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The development of an aquatic bivalve model: Evaluating the toxic effects on gametogenesis following 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) exposure in the eastern oyster (*Crassostrea virginica*)

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Abstract

The objective of this study is to develop a gametogenesis protocol to serve as a model for evaluating the toxic effects of chemicals on oogenesis and spermatogenesis in the eastern oyster (*Crassostrea virginica*). The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) was selected as a "proof of principle" toxicant to examine developmental toxicity in this invertebrate system. The studies were designed to: (1) test the model using 2,3,7,8-TCDD and (2) to use histopathological evaluations to characterize the effects on oocyte and sperm development during stages of gametogenesis. 2,3,7,8-TCDD at 10 pg/g resulted in significant histopathological gonadal lesions by day 14 of gametogenesis in both female and male oysters. These lesions resulted in complete inhibition of gonadogenesis. Studies also showed that a total body dose of 2 and 10 pg/g 2,3,7,8-TCDD caused adverse responses resulting in abnormal gametogenesis in female and male oysters, respectively, such as: (1) incomplete oocyte division, (2) inhibition of oocyte growth and maturation, (3) unsynchronized sperm development, and (4) inhibition of spermatogenesis. The eastern oyster is one of the most responsive invertebrate models tested to date for reproductive effects of chemicals. Therefore, the eastern oyster can be used as a sensitive toxicological model for examining the effects of dioxin-like compounds and other xenobiotics on gametogenesis. The reported studies show that environmentally relevant concentrations of 2,3,7,8-TCDD (2–10 pg/g) have a significant adverse effect on oyster gametogenesis.

Keywords: *Crassostrea virginica*; Oyster model; Gametogenesis; 2,3,7,8-TCDD; Histopathology

1. Introduction

Chemicals found in the environment as industrial byproducts or pollutants can have multiple adverse effects on organisms. An anthropogenic chemical, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), is one of the most toxic environmental pollutants. Public concern about 2,3,7,8-TCDD has stimulated numerous studies to assess TCDD's behavior in the environment and its effect on living organisms (Isensee and Jones, 1975; Brown, 1991; Rune et al., 1991; Bergen et al., 1993; Chevreuil et al., 1996; Rhodes et al., 1997; Chen et al., 2002; Wintermyer and Cooper, 2003; Schultz et al., 2003; Miller et al., 2004). Mammalian models have demonstrated that 2,3,7,8-TCDD significantly impairs and, in a few species, inhibits male and female reproductive capabilities. Studies have shown that 2,3,7,8-TCDD causes a decrease in spermatogenesis and ovulation rate indicating that the most sensitive organs to 2,3,7,8-TCDD exposure are the testis and ovaries (Rune et al., 1991; Johnson et al., 1992; Mably et al., 1992; Bjerke and Peterson, 1994; Sommer et al., 1996; Gray et al., 1997; Heimler et al., 1998; Schultz et al., 2003; Miller et al., 2004; Moon et al., 2004). For example, research has shown, in various female mice, rats, and hamsters, that exposure to low doses of 2,3,7,8-TCDD (0.8–2.5 μg/kg) has resulted in: (1) reduced ovarian weights, (2) ovarian neoplasms, (3) delayed pregnancies, (4) decreased estrogen levels, and (5) reduced or eliminated ovulation (Gray and Ostby, 1995; Chaffin et al., 1996; Gray et al., 1997; Salisbury and Marcinkiewicz, 2002; Miller et al., 2004). Male reproductive studies have shown that *in utero* and *in vivo* exposure of 2,3,7,8-TCDD has resulted in: (1) altered sperm maturation, (2) decreased reproductive organ weights, (3) decreased epididymal sperm reserves, (4) decreased or inhibited spermatogenesis, (6) reduced Leydig cell function and Leydig cell volumes, and (7) increased vacuolar degeneration in germs cells with apoptosis

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in tubules using morphological, histochemical, phase contract cytometry, biochemical, and molecular techniques (Rune et al., 1991; Mably et al., 1992; Johnson et al., 1992; Bjerke and Peterson, 1994; Sommer et al., 1996; Gray et al., 1997; Moon et al., 2004).

Studies involving aquatic organisms have shown that 2,3,7,8-TCDD can bioaccumulate in tissues and adversely effect reproduction. Field and laboratory studies have demonstrated that exposure to 2,3,7,8-TCDD can result in altered gonadal development, egg fertilization success, and embryonic development in fish and invertebrate species (Isensee and Jones, 1975; Brown, 1991; Birnbaum and Tusmisto, 2000; Toomey et al., 2001; Giesy et al., 2002; Butler et al., 2004; Wintermyer and Cooper, 2003; Wintermyer et al., 2005). For example, in adult female rainbow trout (*Oncorhynchus mykiss*), exposure to dietary 2,3,7,8-TCDD (1.8 ng/kg) during the reproductive season resulted in accumulation of 2,3,7,8-TCDD into tissues and eggs, decreased fry survival, and decreased adult survival (Giesy et al., 2002). In adult female bivalves exposure to 2,3,7,8-TCDD during gametogenesis resulted in partially undifferentiated gonads, 2,3,7,8-TCDD accumulation into tissues and oocytes, and a significant reduction in egg fertilization success (Butler et al., 2004; Wintermyer and Cooper, 2003; Wintermyer et al., 2005). In adult male bivalves, it has been shown that 2,3,7,8-TCDD exposure during gametogenesis resulted in 2,3,7,8-TCDD tissue accumulations, reduced spermatogenesis, and reduced sperm viability (Wintermyer and Cooper, 2003). The array of 2,3,7,8-TCDD effects demonstrate some species variability but, many effects are seen in multiple wildlife, domestic, and laboratory species ranging from fish through birds and mammals (Birnbaum and Tusmisto, 2000). The most sensitive adverse effects observed in multiple species appear to be developmental, including effects on the reproductive system. At relatively low dioxin exposures levels, structural malformations are not common in species; however, functional alterations such as in the female and male reproductive systems and in reproductive behaviors are the most sensitive signs of developmental toxicology (Peterson et al., 1993). Cross-species comparisons have shown both female and male reproductive toxicity to have some species variability, but many effects are analogous confirming that 2,3,7,8-TCDD exposure can have a significant effect on the developing reproductive system regardless of species (Peterson et al., 1993).

The mechanism of action (MOA) for 2,3,7,8-TCDD is well documented in mammalian literature and is mediated through the AhR/ARNT receptor complex in mammals and most vertebrate species (Poland and Knutson, 1982; Pohjanvirta and Jouko, 1994; Fernandez-Salguero et al., 1996; Miller et al., 2004). Conversely, in invertebrate species the MOA for 2,3,7,8-TCDD is not understood. Evidence of an Ah-like receptor (96.7 kDa) in invertebrate species has been reported; however, the invertebrate AhR lacks specific, high-affinity binding for 2,3,7,8-TCDD and other prototypical AhR ligands (Butler et al., 2001). Bivalve AhR, as well as other invertebrate AhR homologues do not bind 2,3,7,8-TCDD unlike the mammalian AhR (95–130 kDa) and fish AhR (105–146 kDa) homologues (Hahn et al., 1994; Butler et al., 2001). The difference in AhR binding affinity distinguishes invertebrate from vertebrate AhRs but, as in mammalian tissues, the amount of dioxin that accumulates in aquatic invertebrates varies with the lipid content of tissues (Institute of Medicine, 2003). In oysters, unlike vertebrate species, complete gonadal and gamete resorption is repeated seasonally and recrudescence involves formation of a new gonad prior to the formation of gametes (Loosanoff, 1942; Galtsoff, 1964; Kennedy and Battle, 1964; Eble and Scro, 1996). It is during this time of new development (*i.e.* gonadogenesis and gametogenesis) that bivalves have an increase in gonadal lipid content for the purpose of gamete maturation. Increased body burdens of organic contaminants are a result of tissue lipid content, low phase II metabolism, and slow clearance rates in bivalves (Livingstone and Farrar, 1984; Stegeman, 1985; Rhodes et al., 1997). The accumulation of pollutants in bivalves and fish species can lower their biochemical reserves (*i.e.* protein, carbohydrate, lipid, and glycogen) and can contribute to poor egg quality and fertilization rates (Capuzzo McDowell, 1996; Van, 2002; Sepulveda et al., 2003; Wintermyer and Cooper, 2003).

The reproductive systems of terrestrial and aquatic organisms, both male and female, are sensitive to 2,3,7,8-TCDD exposure during early stages of development (*i.e.* gonadal, gametogenic, embryonic, fetal, and neonatal) suggesting that the reproductive organs may be a target for 2,3,7,8-TCDD-induced responses (ASTM, 1994; Bjerke and Peterson, 1994; Sommer et al., 1996; Gray et al., 1997; Schultz et al., 2003; Wintermyer and Cooper, 2003; Miller et al., 2004; Moon et al., 2004).

This research demonstrated that the eastern oyster is a sensitive toxicological model for evaluating invertebrate gametogenesis. The results indicate that a total body dose as low as 2 pg/g 2,3,7,8-TCDD can adversely affect early stages of eastern oyster gametogenesis. The data presented are the first report of environmentally relevant levels of 2,3,7,8-TCDD having a significant adverse effect on oyster reproduction.

2. Material and methods

2.1. Chemicals

The following chemicals were used in the studies performed: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (98% pure, 50 ± 5 μg/ml in *n*-nonane) was purchased from Cambridge Isotope Diagnostics (Woburn, MA). Toluene (>99% pure) and all histological reagents and chemicals (*i.e.* histological stains, 10% phosphate-buffered formalin, ethanol, glutaraldehyde, OsO_4, acetone, epon, and araldite) were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Animals

Adult eastern oysters (*Crassostrea virginica*) were purchased from the Blue Mussel Company, Prince Edward Sound, Canada. All oysters used in these studies ($N = 605$) were weighed (g), numbered, and notched. The average total oyster weight (including shells) was 45 ± 2.5 g and the average soft tissue body weight (wet weight, w/w) was 5.0 ± 0.60 g (mean ± S.D.). The average oyster dimensions (mm) were: height = 62.1 ± 5.36, length = 38.2 ± 3.61, and width = 17.2 ± 2.68 (mean ± S.D.).

Each oyster was notched on the left side of the valves for access to the adductor muscle. Oysters were injected via the adductor muscle regardless of group treatment to ensure that injections were not the cause of adverse physiological effects.

