator, XRE = xenobiotic response element, ERα = estrogen receptor alpha, ERE = estrogen receptor. ITE = 2-(1'H-indole-3'-carbonyl)-tiazole-4-carboxylic acid methyl ester. Modified from several sources (16, 20, 24, 26–30, 40–42, 44)

affect highly conserved pathways in biological systems, similar effects are often manifested in both wildlife and humans (Figure 1). Gonad development for male and females are highly conserved across eukaryotic species. Reproduction is a target for a number of chemicals because of the synchronization of neuronal and hormonal cues (2). In ecological epidemiologic studies it is
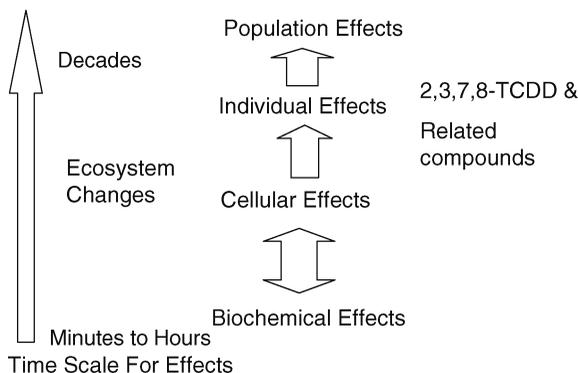
Figure 2: Time scale and effects in organisms within an ecosystem exposed to 2,3,7,8-TCDD.

generally easier to relate an effect in the field when single chemical discharges are involved compared with complex harbor estuarine systems (3). Within Figure 2 is shown the relationship between biochemical effects which may or may not be manifested at the higher levels of organization (cellular, individual, and population) within an ecosystem, along with the time scale at which these events may occur. The effects that are observed at the population level can take decades to be manifested due to a slow decline of viable or behaviorally impaired offspring (4). Any attempt to relate a cause and effect relationship between a specific lesion and a chemical requires that both laboratory controlled studies and field studies result in similar effects at comparable tissue doses.

Bivalve mollusks have served as indicator species for monitoring chemical contamination and chemical effects in coastal environments for many years (5, 6). Because of their sessile life stage and ability to filter large volumes of water, adult bivalve mollusks have the ability to accumulate a wide variety of chemicals through both particle associated chemicals and direct absorption across epithelial surfaces (7). Bivalve mollusks can accumulate metals and organic contaminants through both active and passive uptake mechanisms. In comparison to lower vertebrates and higher vertebrates the phase I metabolic enzymes are between 100- to1,000-fold lower in activity (8), while some of the phase II metabolic enzyme activities for organic compounds are comparable (9). Since bivalves are poikilotherms, their enzymatic and metabolic rates vary dramatically throughout the year. Bivalves do go through seasonal changes that can influence the chemical body burden. For example, during certain periods of the year a bivalve will accumulate lipids, glycogen, and proteins prior to gonad development and at spawning time will lose up to 30% to 40% of its body mass (7).

Dioxin-like compounds (DLC) have been shown to interfere with a number of conserved biochemical pathways, which occur in both wildlife species as well

as in humans (10–12). The toxic effects that are manifested following exposure to DLCs are a result of altering these normal biochemical pathways. DLCs have been grouped together because of their affinity for binding the Aryl hydrocarbon receptor (AhR) and the down stream events that occur (13–16). AhR is a member of the helix-loop-helix/PER-ARNT-SIM (bHLH-PAS) family of transcriptional regulatory proteins and are highly conserved between phyla (15, 16). 2,3,7,8-TCDD has been reported to result in toxic responses in organisms and tissues that do not express or contain a functioning AhR (17–20). Butler et al. (20) described an AhR homologue in *M. arenaria*, which did not bind the 2,3,7,8-TCDD or *β*-naphthoflavone and yet the activated AhR/ARNT dimmer could bind xenobiotic response elements on DNA. Butler et al. (21) described the effect of 2,3,7,8-TCDD as resulting in undifferentiated reproductive tissue following bath exposure at 10 ng/L. These studies and those in mammalian systems attempting to identify the endogenous function and ligand(s) for AhR lend growing support for the hypothesis that 2,3,7,8-TCDD can modulate cell growth and differentiation without direct binding to AhR.

The developing organism, whether it is a lower vertebrate (teleost) or higher vertebrate (avian, mammals), is more sensitive than the adult with respect to DLC exposure (11, 12). Bivalve larval stages have been known for a long time to be more sensitive to chemical exposure than the adult organism both for metals and organic compounds (12, 22). The inappropriate expression (up or down) of genes, proteins, or other factors during embryonic growth and tissue remodeling can result in abnormal development. The abnormal development may be manifested in both physical abnormalities during development (edema, pericardial damage, hemorrhaging, etc.) and altered "biochemical imprinting" that may not be manifested until much later in life (11). There have been a number of recent studies examining the physiological role of AhR in the formation of primordial follicles and maturation in AhR-null mice exposed to 2,3,7,8-TCDD (23). Developing organisms and reproductive organs, whether it is an invertebrate or mammal, are sensitive to chemicals that disrupt normal temporal or spatial expression of developmental cues and cell cycling (24).

