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Toxicity of *o,p'*-DDE to medaka d-rR strain after a one-time embryonic exposure by in ovo nanoinjection: an early through juvenile life cycle assessment

Sergio A. Villalobos [a,1], Diana M. Papoulias [b], Stephanie D. Pastva [c], Alan L. Blankenship [a,d], John Meadows [b], Donald E. Tillitt [b], John P. Giesy [a,*,2]

[a] *Department of Zoology, National Food Safety and Toxicology Center, Institute of Environmental Toxicology, Michigan State University, 203 Natural Science Boulevard, East Lansing, MI 48824, USA*

[b] *Environmental and Contaminants Research Center, Biological Resources Division, USGS, 4200 New Haven Road, Columbia, MO 65201, USA*

[c] *Department of Fisheries and Wildlife, National Food Safety and Toxicology Center, Institute of Environmental Toxicology, Michigan State University, East Lansing, MI 48824, USA*

[d] *ENTRIX, Inc., East Lansing, MI 48823, USA*

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Abstract

The toxicity of *o,p'*-DDE (1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl) ethylene) was evaluated in embryos of medaka (*Oryzias latipes*) following a one time exposure via nanoinjection. Medaka eggs (early gastrula) were injected with 0.5 nl of triolein (vehicle control) or 0.5 nl of 4 graded doses (0.0005–0.5 ng/egg) of *o,p'*-DDE in triolein. Embryos were allowed to develop, and fry were reared. Embryonic survival was monitored daily during the first 10 d until hatching and thereafter, on a weekly basis until day 59, at which time the fish were monitored for sexual maturity until day 107. In general, *o,p'*-DDE caused a dose- and time-dependent mortality. No changes in mortality were observed between the last two time points (day 38 and 59, respectively), and hence a 59 day-LD_{50} of 346 ng *o,p'*-DDE/egg was derived from the linear dose–response relationship. Prior to late stage death, only isolated cases of cardiovascular lesions and spinal deformities were observed, but were not dose-dependent. The lowest observable adverse effect level (LOAEL), based on upper 95% CI for regression line = 0.0018 mg/kg, and the LOAEL based on exposure doses = 0.5 mg/kg. Likewise, the no observable adverse effect level (NOAEL) based on linear extrapolation to 100% survival = 0.0000388 mg/kg, while the NOAEL based on exposure doses = 0.05 mg/kg. The nanoinjection medaka model has potential in the study of hormonally active compounds in the environment.

Keywords: *o,p'*-DDE; Medaka; Nanoinjection; Maternal transfer model; Weak estrogen

* Corresponding author. Address: Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China. Tel.: +852-2788-8984; fax: +852-2788-7406.

E-mail address: jgiesy@aol.com (J.P. Giesy).

[1] Present address: Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419, USA.

[2] Tel.: +1-517-432-6333; fax: +1-517-432-2310.

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1. Introduction

Recent concerns over the potential effects of chemicals with the capability to modulate the endocrine system has resulted in the need to develop and validate high volume throughput screening tests for wildlife, including oviparous organisms, such as fish (Kavlock et al., 1996; Ankley et al., 1998). This need is underscored by recent legislation mandating that chemicals and formulations be screened for potential estrogenic activity before they are manufactured or used in certain processes (Safe Drinking Water Act Amendments of 1995—Bill Number S.1316; Food Quality Protection Act of 1996—Bill Number P.L. 104–170). As a result of this legislation, the Endocrine Disruptor Screening and Testing Committee (EDSTAC) was established to suggest a screening procedure. Subsequently, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has been overseeing a program to evaluate these proposed methods. Because it has been suggested that exposures of organisms during critical stages of development can result in permanent adverse effects, the present study was conducted to evaluate in ovo exposure by nanoinjection as an alternative to longer-term exposures.