2.3. Gametogenesis protocol

Oysters were maintained in a holding phase at 15 °C in a recirculating seawater system with no additional food supplemented. Under these conditions, oysters remained in a dormant reproductive phase. Oysters, regardless of treatment group, were maintained under optimal holding conditions (*i.e.* salinity (26–27 ppt), light (12 h cycle), water flow (7.57 l/min), water chemistry (pH, nitrite, nitrate, and ammonia), and water changes (Davis and Chanley, 1956; ASTM, 1994). Optimal conditions were held constant during both the holding phase and gametogenic phase.

Gametogenesis was initiated in each oyster immediately following treatment injections (*i.e.* seawater, toluene, or 2,3,7,8-TCDD) by transferring oysters to a 20 °C recirculating seawater system and supplementing existing food with 2 l of live cultured algae (*C. isochrisis*; 15 million cells/ml) per day. The studies were conducted over 28 days to allow for full gonadal development. The 28 days time period for gonadal development was determined in previous work (Wintermyer, 1998). Under these gametogenic conditions, the gender of the oysters could be identified as early as 72 h after induction. The 28 days time frame for oyster gametogenesis was used in all of the studies performed.

2.4. Dosing regime

Adult control oysters were injected with 100 μl of filtered seawater (0.45 μ filter mesh) or 100 μl of 4 ppb; ng/g (parts per billion) toluene (solvent carrier). Treated oysters were injected with 2 or 10 pg/g (parts per trillion) 2,3,7,8-TCDD based on soft tissue body weight (w/w). Following injections, all oysters were placed on absorbent paper for 1 h before being returned to a recirculating seawater system. This allowed discharging and recirculation of the injected dose by the oysters with out a significant loss to the recirculating seawater system. Oysters were not fed 24 h before or 24 h after the injections. Each oyster was injected with the respective treatment dose prior to the induction of gametogenesis and re-injected on day 14 ($t_{1/2}$ for TCDD) of the study according to the procedure described above. This dosing regiment was based on the $t_{1/2}$ of elimination determined in previous work (Wintermyer et al., 2005).

2.5. Light microscopy (LM)

Oyster samples were preserved in a10% phosphate-buffered formalin (pH 6.9–7.1) for several days and then transferred into 70% ethanol. Transverse cuts were made with a scalpel through the mid-visceral region of the oyster to obtain a segment approximately 5 mm thick. Segments were embedded in paraffin after processing (dehydration and clearing through an alcohol: xylene series). Sections (6–12 μm) were cut and stained with Harris hematoxylin and eosin (H&E). Sections were examined at 10× and 20× magnifications using a Carl Ziess, compact transmitted-light microscope KM (Howard and Smith, 1983). Gonad condition was graded according to Kennedy (1977):

Stage 0 = resting stage
- Dormant phase of gametogenesis

Stage I = early development
- Pre-vitellogenic oocytes (6–7 μm in diameter) located within the germinal epithelial wall
- Spermatogonia present in the tubules

Stage II = later development
- Vitellogenic oocytes (11–49 μm in diameter) attached to the follicular wall
- Primary and secondary spermatocytes

Stage III = sexual maturity

Stage IIIa = maturity
- Post-vitellogenic oocytes (50–55 μm in diameter) unattached from the follicular wall
- Spermatids and mature sperm

Stage IIIb = spawning

Stage IIIc = redevelopment

Stage IIId = recently spent

2.6. Electron microscopy (EM)

Gonadal samples (1 mm × 1 mm) were fixed for 24 h in 2% glutaraldehyde in 0.1 M phosphate buffer. Post-fixation was completed in 1% OsO_4 using the same buffer for 60 min on a rotating plate at room temperature. The samples were rinsed in buffer, and then dehydrated in ethanol solutions (70, 80, 95, and 100%). All samples were placed on a rotating plate for a total of 30 min for each progressive ethanol solution. Samples were: (1) cleared in acetone, (2) placed in a 1:1 mixture of epon:araldite in ethanol (100%) for 1 h, (3) placed in a 1:3 mixture of epon:araldite in acetone for 1 h, and (4) samples were finally placed in 100% epon:araldite and polymerization was completed at 60 °C for 24 h.

Semi-thin (1 μm) and ultra-thin (60–70 nm) sections were cut with glass and diamond ultramicrotome knives, respectively. The semi-thin sections were stained with 0.5% toluidine blue for light microscopy (LM). The ultra-thin sections were contrasted with uranyl acetate and lead citrate stains and examined using a Ziess 10CH electron microscope (Howard and Smith, 1983).

2.7. Statistical analysis of data

Results were analyzed using 1-way and 2-way analysis of variance (ANOVA) and expressed as the percentage related to the controls (seawater and toluene).

3. Results

3.1. Gametogenesis protocol

The 28 days gametogenesis protocol developed in our laboratory using untreated eastern oysters (*C. virginica*) ($N = 270$) resulted in the time correlated gonadal and gametogenic devel-

opment as follows: (1) the branching of follicles and tubules throughout the gonadal connective tissue in females and males, respectively, by days 1–7, (2) gender identification of individual oysters at day 7, (3) the peak of gamete development by day 14, and (4) the maturation of gametes by day 28. Within the developing follicles at day 7, there were various sizes of pre-vitellogenic oocytes, 6–10 μm in diameter, located within the germinal epithelial (follicular) wall indicative of early stages in oocyte development (Stage I; Fig. 1a). The male gonads had developing/branching tubules with the presence of spermatogonia lining the outer periphery of the inner tubules correlating to early spermatogenic development (Stage I; Fig. 1b). At day 14 of the gametogenesis protocol, the gonads had well defined and differentiated follicles in the female and tubules in the male comprising greater than 90% of the gonadal connective tissue (Fig. 1c and d). Within the developed follicles, there were vitellogenic oocytes ranging from approximately 11 to 50 μm in diameter and attached to the follicular wall correlating to late development and early maturation stages (Stage II; Fig. 1c). The male gonads had well developed and expanded tubules with the presence of primary and secondary spermatocytes and the early developmental stages of spermatids (Stage II; Fig. 1d) also correlating to the maturation stages of spermatogenesis. By day 28, the gonads were fully developed and compact with differentiated follicles in the female and tubules in the male (Fig. 1e and f). With in the densely packed follicles post-vitellogenic oocytes, approximately 50–55 μm in diameter, were unattached from the follicular wall concluding the maturation phase (Stage

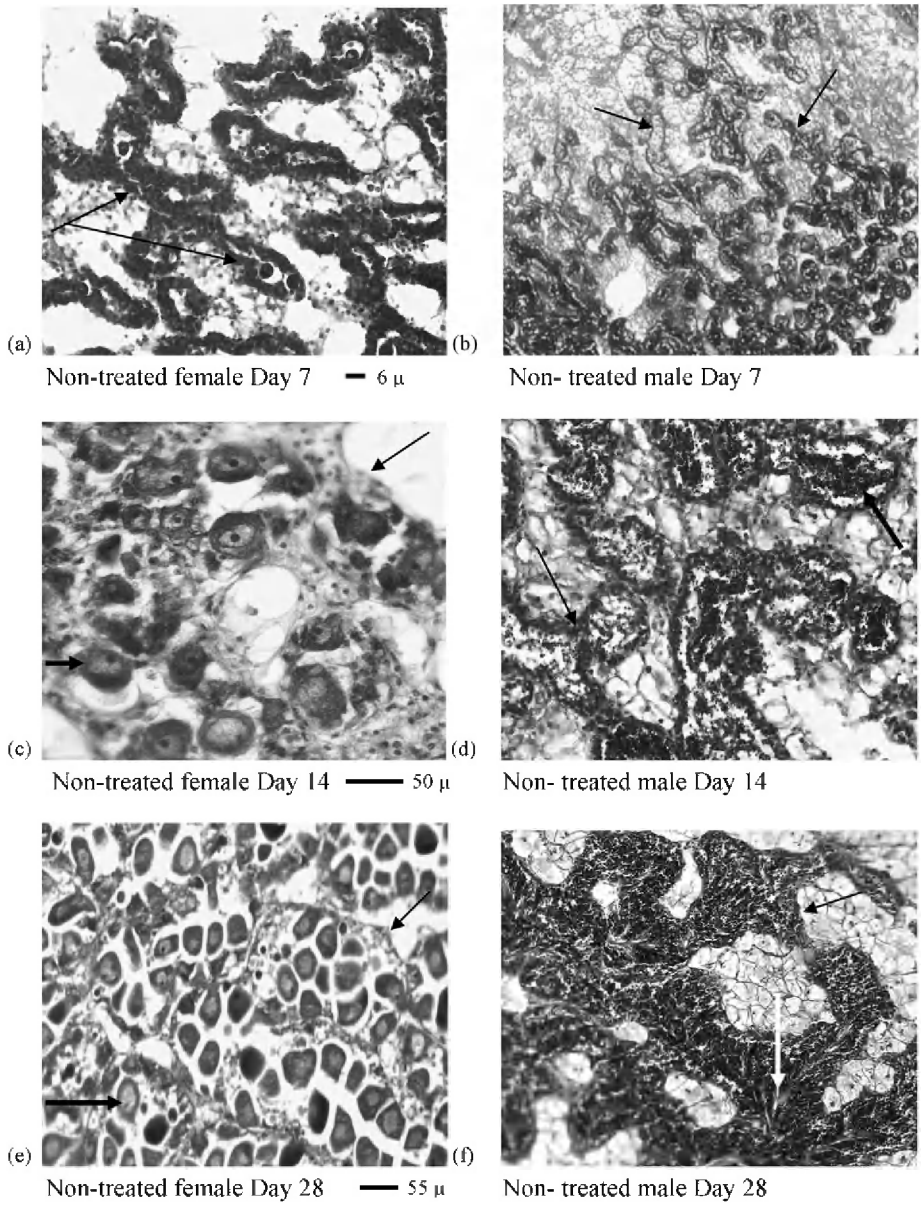

Fig. 1. Gonadogenesis at days 7, 14, and 28 in non-treated eastern oysters (*Crassostrea virginica*): (a) a female and (b) a male gonad illustrating early development (Stage 1) at day 7. (c) A female and (d) a male gonad illustrating late development to early maturation stages (Stage II/IIIa) at day 14. (e) A female and (f) a male gonad illustrating mature gametes at day 28 (Stage IIIb). Thin arrows show follicular and tubular development, thick arrows show oocyte and sperm development and the bar scale indicates oocyte sizes. H&E stain. Similar results were obtained from three independent studies.

IIIb; Fig. 1e). In the male, densely packed tubules were observed with spermatogonia lining the periphery of the tubules followed by primary spermatocytes, secondary spermatocytes, and spermatids. Mature sperm were observed gathered toward the center of the tubules indicating the readiness for spawning (Stage IIIb; Fig. 1f).