In bivalve mollusks, neurohormonal release by ganglia and endogenous steroids produced at the gonad serve as modulators for initiating and sustaining gonad development (25, 26). A number of studies over the past ten years have begun to characterize the role of steroidal hormones in both male and female reproductive cycles (27–30). Figure 3 summarizes information obtained from literature from different bivalve species in order to understand normal cues for female gonad development. Specific cells lining the follicle have been identified that have the ability to synthesize and release reproductive steroids (37–30). In the case of the female, these cells are on the exterior of the follicle, while in the male the cells are on the inside of the follicle (29, 30). The developing gonad in *C. virginica* and other bivalves can be
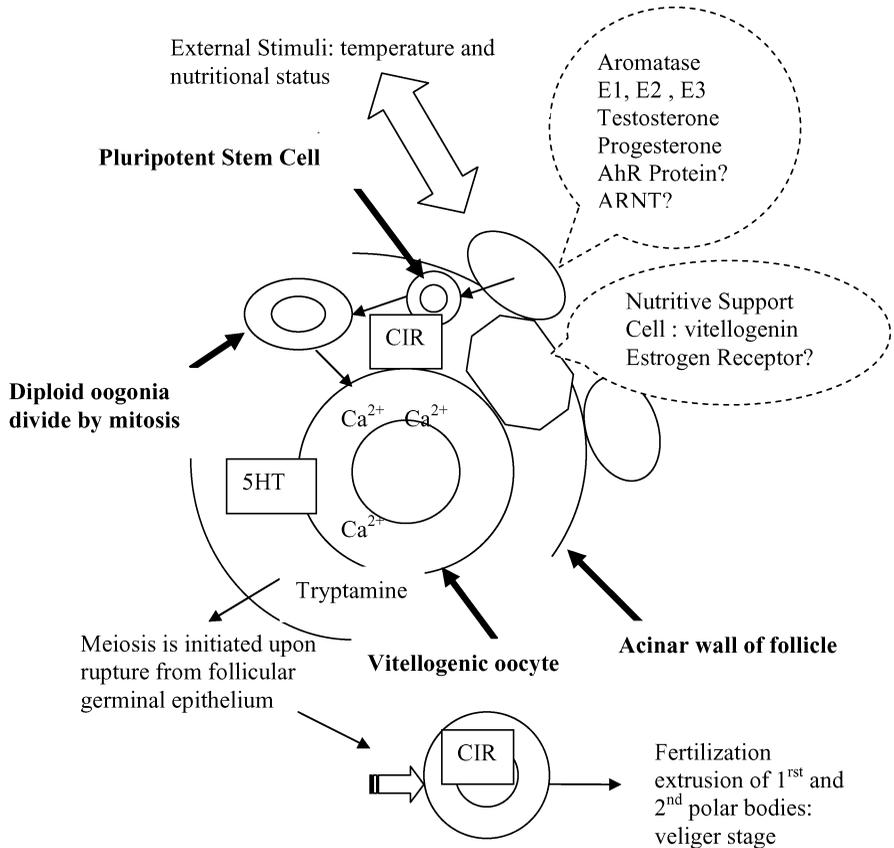
Figure 3: Current knowledge of female gonad development bivalve mollusks and associated paraquine / autocrine signaling factors, neurohormones, and steroid hormones. Components with question marks have not been specifically associated with a cell type. Abbreviations: E1 = estrone, E2 = 17β estradiole, E3 = estriole, AhR = aryl hydrocarbon receptor, 5HT = 5 hydroxytryptamine (serotonin), Ca^2 = calcium CIR= Crassostrea insulin receptor.

divided into various morphological stages, which can be used to assess a chemical's effect on normal gonad development (7). During the spawning season, the gonad is the largest organ ($\sim$30%–40%) in the eastern oyster and other bivalves in terms of mass and surface area. The initiation of gametogenesis in bivalve mollusks depends on several environmental cues both endogenous (adequate glycogen/protein stores/flavonoids) and exogenous (temperature). The resting phase of gametogenesis precedes the spawning season and the gonad can not be distinguished grossly from the surrounding vesicular connective tissue. In bivalves there is no distinct male and female reproductive organ and the germinal stem cells are in direct contact with the surrounding connective tissue. The developmental stages of the sperm follow a similar

morphological pattern as seen in lower and higher vertebrates: spermatogonium, spermatocyte, spermatid, and spermatozoa. Steroid production is believed to be maintained at the follicular level by Leydig-like and Sertoli-like cells (27). The developmental stages in egg development are similar to other oviparous organisms. Egg development and maturation is maintained by varying levels of 17β estradiol, testosterone, and progesterone (25–29). Aromatase (CYP19) has been identified within cells of the female gonad (30). Egg maturation is associated with 5HT membrane receptors associated with calcium uptake. Information on the specific cells involved in production and release of hormones as well as other factors involved in gonad development in bivalves is an area of increasing interest. Figure 3 represents a generalized female gonad constructed from the above studies in several different bivalve mollusks as it relates to location of different cell types and biochemical markers associated with individual cells. Individual male and female oysters synchronize sperm and egg release into the water column allowing for external fertilization (7). Following external fertilization, the embryo rapidly develops into the veliger larval stage and undergoes further differentiation (straight hinge veliger, late veliger, and eyed stage) until settling out onto the appropriate substrate.

The summarized results presented in this paper were taken from research carried out in my laboratory from 1986 until present on the effects of 2,3,7,8-TCDD on soft-shell clams (31), eastern oyster (32–34) and a literature review. This review discusses the current state of our knowledge concerning the effects of 2,3,7,8-TCDD on bivalve mollusk gonad development and survival of veliger offspring.