Like other organochlorine insecticides and their by-products, DDT (1,1-dichlorodiphenyltrichloroethane) and its metabolites are ubiquitous and persistent (Tanabe et al., 1982; Rapaport et al., 1985). Technical grade DDT is made up of roughly 65–73% *p,p'*-DDT and 19–21% *o,p'*-DDT (Crosby, 1998). Once in the environment, weathering of technical grade DDT over time leads to a shift in relative abundance among metabolites, predominantly *p,p'*-DDE. *o,p'*-DDE (1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl) ethylene) is one of several metabolites of the insecticide DDT still found in fish (Kitzman, 1993) and associated biota, particularly near contaminated sites (Spies and Thomas, 1997) but also in remote locations of the world (Kannan et al., 1995). In juvenile rainbow trout (*Oncorhynchus mykiss*), *o,p'*-DDE is weakly estrogenic, as evidenced by induction of plasma vitellogenin and hepatic estrogen binding sites in males (Donohoe and Curtis, 1996). Recently, sex reversal was successfully induced by embryonic nanoinjection of *o,p'*-DDT in medaka eggs, causing permanent and functional changes at doses of 227 ng/egg (Edmunds et al., 2000). Furthermore, fertility and hatching success were more sensitive to estrogenic effects of waterborne *o,p'*-DDT in medaka, than were other typical biomarkers such as vitellogenin and gonadal differentiation (Cheek et al., 2001).

Although extensive work has been conducted on the toxicity of technical mixtures of DDT in general (Crosby, 1998), most of the emphasis on aquatic life has focused on *p,p'*-DDE, the most prevalent metabolite that is bioaccumulated (Schmitt et al., 1990; Kitzman, 1993; Kannan et al., 1995; Spies and Thomas, 1997; Crosby, 1998). *p,p'*-DDE may be the major metabolite responsible for eggshell thinning in birds and associated effects on hatching success and productivity (Blus, 1996), and antiandrogenic activity, including impairment of the development of secondary sexual development of male guppies (*Poecilia reticulate*) (Bayley et al., 2002). In comparison to *p,p'*-DDE, which may account for up to 97% of liver tissue concentrations of the total DDT in feral populations of female kelp bass (*Paralabrax clathratus*) from polluted sites (Spies and Thomas, 1997), *o,p'*-DDE normally accounts for <10% of the total DDE residues found in biota (Pereira et al., 1996; Senthilkumar et al., 1999). However, DDE is recalcitrant and does not easily degrade in the environment (Pereira et al., 1996). To be able to interpret the potential effects of *o,p'*-DDE as an estrogenic compound in complex environmental mixtures of other estrogen agonists, it is necessary to know the relative potency of *o,p'*-DDE to cause effects on fish. Only then can the changing absolute and relative concentrations of the various components from the DDT mixture, including metabolites, be interpreted.

Because little information on the toxicity of *o,p'*-DDE to fish is available, an experiment divided into two parts was designed to assess the long-term effects of this compound on medaka after a one-time exposure at an early embryonic stage simulating maternal transfer of contaminant. In the first part, the objective was to assess the toxicity of *o,p'*-DDE at the embryonic, larval, juvenile and pre-adult stages of medaka. The validity of the nanoinjection technique for rapid screening to predict adverse effects that would be observed in longer-term exposures through different vectors of exposure, was also assessed by comparing LD_{50}, no observable adverse effect level (NOAEL), and lowest observable adverse effect level (LOAEL) values derived from other studies. Additionally, the thresholds for effects observed in the nanoinjection studies were compared with environmental concentrations of *o,p'*-DDE and *o,p'*-DDT. The second part, assessment of effects at sexual maturity on the same tested population of medaka has been presented elsewhere (Papoulias et al., 2003).

2. Methods

Embryos were reared and prepared for injection in the laboratories of the USGS Columbia Environmental Research Center (Columbia, MO) as described in Papoulias et al. (2000) and Villalobos et al. (2000). The term "egg" will be used in the context of this paper in lieu of the more proper term "embryo", in order to facilitate uniformity in doses and endpoint calculations (i.e. LD_{50}) derived from injecting the fertilized egg. The average weight of medaka eggs is 0.88–1 mg, ww (Elo-