This protocol has been validated for gonadal and gametogenic development over 28 days according to specific conditions such as, but not limited to, water temperature and flow, water quality, and live algal food quality and quantity. At day 7, all oysters evaluated ($N=90$; 45 females, 45 males) were at developmental Stage I (100%). At day 14, 86.7% of the females ($N=39$) and 80.0% of the males ($N=36$) evaluated were at developmental Stage II. At day 28, all oysters evaluated ($N=90$; 45 females, 45 males) were at developmental Stage IIIa (100%) (Table 1). There was no significant difference between staged gonadal development in female and male groups at day 7, 14, or 28. This 28 days gametogenesis protocol was used in all the studies performed.

3.2. The effects of 2,3,7,8-TCDD on gonadogenesis and gametogenesis in C. virginica

Gonadal and gametogenic development is shown in Fig. 2a–h of female and male oysters treated with seawater, toluene (4 ng/g), 2 and 10 pg/g 2,3,7,8-TCDD during early gametogenesis (days 7 and 14 of the gametogenesis protocol). Fig. 2a shows the gonadal development of a control seawater female oyster at day 7. Seawater control females ($N=20$) had well-defined and differentiated follicular development with pre-vitellogenic oocytes closely attached to the follicular epithelial wall at day 7 (Stage I). Fig. 2b shows the gonadal development for a female toluene solvent control (4 ng/g) oyster at day 7. The toluene solvent control females ($N=20$) had a slight delay in gonadal development compared to the seawater control females illustrated by the lack of differentiated follicular structures; however, pre-vitellogenic oocytes were present in the follicles of all females examined (Stage I). Fig. 2c shows the day 7 gonadal development for a 2 pg/g 2,3,7,8-TCDD exposed oyster. The 2 pg/g 2,3,7,8-TCDD females ($N=20$) had moderately differentiated follicular structures at day 7 compared to the seawater control group with pre-vitellogenic oocytes showing delayed development indicated by the small (≤ 6 μm), compact, darkly stained oocytes present in the follicles (Stage I). Fig. 2d shows the gonadal development for a 10 pg/g 2,3,7,8-TCDD exposed female oyster at day 7. The 10 pg/g 2,3,7,8-TCDD females ($N=20$) had abnormal gonadal development at day 7 indicated by: (1) delayed follicular growth/branching, (2) lack of follicular definition (thin epithelial wall), (3) delayed pre-vitellogenic oocyte growth (≤ 6 μm), and (4) the appearance of macrophages (*i.e.* brown cells) infiltration in the gonadal tissue of all females examined as early as day 7 of gametogenesis.

Fig. 2e shows the gonadal development of a seawater control male at day 14. The seawater control males ($N=20$) had Stage II gonadal development at day 14 indicated by the well-differentiated and expanded tubules with primary and secondary spermatocytes present (individual spermatocytes not visible from photomicrograph). Fig. 2f shows the gonadal development of a toluene solvent control (4 ng/g) male at day 14. The toluene control males ($N=20$) had Stage II gonadal development similar to the seawater control males. As with the seawater control males, the toluene-treated males had well-differentiated and expanded tubules with advanced stages of spermatogenic cells present in the tubules at day 14. Fig. 2g shows the gonadal development of a 2 pg/g 2,3,7,8-TCDD exposed male oyster at day 14. The 2 pg/g 2,3,7,8-TCDD treated males ($N=20$) appeared to be undergoing normal gonadal development similar to both the seawater and toluene controls. The outer peripheries of the inner tubules appeared to have an enlarged space between the spermatogenic cells and the tubule epithelium (*); however, the tubules were well differentiated and expanded at day 14. Fig. 2h shows the gonadal development of a 10 pg/g 2,3,7,8-TCDD exposed male oyster at day 14. The 10 pg/g 2,3,7,8-TCDD treated males ($N=20$) had abnormal gonadal development at day 14 indicated by: (1) delayed tubular growth/branching, (2) lack of differentiated tubular structures, (3) delayed spermatogenesis compared to control males, and (4) the appearance of macrophages (*i.e.* brown cells) in the gonadal tissue of all males examined on day 14 (macrophage infiltration not pictured).

The percentage of female and male oysters which displayed specific morphological lesions at days 7 (Stage I) and 14 (Stage II), respectively, are shown in Table 2. In the female oysters at day 7, differential follicular development and delayed gonadal development (Stage I) were significantly different in the toluene, 2 and 10 pg/g 2,3,7,8-TCDD groups compared to the seawater control group (ANOVA, $P<0.05$). Macrophage infiltration was significantly different in the 10 pg/g 2,3,7,8-TCDD group compared to the seawater, toluene, and 2 pg/g 2,3,7,8-TCDD groups (ANOVA, $P<0.01$).

The male oysters at day 14 in the 10 pg/g 2,3,7,8-TCDD group had a significant difference in differential tubule development, delayed spermatogenesis, and macrophage infiltration

Table 1
The staging of gonadal and gametogenic development in non-treated oysters (*C. virginica*) using the 28 days gametogenesis protocol

Non-treated oysters	Day 7 (Stage I)	Day 14[a] (Stage II)	Day 28 (Stage IIIa)
Female oysters ($N=135$)	45/45 (100%)	39/45 (86.7%)	45/45 (100%)
Male oysters ($N=135$)	45/45 (100%)	36/45 (80.0%)	45/45 (100%)
Total oysters evaluated ($N=270$)	$N=90$	$N=90$	$N=90$

Results were obtained in three independent studies. At each time point (day 7, 14, and 28), 30 untreated oysters were randomly selected (15 females, 15 males). No significant difference between or among female and male groups at days 7, 14 and 28 (ANOVA, $P>0.05$).

[a] Day 14 females, six oysters had advanced oogenesis; Day 14 males, nine oysters had delayed spermatogenesis.

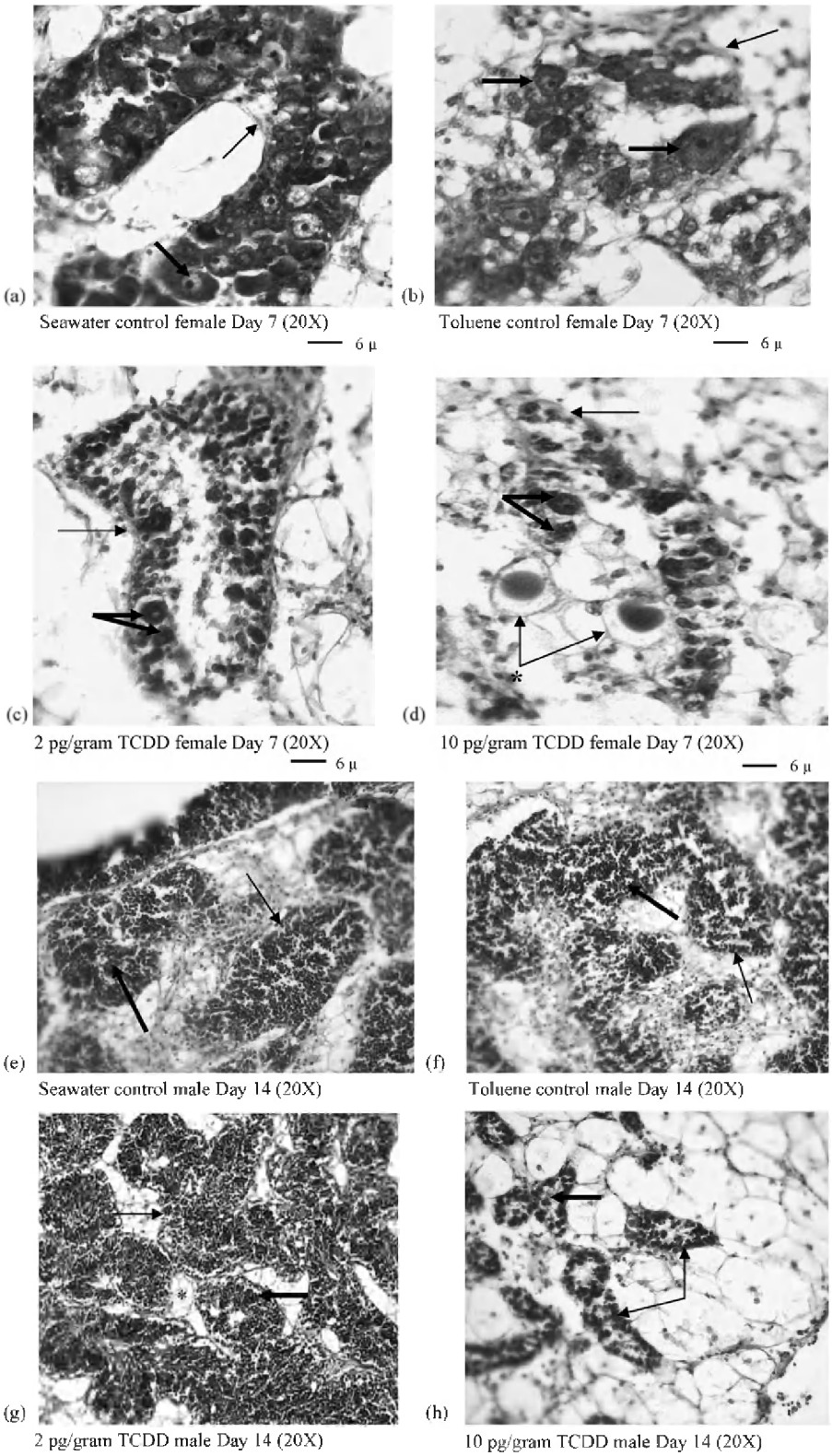

Fig. 2. Female gametogenesis at day 7 and male gametogenesis at day 14 showing treatment groups: seawater, 4 ng/g toluene, 2 and 10 pg/g 2,3,7,8-TCDD in *C. virginica*. (a) A seawater control female illustrating early stages of gametogenesis at day 7 (Stage I). (b) A toluene solvent control female illustrating early stages of development at day 7 (Stage I). (c) A 2 pg/g TCDD female illustrating early gametogenesis at day 7 (Stage I). (d) A 10 pg/g TCDD female illustrating a delay in early gonadogenesis with a delay in oocyte growth (early Stage 1), and invading macrophages (*) at day 7. (e) A seawater control male illustrating normal spermatogenesis at day 14 (Stage II). (f) A toluene solvent control male illustrating normal spermatogenesis at day 14 (Stage II). (g) A 2 pg/g TCDD male illustrating an enlarged intercellular space within tubule (*) at day 14 (Stage II). (h) A 10 pg/g TCDD male showing a delay in gonadogenesis and spermatogenesis at day 14 (Stage 1). Thin arrows indicate development of follicles and tubules, thick arrows indicate oocytes and sperm development, and bar scale represent oocyte sizes. H&E stain. Similar results were obtained in three independent studies.