MATERIALS AND METHODS

For greater detail on the methods used in studies conducted in both *M. arenaria* and *C. virginica* the reader should consult references (31–34).

Chemicals

2,3,7,8-TCDD (98% pure) in n-nonane or toluene was purchased from Cambridge isotope diagnostics (Woburn, MA). Toluene (>99% pure) and all histological reagents were purchased from Fisher Scientific (Fair Lawn, NJ). (^{3}H) 2,3,7,8-TCDD (20.5 Ci/mM or 34.7 Ci/mM, purity 98%) was purchased from Chemsyn Science Laboratories (Lenexa, KA): Calcium-45 (CaCl$_2$ 5 mCi) was purchased from New England Nuclear (Boston, Mass).

Animals

Eastern oysters (*Crassostrea virginica*) were purchased from Blue Mussel Company, Prince Edward Sound, Canada. The average total weight was

$45 + 2.5$ g and the average wet weight was $5.0 + 0.60$ g. All laboratory reared bivalves were maintained under optimum conditions. Soft shell clams (*M. arenaria*) were purchased from Warren Denton Seafood (Prince Frederick, MD). The soft-shell clams were maintained in a recirculation seawater system maintained at $12°C$. the clams were fed daily with ground Tetramin[R]. Field populations of soft-shell clams were collected from Tuckerton, NJ and Elizabeth, NJ.

Crassostrea virginica Studies

Oysters were maintained in a holding phase at $15°C$ in a recirculation seawater system with no supplemental food. Gametogenesis was initiated following treatment by transferring the oysters to a $20°C$ recirculating system and supplementing existing food with *C. isochrisis*: 15 million cells/ml) per day (34). The studies were conducted over 28 days, which allowed for full maturation of the gonads.

The oysters were injected (0.1 ml) into the adductor muscle sinus through a notch in the left side of the shell. All oysters were placed on absorbent paper for 1 hour to allow for uptake of the compound. The oysters were then placed in a 76 liter aquarium for 24 hours, prior to being placed in separate temperature controlled tanks. In order to maintain the desired tissue concentration, the oysters were re-injected at 14 days. This was based on the calculated half-life in the oyster (32). The nominal concentrations were 2.0 and 20 pg/g, and the determined tritium concentrations $(X + SD)$ were $0.996 + 0.054$ and $27.7 + 25.4$ pg/g based on a 50 g oyster.

A strip spawning technique was used to obtain eggs and sperm for fertilization studies. At 28 days, the oysters were sexed by removing a small sample of gonad. The stripped eggs were sieved through 53 and 25 micrometer mesh, counted and then fertilized. Fertilization was allowed to take place for 2 hours prior to being transferred to rearing chambers.

Protein levels were determined from hemolymph samples ($N = 8$ per treatment) collected from the adductor sinus. Phosphate levels or vitellogenic equivalents were determined from hemolymph using a colorimetric determination of organic phosphate.

M. arenaria Studies

The Chesapeake clams were dosed in 10-gallon glass aquaria using a static nonrenewal pulse dosing for 24 hours protocol with (H^3) 2,3,7,8-TCDD (31). Tissue doses were determined by scintillation counting and converted to DPM/g wet weight. The study was repeated using 200 and 240 clams. There was a no treatment control, a solvent control and in experiment 1 (2, 20, and 200 ng/L) and experiment 2 (2.5, 25, 250, and 2,500 ng/L) treatment groups.

Ten clams from each group were removed at 7, 14, 21, and 28 days to evaluate for histological lesions.

Effects of 2,3,7,8-TCDD on weight loss were evaluated by exposing soft shell clams to 200 pg/g TCDD and examination of wet weight versus shell weight of individual clams. Three routes of exposure were examined in this study. Animals were either gavaged down the excurrent siphon of the clam, injected into the cardiac region, or exposed for 24 hrs in a static water-borne exposure scenario. Animals (n = 5) were collected at days 1, 3, 7, 14, 21, and 28 days post exposure.

2,3,7,8-TCDD's effect on calcium incorporation into the shell was done using calcium 45 incorporation. Twenty-five clams from the Chesapeake Bay and the Arthur Kill were exposed in a 20 L aquarium with 0.01 μCi Calcium 45 for 7 days. All the clams were shucked and rinsed in cold running water. Shells were collected and scrubbed and then digested in 10 ml of 15 N nitric acid. A 0.5 ml aliquot was neutralized and prepared for scintillation counting. The total amount of radioactivity in the whole shell was calculated.

Histological

Animals prepared for light microscopy were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections (6 μM) were stained with hematoxylin and eosin. Samples prepared for electron microscopy were fixed in 2% gluteraldehyde in phosphate buffer and post fixed with 1% osmium tetroxide. The tissue was dehydrated in ethanol and embedded in Epon/Araldite.the ultra-thin sections were contrasted with uranyl acetate and lead citrate.