nen et al., 1998). For calculations of approximate doses of *o,p'*-DDE, the weight of medaka eggs was assumed to be 1 mg. *o,p'*-DDE (99.8 % pure, Chem Service, Westchester, PA); was dissolved in methylene chloride to the appropriate serially diluted concentrations of 0.001, 0.01, 0.1, or 1 ng/nl. Then, the appropriate volume of triolein (95% pure, Sigma Chemical, St Louis, MO) was filter sterilized (22 mm cellulose acetate filters, 0.22 μm, Corning Glass, Corning, NY) and then added to vials containing *o,p'*-DDE. Finally, methylene chloride was removed by gentle nitrogen-evaporation and heating. Each egg received a total of 0.5 nl dosing solution resulting in doses of 0.0005, 0.005, 0.05, or 0.5 ng *o,p'*-DDE/egg. Controls consisted of eggs injected with 0.5 nl of triolein, or of uninjected eggs, which in addition served as a reference for embryonic and newly hatched development, as well as an indicator of broodstock health and quality. A total of 36 embryos were injected with each dose. At the time of injection no gender staging of the embryos was performed, since it was not possible to discern sexes. Injected embryos were monitored for 24 h to determine viability and then placed into 150-ml Pyrex side-arm test tubes for rearing as described in Papoulias et al. (2000). There was one side-arm test tube per treatment (no replication). Methods for nanoinjection and post-exposure care of eggs until hatching has been described in Villalobos et al. (2000), except for the following steps. Following the nanoinjection (day 0), survival was monitored on a daily basis for the first 10 day, and thereafter at weekly intervals. During the time of embryonic development (days 0–10) no numerical scoring was performed. Rearing of newly hatched larvae was as described in Papoulias et al. (2000). Fish were kept for a total of 107 day. As stated earlier, the present assessment only accounts for the first 2 month of life, the period of development until juvenile maturation.

The 59-day LD_{50} was calculated using the moving average method (ToxCalc, 1984) from cumulative mortalities using probit analyses. Significance levels were set at $p < 0.05$. Correction of background mortalities was done according to Abbott (Abbot, 1925) to normalize mortality to that observed in injected controls. Each embryo was considered its own replicate since they were all kept together in the same vial following the injection. The lowest observable effect level (LOEL), was calculated as the point where the upper 95% confidence limit of the log-linear least-squares regression (PlotIT, 1994) intersected the concentration axis (Chevre et al., 2002) (Fig. 1).

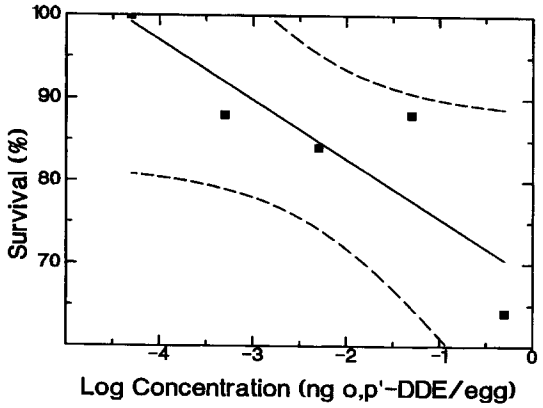

Fig. 1. Survival over time (from embryonic to 59 day) of medaka after a one-time in ovo nanoinjection to *o,p'*-DDE doses of 0.0005–0.5 ng/egg. Mortality for either day 38 or 59 could be predicted by the following equation: Mortality = −7.188[log dose] + 68.29.

3. Results

There was a dose-dependent increase in mortality relative to the triolein control that was statistically significant. Survival of triolein control fish was 69.4%, and that of uninjected controls was 83.3 % (Table 1), indicating healthy broodstock. Survival of all treatment groups remained the same for the last two time points, day 38 and 59, respectively, during the 59-day

Table 1
Percent survival over time in medaka following in ovo exposure to *o,p'*-DDE at early embryonic stage

	Time (days) Developmental landmarks										
	Embryonic stages						Hatching, sex differentiation, growth				
o,p'-DDE (ng/egg)	0	1	2	3	8	10	17	24	31	38	59
Uninjected	100	97.2	97.2	97.2	97.2	83.3	83.3	83.3	83.3	83.3	83.3
Triolein	100	88.9	77.8	77.8	75.0	75	72.2	72.2	72.2	69.4	69.4
0.0005	100	72.2	72.2	69.4	69.4	61.1	61.1	61.1	61.1	61.1	61.1
0.005	100	77.8	69.4	66.7	66.7	58.3	58.3	58.3	58.3	58.3	58.3
0.05	100	86.1	80.6	80.6	80.6	80.6	63.9	63.9	61.1	61.1	61.1
0.5	100	69.4	63.9	63.9	63.9	63.9	50	44.4	44.4	44.4	44.4

experimental period. Mortality for either day 38 or 59 could be predicted by the following equation (Fig. 1):

$$\text{Mortality} = -7.188[\log \text{dose}] + 68.29 \quad (R^2 = 0.756); \quad p < 0.001. \tag{1}$$