Table 2
Histological (LM) evaluation of morphological lesions during gonadogenesis in female oysters at day 7 and male oysters at day 14 exposed to seawater, toluene (4 ng/g), 2 and 10 pg/g 2,3,7,8-TCDD

LM	Females day 7 (Stage I)	Males day 14[a] (Stage II)
Seawater control, $N = 20$ females, 20 males	*&$ Differentiated follicles, 100% !£ Delayed development (early Stage I), 0% Pre-vitellogenic oocytes, 100% Macrophages, 0%	& Differentiated tubules, 100% Delayed development (Stage II), 0% « Enlarged intercellular space, 0% × Macrophages, 0%
Toluene control, (4 ng/g) $N = 20$ females, 20 males	* Differentiated follicles, 40% Delayed development (early Stage I), 60% Pre-vitellogenic oocytes, 100% ? Macrophages, 0%	$ Differentiated tubules, 100% ? Delayed development (Stage II), 0% ‡ Enlarged intercellular space, 0% ¿ Macrophages, 0%
2 pg/g TCDD, $N = 20$ females, 20 males	& Differentiated follicles, 20% ! Delayed development (early Stage I), 100% Pre-vitellogenic oocytes, 100% Macrophages, 0%	* Differentiated tubules, 100% # Delayed development (Stage II), 0% ‡« Enlarged intercellular space, 100% Macrophages, 0%
10 pg/g TCDD, $N = 20$ females, 20 males	$ Differentiated follicles, 0% £ Delayed development (early Stage I), 100% Pre-vitellogenic oocytes, 100% ? Macrophages, 100%	*$& Differentiated tubules, 0% #? Delayed development (Stage II), 100% Enlarged intercellular space, NA ¿× Macrophages, 100%

Results were obtained from three independent studies. Symbols indicate significant difference between treatment groups within females at day 7 (ANOVA, $P < 0.01$) and within the males at day 14 (ANOVA, $P < 0.05$).
[a] Males day 14, enlarged intercellular spaces refer to enlarged intercellular spaces within tubules.

compared to the seawater, toluene, and 2 pg/g 2,3,7,8-TCDD groups (ANOVA, $P < 0.01$). The 2 pg/g 2,3,7,8-TCDD male oysters had tubules with an enlarged intercellular space between the tubule epithelium and spermatogenic cells that was significantly different from the seawater and toluene control males (ANOVA, $P < 0.01$) (Table 2).

Gonadal development in oysters exposed to 2 pg/g 2,3,7,8-TCDD early in gonadogenesis did not appear to be as adversely affected, histologically, compared to the 10 pg/g 2,3,7,8-TCDD treated oysters; however, gametogenesis was impaired at days 7 and 14 in both the female and male oysters, respectively. Histologically, the 2 pg/g 2,3,7,8-TCDD treated females appeared to have similar follicular development (*i.e.* follicular definition and expansion) compared to the controls at day 7 and the males appeared to be undergoing normal gonadal development at day 14 (Fig. 2c and g). Female and male oysters exposed to 10 pg/g 2,3,7,8-TCDD during early gonadal development underwent altered gametogenesis illustrated by the lack of gamete development and maturation by days 7 and 14, respectively (Fig. 2d and h). The end result was an inhibition of gonadogenesis and gametogenesis in both the female and male oysters by day 14 indicated by the significant delay in follicular and tubular branching/expansion, lack of follicular and tubule differentiation, and inhibition of gamete development at Stage I.

3.3. Female gonadogenesis and gametogenesis over 28 days using 10 pg/g 2,3,7,8-TCDD

Female oyster gonadogenesis and gametogenesis was examined in this study due to an alteration in the gender ratio correlated to 10 pg/g 2,3,7,8-TCDD exposure. Inhibition of gametogenesis in the female eastern oyster was observed by day 14 of the gametogenesis protocol as a result of 10 pg/g 2,3,7,8-TCDD exposure during early gonadogenesis. Fig. 3 shows the photomicrographs for follicular and oocyte development in the seawater, toluene (4 ng/g), and 10 pg/g 2,3,7,8-TCDD groups at days 1, 14, and 28 of gametogenesis (female oysters shown only). Fig. 3a shows the follicular as well as the oocyte development of the female seawater control group. At day 1 ($N = 15$ oysters; 9 females, 6 males), the female gonads showed signs of early development (Stage I) indicated by the presence of branching follicles through out the gonadal connective tissue with various sizes of oocytes (5–7 μm) located within the germinal epithelial (follicular) wall (pre-vitellogenic oocytes). Fig. 3b shows at day 14 ($N = 15$ oysters; 8 females, 7 male), the follicular structures were less defined and various sizes of maturing oocytes (11–50 μm) (vitellogenic/post-vitellogenic oocytes; Stage II/IIIa) were present in all females evaluated. Fig. 3c shows at day 28 ($N = 15$ oysters; 6 females, 9 males), the majority of the oocytes were fully matured in each female denoted by the oocyte sizes (50–55 μm) and detachment of the oocytes from the germinal epithelial walls (post-vitellogenic oocytes; Stage IIIa). Fig. 3d–f shows the female gonadal development as well as the oocyte development for the toluene solvent control (4 ng/g) oysters. Fig. 3d shows at day 1 ($N = 15$ oysters; 6 females, 9 males), the gonadal development was slightly delayed in the females compared to the seawater controls illustrated by the delay in follicular development and moderately undifferentiated follicular membranes (Stage 0/I). An early stage of oocyte development was indicated by the oocyte sizes (6 μm) and close adherence to the germinal epithelial base membrane (pre-vitellogenic oocytes). Fig. 3e shows at day 14 ($N = 15$ oysters; 9 females, 6 males), the follicles were well developed and differentiated with various sizes of oocytes (11–50 μm) attached to the germinal base membranes (vitellogenic oocytes; Stage

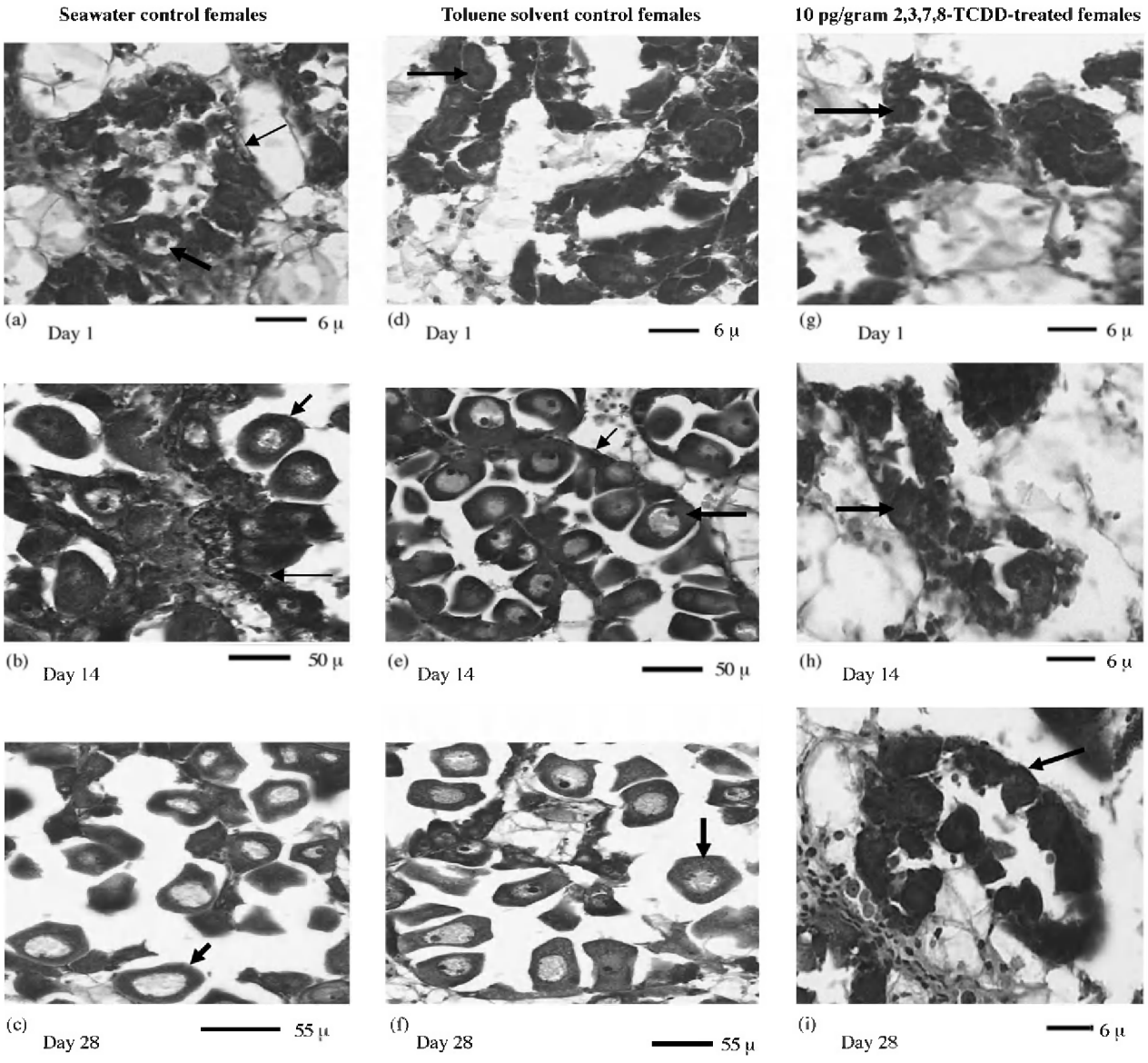

Fig. 3. Female gametogenesis at days 7, 14, and 28 of the 28 days gametogenesis protocol showing treatment groups: seawater, toluene (4 ng/g), and 10 pg/g 2,3,7,8-TCDD. (a) Seawater control group at day 1 illustrating pre-vitellogenic oocytes (Stage I), (b) day 14 illustrating vitellogenic oocytes (Stage II), (c) day 28 illustrating post-vitellogenic oocytes (Stage IIIa), (d) toluene solvent control group at day 1 illustrating pre-vitellogenic oocytes (Stage I), (e) day 14 illustrating vitellogenic oocytes (Stage II), (f) day 28 illustrating vitellogenic and post-vitellogenic oocytes (Stage II/IIIa), (g) 10 pg/g TCDD treatment group at day 1 illustrating pre-vitellogenic oocytes (Stage I), (h) day 14 illustrating pre-vitellogenic oocytes (Stage I), and (i) day 28 illustrating inhibition of oocyte grow and development (pre-vitellogenic oocytes; Stage I). Thin arrows show follicular development, thick arrow show oocyte development, and the bar scale represents oocyte sizes. H&E stain. Similar results were obtained from three independent studies.