Analytical

Tritium labeled samples were counted on a Tracor Mark III liquid scintillation counter (Tracor Analytic, Elk Grove village, IL). All disintegrations per minute were converted to DPM/gram wet weight of tissue following background subtraction. DPMs were converted to pg TCDD equivalents based on the specific activity. Tissue samples from field samples were analyzed for specific congeners using a high resolution gas chromatograph (HP-5890 provided with a 6 m SP2330 column) and a high resolution mass spectrometer (VG 70-2505 at a resolution of 5,000) (31).
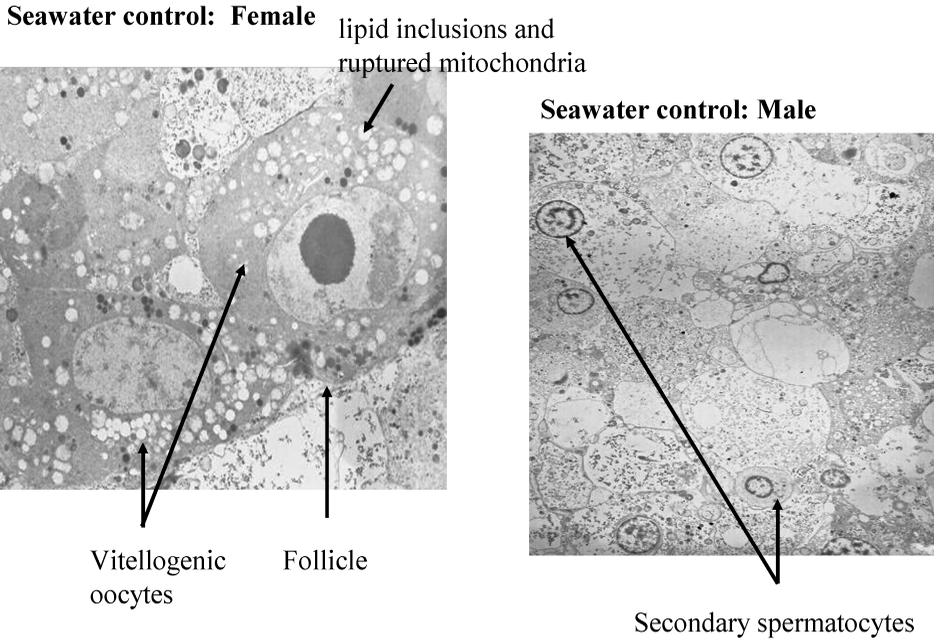
Statistics

All categorical data was examined using a Chi-square followed by a Yates correction for continuity. A one-way analysis of variance (ANOVA) followed by a post hoc test were carried out with a significance level of 0.05.
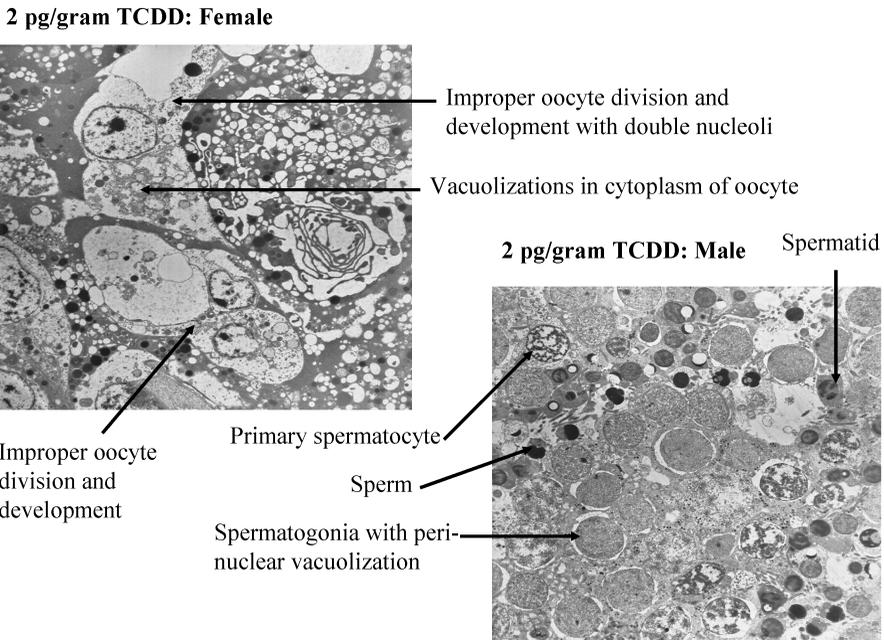
RESULTS

Eastern Oyster: *Crassostrea virginica* Studies

Representative electron micrographs of oysters exposed to seawater, 2.0 pg/g 2,3,7,8-TCDD, and 10 pg/g 2,3,7,8-TCDD are shown in Figure 4. The lesions observed for each group are discussed below. The seawater control injected oysters and the solvent control oysters showed normal male and female gonad development by day 28 (Figure 4a). This included the formation of the follicles and the support cells (Leydig-like cells and Sertoli-like cells). The solvent control oysters did show an initial delay at day 1, but by day 28 the oysters were comparable to the seawater controls. There was a dose-dependent effect on the ovary development with development being halted by day 14 of development with little change by day 28 at the 10 pg/g level (Figure 4c). The eggs in the highest dose appeared to begin development and then halt prior to the vittelogic stage. The 2.0 pg/g group also showed multinucleated oocytes, with vacuolation of the cytoplasm, and abnormal follicular structure (Figure 4b). Examination of alkali-labile phosphate (ALP) in hemolymph from day 30 post-exposure oysters exposed to 10 pg/g 2,3,7,8-TCDD (0.010 + 0.007 mg/ml) resulted in a significant decrease in circulating levels compared with seawater (0.112 + 0.042 mg/ml) and toluene treated (0.082 + 0.032 mg/ml) oysters. Circulating hemolymph protein levels (range 0.652–0.758 mg/ml) were not significantly different between groups. The 2.0 pg/g treated male oysters showed only minor effects, while at 10 pg/g there was altered tubular formation and disrupted primary spermatid structure (Figure 4b,c). The Leydig-like cell population was decreased in both 2,3,7,8-TCDD groups when compared with the control oysters.