From this equation a 59-day LD_{50} of 346 ng/egg or 346 mg/kg, ww, was derived. The LOAEL, based on upper 95% CI for the regression line was 0.0018 mg/kg (Type I model), and the LOAEL based on individual doses was 0.5 mg/kg (Type II model). Likewise, the NOAEL based on linear extrapolation to 100% survival was 0.0000388 mg/kg, while the NOAEL based on individual doses was 0.05 mg/kg.

3.1. Embryonic period (days 1–10)

Mortality, particularly during later stages of development, days 8–10, was the predominant effect. Until day 10, sham control survival was 75%, and that of uninjected controls was 83% (Table 1). Morphological lesions preceding death included isolated cases of hemorrhaging and other cardiovascular lesions in embryos exposed to the greatest and least doses, whereas the mid-level doses caused isolated spinal deformities only. These spinal lesions became distinctive only after hatching. The late mortalities occurred in the group treated with 5 ng/egg on days 9–10 after dosing, a time when the majority of embryos had already hatched. Most of the dead organisms were found autolysed and, in several cases, fragmented at different parts.

3.2. Larval and juvenile period (days 11–59)

Two weeks following hatching, the majority of mortalities were observed only at the two greater doses. After that, the only change in mortality was observed in the triolein treatment, on day 38 by the death of one larva, after which no other changes in mortality were noted. No additional mortalities were observed in the uninjected controls (Table 1).

4. Discussion

Given the limited toxicological data for *o,p'*-DDE, one objective of this study was to assess the toxicity of *o,p'*-DDE to the developing medaka by comparing the LD_{50}, NOAEL, and LOAEL values obtained herein with environmental concentrations or similar exposures from the scientific literature (Fig. 2).

The LD_{50} value for *o,p'*-DDE reported herein (346 ng/egg) is similar to that of nanoinjected *o,p'*-DDT ($LD_{50} = 511 \pm 22$ ng/egg or ppm) (Edmunds et al., 2000), the only other similar study that has been conducted on medaka. This suggests that the toxic potencies

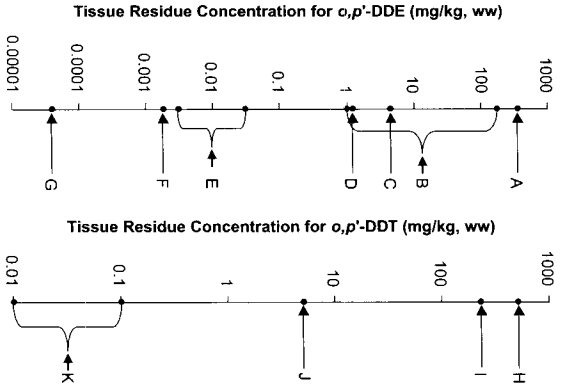

Fig. 2. Tissue residue concentrations (mg/kg, ww) of *o,p'*-DDE and *o,p'*-DDT for medaka and other reported fish species. (A) 59-day LD_{50} ELS nanoinjected medaka, 346 ng/egg = 346 mg/kg, w/w (this study). (B) 1–160 mg/kg dose range causing significant mortalities and possible adverse gonadal effects in injected eyed embryos of chinook salmon and rainbow trout (Carlson et al., 2000). (C) Significant reduction in fecundity and survival of rainbow trout, 4.3 mg/kg tissue residue (Dacre and Scott, 1971). (D) Historical levels in rainbow trout tissues in New Zealand, 1.19 mg/kg (Dacre and Scott, 1971; Edmunds et al., 2000). (E) Range of measured residues in Lake Michigan salmonids eggs, 3–30 μg/kg, w/w (Giesy et al., 1986). (F) LOAEL ELS nanoinjected medaka, 0.0018 mg/kg, w/w (this study). (G) NOAEL ELS nanoinjected medaka, 0.0000388 mg/kg w/w (this study). (H) LD_{50} (no time line given) ELS nanoinjected medaka, 511 ng/egg = 511 mg/kg w/w (Edmunds et al., 2000). (I) ~EC_{80} for sexual reversal ELS nanoinjected medaka, 227 mg/kg, w/w (Edmunds et al., 2000). (J) Ovarian concentration in gonadal Atlantic croaker, 5 mg/kg, w/w (Ungerer and Thomas, 1996). (K) Range of reported developmental abnormalities, including death, for many ELS of fish (Smith and Cole, 1973; Dethlefsen, 1977; Von Westernhagen, 1988).