II). Development was slightly delayed compared to the seawater control females denoted by the majority (>50%) of smaller sized oocytes and closer adherence of the oocytes to the germinal epithelial wall. Fig. 3f shows that by day 28 ($N = 15$ oysters; 9 females, 6 males), a majority (>50%) of the oocytes were fully matured in all females indicated by the oocyte sizes (50–55 μm) and detachment of the oocytes from the germinal epithelial wall (post-vitellogenic oocytes; Stage IIIa). Fig. 3g–i shows the follicular structure as well as the oocyte development for the 10 pg/g 2,3,7,8-TCDD treated oysters. Fig. 3g shows at day 1 ($N = 15$ oysters; 6 females, 9 males), there was a delay in gonadogenesis compared to controls as illustrated by the lack of follicular branching and undifferentiated follicular structures (Stage I). The oocytes were dense and compact denoted by the dark staining, irregular shapes, and small size (6 μm) in all the observed females (pre-vitellogenic oocytes). Fig. 3h shows at day 14 ($N = 15$ oysters; 2 female, 13 males), the follicles in the females were not well differentiated indicated by the thinness of the follicular membranes and lack of follicular expansion. The delay in oocyte development and maturation (Stage I) was severe compared to the seawater and toluene controls at day 14. Fig. 3i shows at day 28 ($N = 15$ oysters; 2 females, 13 males), the follicles in both females were moderately differentiated in structure, but oocyte growth and maturation was inhibited as indicated by

Table 3
Female oyster staging of gonadal development at days 1, 14, and 28 following exposure to seawater, toluene (4 ng/g) and 10 pg/g 2,3,7,8-TCDD

Female oysters	Day 1 (Stages 0 and I)	Day 14[a] (Stage II)	Day 28 (Stage IIIa)
(*) Seawater control ($N=23$)	9/9 (100%)	6/8 (75.0%)	6/6 (100%)
(§) Toluene solvent control ($N=24$)	6/6 (100%)	5/9 (55.5%)	9/9 (100%)
(*§) 10 pg/g TCDD ($N=10$)	6/6 (100%)	0/2 (0%)	0/2 (0%)

Results were obtained in three independent studies. At each time point (days 1, 14, and 28), five oysters were randomly sampled from each control and treatment group in which only the females were histologically evaluated. Symbols indicate significant difference in female development between treatment groups (day 1–28) (ANOVA, $P<0.05$). Significant difference in female development between treatment groups (days 1–28) (ANOVA, $P<0.05$).

[a] Day 14 females, seawater control group had two oysters with advanced oogenesis; toluene solvent control group had four females with delayed oogenesis; 10 pg/g TCDD group had two females with inhibited oogenesis.

the oocyte sizes (6 μm) and lack of oocyte detachment from the germinal epithelium walls by day 28 (Stage I, pre-vitellogenic oocytes).

The percentage of female oysters at Stages I, II, and III at days 1, 7, and 28, respectively, are shown in Table 3. The seawater, toluene, and 10 pg/g 2,3,7,8-TCDD female oysters had Stage I gamete development at day 1 (100%). At day 14, the seawater-treated females had 75.0%, the toluene-treated females had 55.5%, and the 10 pg/g 2,3,7,8-TCDD-treated female oysters had 0% Stage II oocyte development. At day 28, the seawater and toluene-treated female oysters had 100% Stage IIIa oocyte development while the 10 pg/g 2,3,7,8-TCDD-treated females had 0% Stage IIIa oocyte maturation. There was a statistical significance between the controls and the 10 pg/g 2,3,7,8-TCDD treatment group development over 28 days (ANOVA, $P<0.05$).

The percentage of oysters that developed as a female following 10 pg/g 2,3,7,8-TCDD exposure are shown in Table 4. The seawater control group had a higher percentage of oysters that developed as females (51%) compared to the percentage of oysters that developed as males (49%). The toluene (solvent control) group also had a higher percentage of oysters that developed as females (53%) compared to the percentage of oysters that developed as males (47%), and the 10 pg/g 2,3,7,8-TCDD treatment group had a lower percentage of oysters that developed as females (22%) compared to the percentage of oysters that developed as male (78%). The percent of oysters that developed into females in the control groups was significantly different from the 10 pg/g 2,3,7,8-TCDD group (ANOVA, $P<0.05$). There was also a significant difference between the gender of oysters in the 10 pg/g group (ANOVA, $P<0.001$).

3.4. Electron microscopy of gametogenesis during early stages of gonadogenesis following exposure to 2 and 10 pg/g 2,3,7,8-TCDD

To further examine the effects of 2,3,7,8-TCDD on early gametogenesis, gonadal tissues were evaluated using semi-thin and ultra-thin electron microscopy. Fig. 4 shows the EM photomicrographs of gametogenesis at days 7 and 14 in female and male oysters exposed to seawater, toluene (4 ng/g), 2, and 10 pg/g 2,3,7,8-TCDD. Fig. 4a and b shows the semi-thin section EM photomicrographs for the seawater control group ($N=5$ females, 5 males). The seawater control females (Fig. 4a) showed early stages of gametogenesis at day 7 denoted by the well defined and differentiated follicular development and the presence of pre-vitellogenic oocytes (early Stage I). Fig. 4b seawater control males also showed early stages of gonadal development at day 7 (Stage I) denoted by the well-differentiated tubules and the presence of early spermatogenic cells (Stage I).

Fig. 4c and d shows the semi-thin section EM photomicrograph for the toluene solvent control group ($N=5$ females, 5 males). The toluene control females (Fig. 4c) showed early stages of gametogenesis at day 7 similar to the seawater control group; however, the toluene females appeared to be more advanced in development (late Stage I) compared to the seawater control females (early Stage I) illustrated by the larger oocyte sizes and expanded follicles. Fig. 4d shows that the toluene males were also in an early stage of gonadal development similar to the seawater control males exhibiting well defined and expanded tubules. Spermatogenesis (Stage I) appeared to be comparable to the control males at day 7.

Table 4
The percent of oysters that developed as females in the seawater control group, toluene solvent control group (4 ng/g), and 10 pg/g 2,3,7,8-TCDD treatment group using the 28 days gametogenesis protocol

Treatment groups	# Females			# Males			%Females
	Day 1	Day 14	Day 28	Day 1	Day 14	Day 28	
Seawater control ($N=45$)	9	8	6	6	7	9	51 (**)
Toluene control (4 ng/g) ($N=45$)	6	9	9	9	6	6	53 (§)
10 pg/g TCDD* ($N=45$)	6	2	2	9	13	13	22 (** §)

Results were obtained in three independent studies. At each time point (days 1, 14, and 28), five oysters were randomly selected from each control and treatment group. Symbols indicate significant difference between percent females (ANOVA, $P<0.05$). Significant difference between percent females (ANOVA, $P<0.05$).

* Significant difference between 10 pg/g TCDD # females and # males over 28 days (ANOVA, $P<0.001$).