Studies carried out both in the field transplanted oysters and laboratory injected oysters demonstrated that 2,3,7,8-TCDD affected veliger larval survival in the 2.0–10 pg/g range. Oyster veliger larvae survival from Newark Bay (3.2 pg/g 2,3,7,8-TCDD/2.1 pg/g 2,3,7,8-tetrachlorodibenzofuran, 2,3,7,8-TCDF), Arthur Kill (1.3 pg/g 2,3,7,8-TCDD/1.7 pg/g 2,3,7,8-TCDF), and Sandy Hook (0.15 pg/g 2,3,7,8-TCDD/2.3 pg/g 2,3,7,8-TCDF) oysters that were transplanted from September to June were 3.9%, 7.5%, and 73%, respectively (33). In laboratory studies conducted over the 28-day gametogenesis cycle resulted in control oysters having 80.3% survival (D-stage), while the 2 and 20 pg/g 2,3,7,8-TCDD oyster had 0% survival to the straight hinge stage. For oyster larvae spawned from non-treated oyster (ex vivo) and exposed to rearing solutions dosed with no 2,3,7,8-TCDD, 2 and 10 pg/ml resulted in 76%, 2.3%, and 1.1% survival to the trochophore and D-stage. 2,3,7,8-TCDD has deleterious effects on the developing larvae both from field exposed and laboratory exposed oyster, as well as post spawned veliger larvae.

Seawater control: Female

lipid inclusions and
ruptured mitochondria

Seawater control: Male

Vitellogenic
oocytes

Follicle

Secondary spermatocytes

A.

2 pg/gram TCDD: Female

Improper oocyte division and
development with double nucleoli

Vacuolizations in cytoplasm of oocyte

2 pg/gram TCDD: Male

Spermatid

Improper oocyte
division and
development

Primary spermatocyte
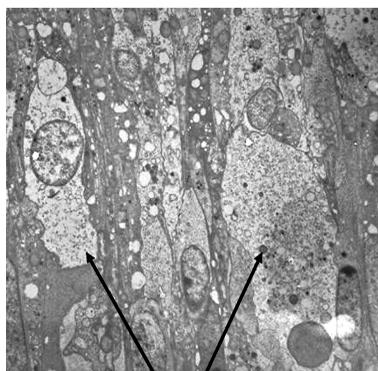
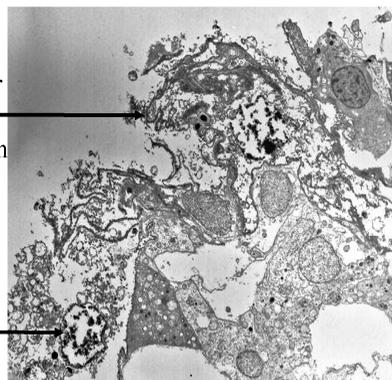Sperm

Spermatogonia with peri-
nuclear vacuolization

B.

Figure 4: Representative electron micrographs of both male and female gonad
development following 2,3,7,8-TCDD (B and C) compared to the seawater control (A.) These
figures were previously published in Wintermyer and Cooper (41). (*Continued*)

10 pg/gram TCDD: Female

10 pg/gram TCDD: Male

Improper tubular formation

Improper structure of primary spermatocyte

Improper pre-vitellogenic oocyte development

C.

Figure 4: *(Continued.)*

Soft Shell Clam: *Mya arenaria* Studies

The moderate to severe hyperplasia/metaplasia observed from field collected soft-shell clams are summarized in Table 1. The percent of lesions found in the Elizabeth, NJ population (Arthur Kill) were in the majority of cases

Table 1: Summary of moderate/severe histological lesions from soft shell clams collected from Elizabeth, NJ (contaminated harbor 4.8-20 pg/g 2,3,7,8-TCDD), Tuckerton, NJ (reference site 0.5-0.6 pg/g 2,3,7,8-TCDD) and Chesapeake Bay (0.1 pg/g 2,3,7,8-TCDD).[a]

Organ	Elizabeth, NJ	Tuckerton, NJ	Chesapeake Bay, MD
Siphon	48% (61/121)[b]	0% (0/27)	8% (7/92)
Kidney	42% (41/98)	20% (3/15)	18% (10/57)
Gill	23% (30/129)	10% (3/29)	23% (23/100)
Mantle	30% (30/99)	12% (3/26)	10% (7/72)
Heart	1% (1/73)	0% (0/15)	5% (2/39)
Intestine	22% (29/131)	3% (1/30)	17% (17/100)
Stomach	14% (18/125)	0% (0/29)	7% (7/98)
Digestive Gland	27% (22/82)	12% (3/25)	23% (17/73)
Gonad	12% (16/130)	3% (1/30)	9% (9/100)
Foot	41% (49/119)	24% (7/29)	23% (21/92)
Dermis	15% (17/117)	4% (1/27)	4% (4/96)