of *o,p'*-DDE and DDT are similar for this species. Minor differences in the experimental and statistical methods between these two studies, however, may preclude a direct comparison. First, the doses of *o,p'*-DDT and volumes of toxicant in triolein injected chosen by Edmunds et al. (2000), were greater (20, 35, 225, 580, 1000 ng/egg with a volume of 1–3 nl/egg) than those used here. For *o,p'*-DDE, doses ranged from 0.0005 to 0.5 ng/egg, at a 10-fold dilution between one dose and the next greater dose, with a fixed volume of 0.5 nl/egg triolein. Second, their LD_{50} was calculated by the least-squares regression method, and it is unknown whether they subtracted the control injected mortality from the treatment related ones. On the other hand in this study, the calculated LD_{50} was extrapolated beyond the experimental range of doses, after using Abbott's correction factor for sham mortality, leading to uncertainty in this value. However, when the Abbott's factor was applied to their data, the corrected LD_{50} value was still near 580 ng/egg. Third, their embryos were injected in

the yolk sac, whereas the ones used here were injected in the oil droplet, a compartment where the likelihood of toxicant's loss is minimized; in some cases the authors were not sure of leakage into the perivitelline space, which is something we did not observe. Edmunds et al. (2000) however, were confident that the material stayed in the embryo since recoveries were within 80%. Despite these differences in our methods, our corrected *o,p'*-DDE LD_{50} value (346 ppm) was slightly less than that of Edmunds et al. (2000) for *o,p'*-DDT; our estimated LOAEL and NOAEL values are 0.0018 ng/egg (1.8 ppb) and 0.000039 ng/egg, or 39 pptr, respectively. Our results are however, based on linear extrapolation and may overestimate toxicity (underestimate the concentration associated with the NOAEL). Edmunds et al. (2000) did not estimate *o,p'*-DDT LOAEL or NOAEL values, although they indicated that 227 ± 22 ng/egg caused permanent and functional sex reversal in 86% (six out of seven) of the genetic males nanoinjected. When Carlson et al. (2000) injected eyed embryos of chinook salmon and rainbow trout with doses between 1 and 160 mg *o,p'*-DDE/kg they observed mortality at first feeding of 23–57% and 13–75% for each species, respectively, perhaps indicating that medaka are less sensitive (considering the correction factors) to the toxicity of *o,p'*-DDE than these salmonids.

When comparing effects of *o,p'*-DDE observed in the early to pre-adult stages up to 59 day (this work) versus those on sexual maturity up to 107 day (Papoulias et al., 2003), there appeared to be no early signs that were predictive of effects on later stages, including sexual development in the medaka. Abnormal effects in male and female gonads, suggesting possible impairment of reproduction at doses less than 0.5 ng/egg (= 0.5 mg/kg, our LOAEL), were reported. Similar conclusions were obtained for chinook salmon (*O. tshawytscha*) and rainbow trout 6 month after being microinjected with *o,p'*-DDE at doses between 1 and 160 mg/kg w/w (Carlson et al., 2000). However, medaka larvae exposed to flow-through waterborne measured concentrations of *o,p'*-DDT between 2 and 4 μg/l showed a female-skewed sex ratio, with longer exposures (8 week, not 2 week) causing ovo-testis in up to 10% of the surviving population (Cheek et al., 2001), a value similar to the LOEC (5 μg/l) reported by Metcalfe et al. (2000), for waterborne *o,p'*-DDT in medaka. Thus, while earlier stages of development may be a more sensitive life stage than adults, early signs related to sex differentiation may be too subtle to detect until later stages of development, at least in medaka.