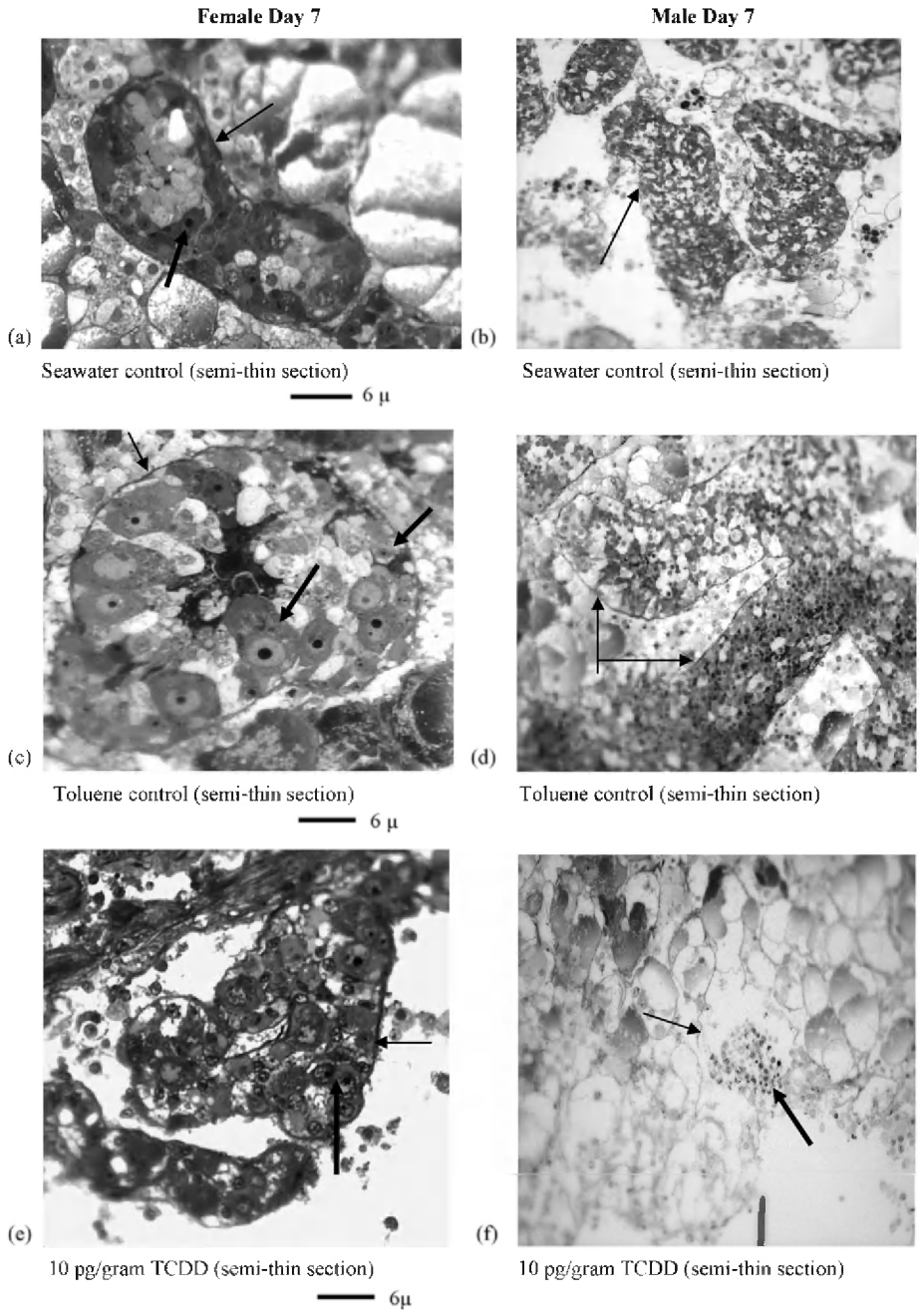

Fig. 4. Electron microscopy (EM) of gametogenesis at days 7 and 14 in the eastern oyster *Crassostrea virginica* showing treatment groups: seawater, toluene (4 ng/g), 2 and 10 pg/g 2,3,7,8-TCDD. Semi-thin sections: (a) a seawater control female and (b) male illustrating early stages of development (Stage I) at day 7. (c) A toluene solvent control female (Stage I/II) and (d) male (Stage I) illustrating early stages of development at day 7. (e) A 10 pg/g TCDD exposed female illustrating altered gonadogenesis and altered pre-vitellogenic oocyte development (Stage I) and (f) a male illustrating delayed gonadogenesis and early stages of spermatogonia development (Stage I) at day 7. Ultra-thin sections: (g) a seawater control female and (h) male illustrating oocyte and sperm maturation (Stage II) at day 14. An oocyte containing lipid granules in the cytoplasm (arrow) and a male tubule containing secondary spermatocytes (arrow). (i) A toluene solvent control female and (j) male illustrating early to late stages of development at day 14 (late Stage I). Oocytes contained few lipid granules in the cytoplasm (arrow) and in the male tubule: (1) spermatogonia with peri-nuclear vacuolization and (2) primary spermatocytes were present (Stage II). (k) A 2 pg/g TCDD exposed female and (l) male at day 14. A follicle with: (1) altered oocyte division and (2) development, and a (3) membranous swirl within the follicle. A male tubule with unsynchronized spermatogenesis (*i.e.* (1) sperm, (2) spermatid, (3) primary spermatocyte, and (4) spermatogonia) in same area of tubule and spermatocytes exhibiting (5) peri-nuclear vacuolization at day 14. Thin arrows indicate follicular and tubular development, thick arrows indicate oocyte and sperm developmental stages, and bar scales represent gamete sizes. Semi-thin sections were stained with toluidine blue and the ultra-thin sections were contrasted with uranyl acetate and lead citrate stains. Similar results were obtained in three independent studies.

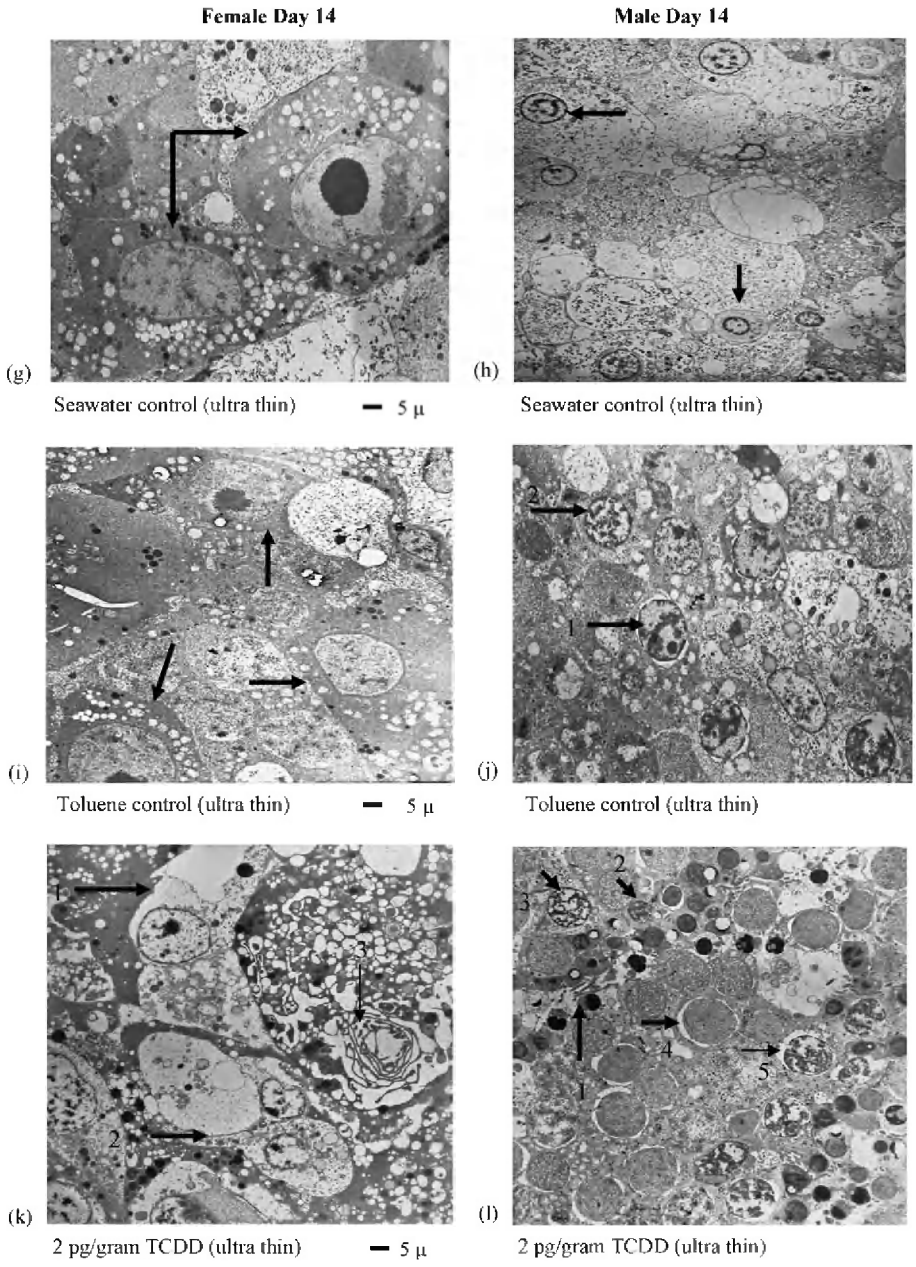

Fig. 4. (*Continued*).

Fig. 4e and f shows the semi-thin section EM photomicrograph for the 10 pg/g 2,3,7,8-TCDD group ($N = 10$ females, 10 males) at day 7. The 10 pg/g 2,3,7,8-TCDD-treated females (Fig. 4e) showed altered gonadal development denoted by the disorganization of the developing follicles and surrounding supportive gonadal tissue. The developing follicles were moderately defined; however, follicular organization and expansion were not comparable to that of the seawater and toluene-treated oysters. In each 10 pg/g 2,3,7,8-TCDD-treated female oyster examined ($N = 10$), oogenesis was not progressing in an organized matter with in the follicles. Pre-vitellogenic oocytes appeared to be undergoing altered development indicated by the lack of oocyte structure and definition, altered oocyte cleavage, and mitotic activity in many (>50%) of the follicles indicative of improper gametogenesis. Fig. 4f shows the 10 pg/g 2,3,7,8-TCDD exposed males with delayed gonadal development as indicated by the delay in branching tubules throughout the gonadal tissue at day 7. The tubules were not well defined and spermatogenesis was delayed compared to the seawater and toluene controls resembling the beginning stages of gametogenesis (early Stage I).

Fig. 4g and h shows the ultra-thin section EM photomicrograph for the seawater control group ($N = 5$ females, 5 males) at day 14. The seawater control females (Fig. 4g) showed signs of oocyte maturation at day 14. In the female gonads, the oocytes contained lipid granules in the cytoplasm indicative of the vitellogenic phase of oocyte development (Stage II). Fig. 4h shows the seawater control males also with stages of spermatogenic cell

maturation at day 14. In the male gonads, the tubules contained secondary spermatocytes evidence of advanced spermatogenesis (Stage II).

Fig. 4i and j shows the ultra-thin section EM photomicrograph for the toluene solvent control group ($N = 5$ females, 5 males) at day 14. The toluene exposed females (Fig. 4i) showed a slight delay in oocyte maturation at day 14 (late Stage I) compared to the seawater controls. The oocytes appeared to have less lipid accumulation compared to controls indicating an earlier stage of oocyte maturation at day 14 (early Stage II). Fig. 4j shows in the toluene-treated male gonads, spermatogonia were the majority (>50%) of sperm present indicative of early sperm development (late Stage I; early Stage II) compared to the seawater control group. In addition, the toluene male group also displayed peri-nuclear vacuolization around spermatogonia in the tubules at day 14 compared to the seawater control males.

Fig. 4k and l shows the ultra-thin section EM photomicrograph for the 2 pg/g 2,3,7,8-TCDD group ($N = 10$ females, 10 males). The 2 pg/g 2,3,7,8-TCDD exposed females and males showed delayed and altered gamete maturation at day 14. In Fig. 4k, the female gonads showed improper oogenesis indicated by altered cleavage resulting in oocytes with two nuclei and two nucleoli, and a significant alteration in oocyte sizes and shapes giving the appearance of disfigured oocytes (Stage II). In the male gonads (Fig. 4l), sperm development appeared comparable to the seawater and toluene controls as indicated by the well structured spermatogenic cells and advanced stages of spermatogenesis; however, several stages of spermatogenesis were observed within the same area, implying that development was not synchronized at day 14 (late Stage II). Peri-nuclear vacuolization was also observed around the spermatogonia similar to what was observed in the toluene treatment group.

The results of the EM photomicrographs illustrate that 2 and 10 pg/g 2,3,7,8-TCDD can interfere with gamete development early in gonadogenesis (days 1–7) according to the 28 days gametogenesis protocol. At day 7, the 10 pg/g 2,3,7,8-TCDD female and male oysters each exhibited altered and delayed gametogenesis with the females displaying an altered affect, histologically, in gamete development and organization compared to the 2,3,7,8-TCDD males at day 7 (Fig. 4e and f). The 2 pg/g 2,3,7,8-TCDD female and male oysters exhibited altered and unsynchronized gamete development at day 14. The female gonads exhibited altered oocyte cleavage resulting in improper oocyte growth and development, and the males displayed unsynchronized sperm development with peri-nuclear vacuolization around the developing spermatogonia (Fig. 4k and l). Oogenesis in the 2 pg/g 2,3,7,8-TCDD females appeared to the more adversely affected, histologically, compared to spermatogenesis in the 2 pg/g 2,3,7,8-TCDD male gonads at day 14 (Fig. 4k and l). This observation was similar in the 10 pg/g 2,3,7,8-TCDD females and 10 pg/g 2,3,7,8-TCDD males at day 7 (Fig. 4e and f).