[a]Lesion prevalence taken from Brown (39).
[b]Values within the table are the percent lesion and the values in () are the number with lesions compared to number of animals with this organ.

higher then those observed from Tuckerton, NJ or the Chesapeake Bay clams. This is not surprising since the Elizabeth, NJ site is a highly industrialized area with a diverse list of chemicals being present (5). The lesions observed on the siphon showed ulcerations of the epithelium and dysplasia of the epidermal cells. Similar lesions were not seen in either of the reference groups. The kidney lesions either were kidney "lakes," nests of hyperplastic cells, or hyperplasia of the entire kidney. In both reference sites kidney "lakes" were present and no hyperplasia was present. The gill lesions in Elizabeth, NJ primarily were lamellar hyperplasia, however in some clams there was extensive gill fusion and several clams had 3, 4, and in one case 5 separate gill plates. The lesions in the reference sites were exclusively focal hyperplasia in association with parasites. The lesions observed in the mantle consisted of hyperplasia along the inner wall and metaplasia, hyperplasia, and vacuolation along the outer mantle wall. The mantle epithelium changed from an eosinophilic columnar epithelium into a basophilic cuboidal epithelium. The metaplasia along the outer epithelium of the mantle was not observed in either the Chesapeake Bay or Tuckerton clams. There were no major differences observed between the heart, intestine, stomach, digestive gland, foot, or dermis lesions between the sites. In the Elizabeth clams, there was evidence of multinucleated ova, and altered follicular structure similar to those observed in the oyster described above. Similar gonad lesions were not observed in any of the reference sites.

Field collected clams from the Arthur Kill at Elizabeth, NJ had thin shells compared with reference organisms (31). A study was carried out to determine if a major component of the shell matrix, calcium was affected in the clams from the Arthur Kill. Calcium 45 was used as a marker for determining calcium incorporation into the shell matrix of clams collected from Chesapeake Bay (reference) and the Arthur Kill (contaminated). There was a significant decrease in incorporation (mean and SD) into the shell matrix of Calcium 45 between the Chesapeake Bay (1384 + 444) and the Arthur Kill (814 + 234) clams.

As shown in Table 2, there was a significant decrease in the wet weight of the oyster when compared with the shell weight by day 14 post-excurrent injection. The shell weight would not change over this period and allowed for the normalization of the weight loss to a constant value. In both the muscle-injected animals and in the water-borne exposed animals there was a decrease in wet weight but it was not significant by day 21.

Within Table 3 is reported the distribution of (^{3}H) 2,3,7,8-TCDD following a bolus dose being injected into the excurrent siphon. The heart/kidney, gill and digestive gland were the highest tissues 24 hours after dosing. These same tissues on a per cent basis remained elevated throughout the 28-day study. By day 7 post-exposure, the gonad was the highest percent of the dose recovered compared to the other tissues. The gonad steadily increased over the 28-day period, reaching the highest by day 21 (35.8%). A similar accumulation into

Table 2: Comparison of body weight to shell weight ratio following a single bolous dose of 2,3,7,8-TCDD (200 pg/g) in soft shell clams, M. arenaria[a]

Exposure Route	Day 1	Day 2	Day 7	Day 14	Day 21	Day 28
Siphon Gavage[b]	1.330 (0.145)	1.326 (0.145)	1.168 (0.092)	1.023* (0.181)	1.017* (0.051)	0.898* (0.104)
Muscle Injection	1.209 (0.133)	1.201 (0.176)	1.205 (0.171)	1.092 (0.136)	1.081 (0.075)	
Water-borne Exposure (24hr)	1.268 (0.202)	1.257 (0.186)	1.153 (0.146)	1.144 (0.134)	1.222 (0.243)	1.021 (0.135)

[a]Values in the table mean and standard deviation, and * indicates significant difference from Day 1 value.
[b]Five animals were sampled at each time point, with the exceptions of Day 21 and 28 for gavage and day 21 for injection where 4 animals were sampled.

the gonad of 2,3,7,8-TCDD was reported by Wintermyer (32) in oysters and by Rhodes (35) in clams.

DISCUSSION

Invertebrates have been considered to be recalcitrant to 2,3,7,8-TCDD toxicity when compared with lower vertebrates or higher vertebrates. However, this misconception grew out of early studies that only examined the compounds effect on adult organisms (12). It is generally recognized that the developing organism and rapidly differentiating tissues respond to DLC exposure (11, 13, 14). Within Table 4 are summarized 2,3,7,8-TCDD's effects that have been reported in bivalve mollusks. Following exposure to 2,3,7,8-TCDD, there is a time-dependent loss in body mass in both oysters and soft-shell clams. Following direct injection of 2,3,7,8-TCDD hyperplasia was observed in the epithelial

Table 3: Distribution of (^{3}H) 2,3,7,8-TCDD in M. arenaria (N = 5) given as a bolus dose through the excurent siphon[a]

Tissue	Day 1 (19.9 pg)	Day 7 (26.4 pg)	Day 21 (23.6 pg)	Day 28 (32.5 pg)
Siphon	5.3%	6.7%	4.5%	5.5%
Heart/Kidney	13.5%	14.7%	14.4%	15.9%
Gill	33.1%	12.9%	9.5%	9.0%
Mantle	6.9%	8.2%	7.4%	10.6%
Dig. Gland	24.3%	20.2%	18.2%	19.3%
Muscle	3.5%	5.6%	4.7%	6.2%
Shell Gland	9.0%	7.8%	5.5%	6.9%
Gonad	3.5%	23.7%	35.8%	27.5%

[a]Values in the table are per cent of recovered 2,3,7,8-TCDD equivalents. The values in parenthesis are total recovered 2,3,7,8-TCDD equivalents.