Concentrations of *o,p'*-DDE found to be toxic in medaka eggs in the present investigation were greater than the range of reported concentrations for *o,p'*-isomers of DDT and DDE in biota, associated with deleterious effects (Fig. 2). In eggs of salmonid fishes from Lake Michigan, measured concentrations of *o,p'*-DDE ranged from 3 to 30 μg/kg (ww) (Giesy et al., 1986). Historical levels of *o,p'*-DDE in rainbow trout tissue were 1.19 ppm (mg/kg w/w), representing approximately 25% of total DDT analyzed-*o,p'*-DDT, *o,p'*-DDE, and *p,p'*-DDE (Dacre and Scott, 1971). The majority of studies that have reported reproductive effects are based on total DDTs, generally from environmental mixtures. For example, significant effects on the numbers of rainbow trout eggs produced by a female, and subsequent survival of fertilized eggs, were observed when the concentrations of DDT reached levels of 4.36 ppm (mg/kg w/w). The relative proportion of *o,p'*-DDE in this mixture was 25% (Dacre and Scott, 1971). Tissue residues (sum of DDTs, including metabolites) of 1.27 mg/g w/w were present in rainbow trout (Hopkins et al., 1969), representing approximately 25% of the total DDT. In a separate study, survival of progeny of adult coho salmon (*O. kisutch*) was significantly, although not quantified to a given percentage, diminished when embryos and fry were exposed to DDT concentrations of 50 μg/l for a period of 56 day (Johnson and Pecor, 1969). Tissue residues from this study were between 1.09 and 2.76 μg/g w/w *p,p'*-DDT in the fry.

One unique feature of the effect of *o,p'*-DDE was its lethality and consequent failure of the embryos to hatch. The majority of embryonic deaths occurred late in development, when the embryos were fully formed and close to hatching. When a dead larva was found its body was fragmented and autolysed beyond recognition. The morphological defects observed in medaka embryos, despite being isolated, appear similar to those described in other species of fish. Exposure of early developing eggs of cod (*Gadus morhua*) or winter flounder (*Pseudopleuronectes americanus*) to DDT and DDE (10–100 μg DDT/l) caused aberrations in the pattern of development (Smith and Cole, 1973; Dethlefsen, 1977). Several days later, those disruptions translated into morphological defects that included abnormal spinal columns and abnormal hatch in cod. The similarity in our results, injected doses in the range of μg/kg w/w, and the rapid autolysis and fragmentation of the dead hatchlings, coincide with the symptoms of abnormal hatch and subsequent mortalities described in fish eggs and larvae exposed to organochlorine compounds (Von Westernhagen, 1988). Mortality and gonadal effects of *o,p'*-DDE observed in our study are consistent with the findings of Greenlee et al. (1999), who reported that in vitro exposure of murine embryos to low concentrations of *o,p'*-DDT resulted in concentration-related increases in embryo mortality and decreases in cell number. It is thus possible that organochlorines, *o,p'*-DDE included, can increase a process of programmed cell death known as apoptosis.

Both DDE isomers are considered to be weak estrogen agonists in fish, based on life cycle experiments. Several in vitro assays suggest that *o,p'*-DDT is a