The percentage of female and male oysters, which displayed specific morphological lesions at days 7 (Stage I) and 14 (Stage II) using EM, are shown in Table 5. The 10 pg/g 2,3,7,8-TCDD treated females did not show a significant difference in follicular and oocyte development compared to the seawater and toluene controls at day 7 (Table 5). The 10 pg/g 2,3,7,8-TCDD treated males at day 7 did not show a significant difference in tubule development and spermatogenesis compared to the seawater and toluene controls (Table 5). At day 14, the 2 pg/g 2,3,7,8-TCDD treated females were not significantly different in follicular development and oogenesis, and the males at day 14 did not have a significant difference in spermatogenesis and synchronized development compared to controls (Table 5).

4. Discussion

4.1. Gametogenesis protocol study

The 28 days gametogenesis protocol was developed and validated using untreated oysters and then tested using 2,3,7,8-TCDD exposed oysters. Studies were performed using the characterized 28 days gametogenic cycle with time points at days 1, 7, 14, and 28. Untreated oysters, as well as seawater and toluene exposed oysters, displayed similar gonadal and gametogenic development. At day 7 of gametogenesis, the gender of the oyster, as well as developmental stage (Stage I), could be identified using light microscopy. Day 14 was the peak of gonadal development; the developing gametes began to mature into primary oocytes in the female and mature sperm in the males. This stage (Stage II) could be identified best by using electron microscopy. Day 28 of the gametogenic cycle resulted in a fully developed gonad ready for spawning under normal, non-treatment conditions. This stage (Stage IIIa) could be identified by light or electron microscopy. Validating this protocol with untreated oysters showed that day 7 represented developmental Stage I, day 14 represented Stage II, and day 28 represented Stage IIIa. Typically, untreated female oysters were observed to be slightly more advanced in gonadal development at days 7 and 14 compared the male oysters under the same conditions; however, at day 28, both genders were fully matured. Testing this 28 days protocol using 2,3,7,8-TCDD exposed oysters showed that: (1) a total body dose of 2 and 10 pg/g 2,3,7,8-TCDD could cause abnormal gonadal development resulting in inhibition of gamete development and maturation by day 14, (2) morphological lesions in the gonads were permanent through day 28, and (3) morphological lesions were repeatable and proven consistent with 2,3,7,8-TCDD exposure in both female and male eastern oysters. This 28 days gametogenesis protocol can be used as a dependable and sensitive toxicological tool in evaluating reproductive toxicology in bivalve species.

4.2. The effects of 2,3,7,8-TCDD on gonadogenesis and gametogenesis in C. virginica

Studies using the 28 days gametogenesis protocol revealed that oogenesis in the oyster appeared to be more sensitive to 2,3,7,8-TCDD exposure compared to spermatogenesis when viewed using light microscopy (Fig. 2c, d, g, and h). Morphologically, oocyte development was easier to evaluate due to the rapid increase in oocyte sizes during maturation and their distinctive shapes. Although the stages of spermatogenesis in the male appeared to be delayed/inhibited as a result of

Table 5
Histological (EM) evaluation of morphological lesions during gonadogenesis in female and male oysters at days 7 and 14 exposed to seawater, toluene (4 ng/g), 2 and 10 pg/g 2,3,7,8-TCDD

EM	Day 7 (Stage I)	
	Female	Male
Seawater control, $N=5$ females, 5 males	Differentiated follicles, 100% Undifferentiated follicles, 0% Pre-vitellogenic oocytes, 100% Delayed development, 0%	Differentiated tubules, 100% Undifferentiated tubules, 0% Stage I (spermatogonia), 100% Delayed gonadogenesis, 0%
Toluene control (4 ng/g), $N=5$ females, 5 males	Differentiated follicles, 100% Undifferentiated follicles, 0% Pre-vitellogenic oocytes, 100% Delayed development, 0%	Differentiated tubules, 100% Undifferentiated tubules, 0% Stage I (spermatogonia), 100% Delayed gonadogenesis, 0%
10 pg/g TCDD, $N=10$ females, 10 males	Differentiated follicles, 0% Undifferentiated follicles, 100% Pre-vitellogenic oocytes, 100% Delayed development, 100%	Differentiated tubules, 0% Undifferentiated tubules, 100% Stage I (spermatogonia), 100% Delayed gonadogenesis, 100%
EM	Day 14 (Stage II)	
	Female	Male
Seawater control, $N=5$ females, 5 males	Differentiated follicles, 100% Vitellogenic oocytes, 100% Abnormal oocyte development, 0% Delayed development, 0%	Differentiated tubules, 100% Primary and secondary spermatocytes, 100% Unsynchronized sperm development, 0%
Toluene control (4 ng/g), $N=5$ females, 5 males	Differentiated follicles, 100% Vitellogenic oocytes, 100% Abnormal oocyte development, 0% Delayed development, 0%	Differentiated tubules, 100% Primary and secondary spermatocytes, 0% Unsynchronized sperm development, 0%
2 pg/g TCDD, $N=10$ females, 10 males	Differentiated follicles, 20% Vitellogenic oocytes, 0% Abnormal oocyte development, 100% Delayed development, 100%	Differentiated tubules, 100% Primary and secondary spermatocytes, 100% Unsynchronized sperm development, 100%

Results were obtained from three independent studies. Oysters were randomly sampled at days 7 and 14 from each control and treated group to obtain the same number of females and males per group. No significant difference between treatment groups within females or within males at day 7 or day 14 (ANOVA, $P>0.05$).

2,3,7,8-TCDD exposure, the developing tubules and sperm did not appear disfigured as with the oocytes. Upon viewing male gametogenesis using electron microscopy, it was evident that not only were spermatogenic cells morphologically altered, but sperm development was not synchronized. Peterson et al. (1993) reported that structural malformations in the male reproductive system were not common in species; however, functional alterations such as in Leydig cell function, daily sperm production, and sperm maturation were sensitive signs of developmental toxicity. There are reports of altered sperm maturation, germ cell vacuolization, sperm phagocytosis, and inhibition of spermatogenesis occurring in mammalian male species exposed to 2,3,7,8-TCDD, in agreement with the findings in the male oysters of this study (Rune et al., 1991; Johnson et al., 1992; Mably et al., 1992; Bjerke and Peterson, 1994; Sommer et al., 1996; Gray et al., 1997; Moon et al., 2004).

These studies have shown using both light microscopy and electron microscopy that the morphological lesions in the 2,3,7,8-TCDD-treated groups occur as early as day 7 of gonadogenesis and gametogenesis and persist through day 14. Our studies have shown that at day 7 the females exposed to 2,3,7,8-TCDD (2 and 10 pg/g) had a significant delay in gonadal development and oocyte growth as well as macrophage infiltration. These findings are comparable to reports of undifferentiated gonads, decreased oocyte number, and reduced oocyte viability in fish and other invertebrate species exposed to 2,3,7,8-TCDD (Isensee and Jones, 1975; Brown, 1991; Birnbaum and Tusmisto, 2000; Toomey et al., 2001; Giesy et al., 2002; Wintermyer and Cooper, 2003; Butler et al., 2004; Wintermyer et al., 2005). In the 2,3,7,8-TCDD exposed males at day 14, there was a significant delay in gonadal development and spermatogenesis, increased macrophage infiltration, and enlarged intercellular spaces between the germinal epithelium and the spermatogenic cells. Rune et al. (1991) reported that in the marmoset (*Callithrx jacchus*), there was a decrease in the intercellular contact in the germinal epithelium, as indicated by the enlarged intercellular spaces between the Sertoli's cells and the spermatogonia after 2,3,7,8-TCDD treatment. They also reported that there was an accumulation of premature spermatocytes and spermatids in the tubular lumen as a result of decreased intercellular contact and an inhibition of sperm maturation caused by 2,3,7,8-TCDD treatment (Rune et al., 1991). The observations of enlarged intercellular spaces with in the tubules and unsynchronized spermatogenesis in the monkey are similar to our findings in

the male oysters exposed to 2,3,7,8-TCDD; however, a more in depth evaluation regarding the decrease in the spermatogonia population or a reduction in intercellular contact between spermatogenic cells is needed.

Electron microscopy allowed a closer evaluation of the morphological lesion in the female and male oysters exposed to 2,3,7,8-TCDD. These studies showed that *in vivo* exposure to 2,3,7,8-TCDD prior to gonadogenesis could result in undifferentiated follicles and tubules, delayed gonadal development, and abnormal gamete development in both female and male oysters. For example, it was apparent in the 10 pg/g 2,3,7,8-TCDD female oysters at day 7 that the pre-vitellogenic oocytes were not undergoing proper growth and development as illustrated by their sizes, shapes, and orientation with in the developing follicles compared to controls (Fig. 4e). The male oysters exposed to 10 pg/g 2,3,7,8-TCDD were altered compared to the controls in terms of tubular branching and expansion, and spermatogenesis. It was also apparent from the EM photomicrographs that spermatogenesis was delayed in the 10 pg/g 2,3,7,8-TCDD males compared to controls (Fig. 4f). At day 14, there was an adverse difference in gonadal development as well as oocyte growth and maturation in the 2 pg/g 2,3,7,8-TCDD females indicated by the altered oocyte cleavage and an alteration in oocyte sizes and shapes. Electron microscopy allowed for a more in depth look at spermatogenesis in the 2 pg/g 2,3,7,8-TCDD treated group. In the males, there was a difference in synchronized sperm development compared to controls. It was observed in all males in the 2 pg/g 2,3,7,8-TCDD groups that various stages of spermatogenesis were within the same area, implying that development was not synchronized. This resulted in multiple stages of spermatogenesis throughout the tubules at day 14 (Fig. 4l).