Table 4: Summary of 2,3,7,8-TCDD effects observed in bivalve mollusks[a]

Observed Effect	*M. arenaria* Soft shell clam	*C. virginica* Eastern oyster
Wasting Syndrome	+ (200 pg/g)	+ (2.0 pg/g)
Epithelial hyperplasia	+ (2.5 pg/g)	+ (2.2–4.3 pg/g TEF)
Gonad lesions		
Male	-	+/− (2.0–10 pg/g)
Female	+ (4.8-20 pg/g)	+ (2 pg/g)
Survival Offspring	ND	+ (2 pg/g)
Decrease in % female		
gonad development	ND	+ (2 pg/g)
Reduced Calcium deposition shell	+ (2500 pg/g)	ND
Preferential accumulation into Gonad	+	+
Altered vitellogen-like levels	ND	+

[a]Plus (+) or negative (-) signs indicate whether the effect was observed in laboratory controlled studies. ND indicates the endpoint was not evaluated. The value in () are doses resulting in significant differences from controls

tissues. In field populations, hyperplasia was also observed in similar tissues. However, due to multiple contaminants being present, these lesions could not be solely attributed to 2,3,7,8-TCDD. In a field study where clams were transferred from a heavily impacted location into a reference area the mantle, intestine, digestive gland, and kidneys had a significant increase in hyperplasia and metaplasia (31). This increased prevalence of epithelial lesion could be attributed to DLC elimination through macrophage diapedesis or passive diffusion through these tissues.

There was delayed female gonadal development in all oysters at the 2.0 and 10 pg/g dosed animals. A number of oocytes contained two nuclei that indicated altered cell division. Similar multi-nucleated oocytes were observed in clams collected from Elizabeth, NJ. The lesions in both the male and female oysters at 10 pg/g wet weight were more severe than at the 2.0 pg/g wet weight. There was also a shift in the male female ratio of oysters exposed to 2,3,7,8-TCDD, with a decrease in the number of females present at the end of 28 days. Vitellogen equivalents were significantly reduced in the female oysters. This could be used as an indicator for decreased estrogenic activity within the treated oysters. Field studies in clams have shown this biomarker to be decreased in polluted locations (36). Based on the histopathology of the oyster testis, it would appear that sperm development is less affected than egg development. Studies carried out by Butler et al. (21) in soft shell clams at comparable 2,3,7,8-TCDD concentrations also reported a lack of proper gonad development in both female and male clams.

Studies both by our group and those reported in *M. arenaria* by Rhodes et al. (35) indicate that 2,3,7,8-TCDD preferentially accumulates into the gonad and digestive glands. Based on our studies using physiological based pharmacokinetic modeling the uptake is not driven solely by lipid and is best

explained by 2,3,7,8-TCDD binding to a tissue-specific receptor (32). Future studies need to better characterize what is the cause of this apparent increased binding and what relationship it has to the observed toxicity. Studies are also needed to determine what effect other DLC congeners have on the ability of bivalves to successfully reproduce. The role of the sex steroids (estradiol-17B, testosterone, progesterone) and neurohomones in bivalves as it relates to specific cell synthesis and release at different stages of gonad development need to be better characterized. Neurohormones have been cited as the primary messengers involved in the reproductive cycles in mollusks, but a series of recent papers have pointed out the role of steroid hormones in gonad development (27–30). Endocrine effects involving altered hormone levels and gonad development have been reported from mollusks living in contaminated areas when compared to reference locations (36–38). The development of the 28-day oyster protocol (34), which allows for initiation of gonad development from the pluripotent germ cell to mature gonads in 28 days will allow for these questions to be addressed.

The traditional model of how AhR interacts with 2,3,7,8-TCDD and elicits toxicity is based on mammalian systems (14). It is unlikely that the highly conserved AhR and ARNT evolved solely as a means of interacting with polycyclic Aryl hydrocarbons, but the physiological roles for the bHLH-PAS gene superfamily are still mostly unknown. In several studies, it has been demonstrated that exposure to 2,3,7,8-TCDD in animals or cells with non-binding AhRs results in abnormal reproduction and developmental effects (17), altered estrogenic responses (24), activation of cell proliferation pathways (18), interference with the transcription of the estrogen receptor (ER) or binding, and accelerated steroidal metabolism or conjugation reactions (9, 24).

In bivalve mollusks, it has been reported that an AhR receptor does exist and has similar associated cofactors as those reported in higher vertebrates (16). A unique characteristic of the bivalve AhR is that it does not appear to bind 2,3,7,8-TCDD (20). Several forms or splice variants of ARNT have been reported to occur in mollusks, and there does appear to be tissue specificity (Van Beneden personal communication). The AhR/ARNT complex in *M. arenaria* has been shown to interact with XREs (20). The lack of binding to the homologous invertebrate AhR receptor does not mean that 2,3,7,8-TCDD will not result in toxicity as evidenced by our results in both *M. arenaria* and *C. virginica* (31–34). The reproductive system and the developing eggs and sperm are very sensitive to 2,3,7,8-TCDD's effects (33, 34). This observation raises the question of how does 2,3,7,8-TCDD elicit the responses that are observed without being able to bind to the homologous invertebrate AhR. One explanation could be that there are AhR independent pathways that can contribute to 2,3,7,8-TCDD toxicity due to cross talk, competition for ARNT with other independent pathways, or possibly direct membrane receptor activation.

Another possibility is that in mollusks AhR/ARNT activation is though a temperature sensitive mechanism that could change the configuration to allow binding (Figure 1). One of the most intriguing questions concerning the AhR is what is the natural occurring ligand (14, 15). Currently it is not clear as to what the endogenous ligand or phylogenetic purpose of the AhR/ARNT pathway plays in organisms (16). In mollusks, it may be that 2,3,7,8-TCDD plays some role in altering paraquin and/or autoquin signals in the developing gonad. The two major cues in initiating gonad development are temperature and nutritional status. One could hypothesize that temperature cues could modify the AhR protein structure to allow binding to endogenous ligands or ARNT. Since there is an important temporal sequence for proper gonad development, endogenous ligand binding may benefit from lower affinities. Tryptophan and related indole containing compounds are present in bivalve gonad tissue and may be the endogenous ligand for the AhR (14). It has been reported that tryptophan and related compounds can bind to the AhR and activate XREs (20). The clam AhR/ARTNT complex has been shown to be able to bind to XREs, and may be another means to activate genes in the gonad that are involved in steroid catabolism. The role of endogenous flavinoids in the gonad also needs to be examined in activating aromatase. Pivotal research by Ohtake et al. (40) has shown in mammalian systems that both dioxin activated and non-activated AhR can modulate estrogen and androgen signaling and promote proteasomal degradation of both receptors. This work demonstrates the role of cross-talk between the AhR and estrogen hormone signaling and the complex interplay between these systems. This common mechanism for gonad maturation and development is present in both wildlife and humans (Figure 1) and can explain why female gonad development is a critical target for 2,3,7, 8-TCDD.

A second highly conserved pathway (Figure 1), a homologous insulin receptor-related receptor (CIR), that may be affected by 2,3,7,8-TCDD is involved in germ cell mitosis, maturing oocytes and veliger larval development (41). In addition to being involved in gonad proliferation and maturation the CIR has been reported in *C. gigas* to be implicated in proliferation of epithelial cells in the outer fold and outer mantle epithelium, which are both involved in shell formation (42). This receptor belongs to the class II of receptor protein-tyrosine kinase family, and is stimulated by hrIGF-1. Alterations in this pathway caused by 2,3,7,8-TCDD could explain the decreased shell thickness in *M. arenaria* and poor viability of veliger larval survival. Further studies need to be carried out to better characterize the effects of 2,3,7,8-TCDD on this pathway in bivalve mollusks.

In summary, successful reproduction is a necessary process for the survival of any species. Compounds that interfere with normal male and female gonad development and/or post-fertilization development will alter the population structure (Figure 2). 2,3,7,8-TCDD alters normal development of

reproductive organs and early development in bivalve mollusks at tissue concentrations (2–20 pg/g wet weight) being observed in field populations. In both *Crassostria virginica* and *Mya arenaia*, 2,3,7,8-TCDD preferentially accumulates into the gonads. A wasting type syndrome was observed as well as hyperplasia of epithelial tissues. Thin shells in field populations of *M. arenaria* inhabiting Newark Bay could be attributed to decreased calcium deposition to the shell from the mantle and shell gland. As with lower vertebrates (fish embryos) and higher vertebrates, bivalve mollusks would appear to be equally sensitive to 2,3,7,8-TCDD's effect on gonad development, embryonic development and epithelial lesion occurrence. Altered gonad development, decreased veliger larval survival, and altered calcium deposition to the shell can in part explain the lack of self-sustaining bivalve populations in 2,3,7,8-TCDD contaminated estuaries. Future studies need to further elucidate the relationships between 2,3,7,8-TCDD and other DLCs effects on hormonal control involved in gonad development and veliger survival. The use of the 28-day oyster gonad development protocol allows for examination of the role of steroidal hormones, neuroehormones, and related metabolic enzymes as reproductive signals that may be affected by DLC compounds as well as other compounds that target critical signals in sustaining egg and sperm development.

The results presented in this review provide evidence that reproduction in bivalve mollusks is highly sensitive to 2,3,7,8-TCDD exposure. The body burdens (2–10 pg/g wet weight) that resulted in altered gonad development and veliger survival in the laboratory were comparable to those observed in field populations (2,3,7,8-TCDD *M. arenaria* 0.5–20 pg/g wet wt. (43) and *C. virginica* 0.15–3.2 pg/g wet weight (33)). What is the mechanism by which 2,3,7,8-TCDD disrupts gonad development needs to be examined further in bivalves with respect to the findings reported by Ohtake et al. (40) and as presented in Figures 1 and 3. Polychlorinated biphenyls (PCBs) have also been reported to concentrate in gonads of bivalves and result in gonadal atrophy, and reduced reproductive output (45–46–47). PCBs may be acting through a similar mechanism. Altered reproductive ability of bivalves following chemical exposure to reproductive endocrine disruptors such as 2,3,7,8-TCDD and PCBs are likely to be one of the major reasons that bivalve populations both in freshwater and estuaries have steadily declined both here in the United States and around the world.

ACKNOWLEDGEMENTS

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