The effects of 2,3,7,8-TCDD during stages of oyster gametogenesis were characterized using LM and EM histological evaluations. The results showed that gonadal development as well as gametogenesis was adversely affected early in development (day 7) and that over time (day 28), there is no recovery of gonadogenesis or gametogenesis to early 2,3,7,8-TCDD insult. Improper gonadal and gamete development such as observed in the 2 and 10 pg/g 2,3,7,8-TCDD-treatment groups would result in early phagocytosis and resorption of gonadal and gamete material. This was evident in our studies by a significant increase in the macrophage infiltration in to the gonads of the 10 pg/g 2,3,7,8-TCDD treated females at days 7 and 28. In a study where 2,3,7,8-TCDD (10 pg/g) exposed female oysters were strip spawned on days 14 and 28, respectively, and the oocytes collected, results showed that oocytes were adversely affected indicated by membrane blebbing, irregular oocyte shapes and sizes, and irregular interior lipid localization. At day 28, oocyte resorption was evident due to observed degradation of oocytes (Wintermyer, 2004). Gamete resorption early in gonadogenesis is a classic sign of toxicant insult in invertebrates (Bayne, 1972; Vashchenko and Zhadan, 1993). Phagocytosis and resorption of gonadal tissue and gametes is a natural phenomenon in bivalves that occurs in both females and males at the conclusion of the spawning season. Complete gonadal and gamete resorption is repeated seasonally and recrudescence involves formation of a new gonad prior to the formation of gametes (Loosanoff, 1942; Galtsoff, 1964; Kennedy and Battle, 1964). In the male oysters, unsynchronized sperm development was observed in the 2 and 10 pg/g 2,3,7,8-TCDD groups. This would pose a problem during external fertilization, since both mature and immature sperm would be released, resulting in decreased egg fertilization. This would pose effects on the natural oyster population, as a result of reproductive stress.

4.3. Female gonadogenesis and gametogenesis over 28 days using 10 pg/g 2,3,7,8-TCDD study

This study was performed using 2,3,7,8-TCDD as a "proof of principle" (*i.e.* positive control) for the impacts of low-level, chronic exposure on female gametogenesis. Recent studies have shown that dioxin has a significant adverse effect on developing female reproductive systems in both mammals and non-mammal species (Chaffin et al., 1996; Gray et al., 1997; Giesy et al., 2002; Wintermyer and Cooper, 2003; Miller et al., 2004). In fish and bivalves, dioxin was shown to be prevalent in gonadal tissue and oocytes and to significantly decrease oocyte viability as a result of dioxin exposure before and during spawning (Giesy et al., 2002; Wintermyer and Cooper, 2003; Butler et al., 2004). This study is in agreement with other studies reporting TCDD reduced reproductive capabilities in females (Gray and Ostby, 1995; Chaffin et al., 1996; Gray et al., 1997; Salisbury and Marcinkiewicz, 2002; Miller et al., 2004). The gonadal and oocyte development of female oysters exposed to 10 pg/g 2,3,7,8-TCDD clearly showed inhibited growth and maturation in pre-vitellogenic oocytes at day 14 (Fig. 3h). Also, in the 10 pg/g 2,3,7,8-TCDD group, gonadogenesis and gametogenesis at day 28 was very similar, histologically, to day 14 showing lack of gonadal recovery and complete inhibition of gametogenesis (Fig. 3i). Oocytes of dioxin exposed oysters were small, compact, and densely stained at days 14 and 28 due to very little vitellogenin deposition, which was shown histologically (LM and EM) and proven biochemically using alkaline-liable phosphate (ALP) (Wintermyer, 2004). All studies using 10 pg/g 2,3,7,8-TCDD resulted in the same oocyte morphology by day 14 of the 28 days gametogenesis protocol. A slight delay in early gonadal and oocyte development in the toluene solvent control groups was observed. This slight delay was the result of a toluene solvent effect (days 1–14) and is a consequence of membrane disruption during gonadal development (Magnusson et al., 1998; Garbe and Yukawa, 2001). In all studies using toluene as the solvent control, both female and male oysters reached gamete maturity by day 28.

Oysters exposed to 2,3,7,8-TCDD prior to gametogenesis resulted in a higher number of oysters that developed as males. The seawater control group and the toluene solvent control group had a sex ratio of approximately 50:50 (females:males) with 51% females:49% males in the seawater group and 53% females:47% males in the toluene group. The 10 pg/g 2,3,7,8-TCDD treatment group had a sex ratio of 22% females:78% males (Table 4). The decrease or depletion in biochemical reserves (*i.e.* proteins, lipids, carbohydrates, and glycogen,) required for proper gametogenesis may have contributed to the increase in the male ratio

observed in the 10 pg/g 2,3,7,8-TCDD group. Invertebrate studies have shown that due to nutritional stress, wound repair, and environmental stressors such as pollution, biochemical reserves will go toward survival and; therefore, result in an alteration in the sex ratio toward males due to the high energy cost of producing oocytes (Coe, 1932; Tranter, 1958; Bahr and Hillman, 1967; Davis and Hillman, 1971; Russell-Hunter, 1979; Capuzzo McDowell, 1996). This bivalve model can be used to confirm that 2,3,7,8-TCDD, at low environmental doses, can adversely alter oocyte development and maturation as well as alter the sex ratio by using histopathology (LM).

5. Summary

The results presented in this study provide evidence that the oyster can be used as a sensitive toxicological tool in evaluating reproductive toxicity. We have shown through laboratory studies that gonadal development as well as egg viability is significantly reduced as a result of 2,3,7,8-TCDD exposure prior to and during gametogenesis. The dogma in literature has been that invertebrates have little to no adverse effects to 2,3,7,8-TCDD exposure. This assumption is based on several factors: (1) invertebrate species lack an Ah receptor like that observed in mammals and fish (Denison et al., 1985, 1986; Hahn et al., 1994), (2) lack of low-dose, chronic exposure studies evaluating reproductive toxicity in bivalves or other invertebrate species (Cooper, 1989), and (3) the lack of structural malformations in invertebrate species exposed to 2,3,7,8-TCDD (Peterson et al., 1993; West et al., 1997).

There are several hypotheses relating to how 2,3,7,8-TCDD adversely affects gametogenesis in the literature. The principal hypothesis is that 2,3,7,8-TCDD disrupts the cell cycle causing alterations in cell division and ultimately development (*i.e.* differentiation) through inhibition of various cell cycle factors and cell cycle phases (Weber et al., 1997; Puga et al., 2000; Oikawa et al., 2001; Buchanan et al., 2002). Although cell cycle factors have not been studied in the oyster, it is likely that they are highly conserved and would be affected in a similar fashion to these observed in amphibian and mammalian systems. 2,3,7,8-TCDD toxicity is mediated through the AhR/ARNT receptor complex in mammals; however, in lieu of the present evidence that bivalves do not have an Ah receptor similar to mammals or fish, it has been suggested that the changes in reproductive tissue due to inhibition of cell differentiation and/or development is by an AhR-independent mechanism (Lin et al., 2001; Butler et al., 2004; Wintermyer et al., 2005). In the mammalian system, there are proteins that are multi-functional and can be used to aid in mechanisms unrelated to their intended physiological purpose; for example, cytokines, peptides, lipoprotein receptors, xenobiotics receptors (*i.e.* nuclear receptors), and the Ah receptor (Ma and Whitlock, 1996; Weib et al., 1996; Kornmann et al., 1999; Herz et al., 2000; Herz and Bock, 2002; DiCicco-Bloom et al., 2004; Puga et al., 2000). It is not unreasonable to consider that a receptor (*i.e.* xenobiotic receptor) or perhaps a protein (*i.e.* heat shock protein) may be capable of mediating the toxic effects of 2,3,7,8-TCDD in a region-and stage-specific role during gonadal development.

Based both on our findings and the reports in the literature, we propose that the oyster is a useful model to use for studying the effects of 2,3,7,8-TCDD on gametogenesis. We base our reasoning on five factors: (1) the model is sensitive to environmentally relevant low levels of dioxin (2–10 pg/g TCDD), (2) the model has displayed the same gonadal lesions as a result of dioxin exposure both in field studies and in the laboratory (Wintermyer and Cooper, 2003; Wintermyer, 2004), (3) gametogenesis in the oyster can be induced repeatedly and without chemical manipulation (Davis and Chanley, 1956; Loosanoff and Davis, 1963), (4) the model is reliable in terms of the histopathological alterations displayed, and (5) toxicity test manuals and protocols for oyster maintenance/care are well documented (Galtsoff, 1964; ASTM, 1994). Future advancements in molecular techniques critical for invertebrate research will enable this bivalve to be used both as a sentinel organism and an essential laboratory-based model for the effects of toxicants on gametogenesis.

6. Conclusion

In conclusion, these studies were designed to undertake two goals: (1) test the bivalve model using low-level, sub-chronic exposures of 2,3,7,8-TCDD and (2) to use histopathological evaluations to characterize the effects on gamete development during selected stages of oyster gametogenesis. The sampling time points for histologically evaluating gametogenesis were days 7, 14, and 28. The advantage of using the oyster model for reproductive studies is that gametogenesis can be monitored repeatedly from the very early stages of primordial germ cell division to a fully developed gonad in a short period of time (*i.e.* 7 days; Loosanoff and Davis, 1963). Our protocol for oyster gametogenesis was 28 days based on standard bioaccumulation methods for aquatic and terrestrial monitoring, sub-chronic testing and metabolism studies, an oyster pharmacokinetic model, and standard safety assessment protocols (Brown, 1991; Bergen et al., 1993; Eaton and Klaassen, 1996; Johnson, 2001; Mussel, 2003; Steensma et al., 2004; Yu et al., 2004; Wintermyer et al., 2005). The levels of 2,3,7,8-TCDD used in this research (2–10 pg/g) were within the environmental range of sediments and bivalve tissue body burdens (Newark Bay, NJ; 11–20 pg/g) (NJEPA, 1993; NJDEP, 1996).

The effects of 2,3,7,8-TCDD on oyster reproduction occur early in gonadogenesis and persist throughout the 28 days gametogenic cycle. 2,3,7,8-TCDD at 2 pg/g showed delayed and altered gamete development and maturation, and 10 pg/g 2,3,7,8-TCDD showed inhibition of gametogenesis by days 14–28. The data presented suggest that the eastern oyster can be used as a toxicological model for evaluating gametogenesis by utilizing the characterized 28 days gametogenic cycle in conjunction with histopathological evaluations to describe the effects on gamete development. This model can be used to: (1) examine early to late stages of gametogenesis by using LM and/or EM techniques and (2) the model can be used for both field studies and controlled laboratory studies. By incorporating the characterized 28 days gametogenic cycle as an endpoint for reproductive toxicity in invertebrates, the usefulness of the oyster as an aquatic model is markedly expanded.

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