stronger agonist than is *o,p'*-DDE (Coldham et al., 1997) and that in general, the relative potencies of in vitro versus in vivo assays, despite agreement, may not be comparable (Metcalfe et al., 2000). In intraperitoneally injected rainbow trout, both *o,p'*-DDE and *o,p'*- and *p,p'*-DDT evidenced weak estrogenicity by elevating plasma vitellogenin and affinity for hepatic estrogen receptors (ER) (Donohoe and Curtis, 1996). Quantification of this estrogenicity in feral populations of female kelp bass exposed to polluted areas near Palos Verdes (CA) by Spies and Thomas (1997), indicated that *o,p'*-DDE was 100-fold less potent than E2, while *o,p'*-DDT was approximately 1000-fold less potent. More recently, intersex in the form of testis-ova was observed in medaka exposed constantly for 100 day to micrograms per liter concentrations of *o,p'*-DDT, with a LOEC of 1.2 μg/l, measured concentration (Metcalfe et al., 2000). These researchers also found that progeny of previously exposed females (2.5 μg/l, nominal) were capable of spawning and producing their own viable offspring, with even better performance than controls. Waterborne flow-through exposures of medaka larvae to *o,p'*-DDT showed that this compound is estrogenic to medaka because it stimulated vitellogenesis in juveniles and adults at concentrations greater than 0.3 μg/l, feminized males, and reduced reproductive success at concentrations between 1.3 and 4.3 μg/l (Cheek et al., 2001). Regardless of the differences in relative potencies of one isomer to another, it is surprising to see the "permanence of in vivo effects" such as functional reversal of the gonad after *o,p'*-DDT treatment (Edmunds et al., 2000), development of testis-ova in medaka males (Metcalfe et al., 2000; Cheek et al., 2001), and gonadal abnormalities in both sexes (Papoulias et al., 2003) of these rather "weak" ER agonists occurs. This "permanence of effects" may contrast with the recent temporality of effects of the much more potent ethynyl estradiol (EE2) reported by Van den Belt et al. (2002). Zebrafish (*Danio rerio*) exposed to 10 and 25 ng/l of EE2 for 24 day followed by a 6–24 day period of EE2 free recovery showed the possibility of fully reversing transient effects.

It has been suggested that the estrogenic effect of *o,p'*-DDT in gonads of the Atlantic croaker is facilitated by its ability to interact with very low density lipoproteins (VLDLs), which carry the chemical through the bloodstream in a way similar to how vitellogenin is carried from the liver to the oocytes of females (Ungerer and Thomas, 1996). These authors found that after traveling in the plasma via VLDLs, *o,p'*-DDT was compartmentalized within the oocyte, with more than 95% ultimately deposited in the triglyceride-rich oil globule. Considering the similar estrogenic affinities of *o,p'*-DDE described by Donohoe and Curtis (1996) and Spies and Thomas (1997), this isomer may also be incorporated into the developing oocytes in a similar way. The implications of this hypothesis could be detrimental for an oviparous organism during specific and sensitive developmental periods, such as preparation of the late embryo for hatching. These lipophilic toxicants may be mobilized from the last larval energy reservoir, the oil droplet, prior to exogenous feeding, during a period of high sensitivity to hormones (Ungerer and Thomas, 1996).

To date three attempts of microinjecting DDT or DDE into fish eggs have been reported. The first in the late 1960s with eggs of the killifish, *Fundulus heteroclitus*, was unsuccessful due to the difficulties and limitations associated with the relative toughness of the egg's chorion (Weis and Perlmutter, 1967). With the advent of modern technology, problems such as high perivitelline osmotic pressure and/or tough chorion or periblast associated with small eggs have been overcome. In fish with larger eggs, i.e. salmonids, these problems are less of a limiting factor. As a result, Carlson et al. (2000), injected embryos of rainbow trout and chinook salmon with 1 μl/egg of doses ranging from 1 and 160 mg *o,p'*-DDE/kg, respectively (average weight 110 mg/egg). The authors conducted several exposures (at different dose ranges each) and observed male dominant sex ratios and one case of intersex in one out of three trials, but found that sexual development of these salmonids was not consistently altered by embryonic exposures. The effects could not be replicated in the other experiments, and the mortality associated with the experimental procedure was relatively great (approximately 45% in the sham injected group).

Nanoinjection of the medaka embryo for further full life cycle assessment is a potential model for evaluating hormonally active chemicals. The model has potential for the study of hormonally active compounds in the environment and may be used as a means to elucidate mechanism(s) of action, as well as a screening tool. Additional benefits of using this teleost are the capacity of replication due to small size of medaka, relative short time to generate data (two generation assay in 6–8 month), certainty in the dose received while simulating maternal transfer, and ability to contrast or compare to waterborne exposures. However, refinements to the model need to be considered if such technique is to be adopted in ED screening or validation exercises. This is particularly true if biochemical or histochemical parameters can be incorporated to improve the predictiveness of certain biomarkers. For instance, in addition to the diversity of reproductive strategies developed by fish species, ranging from synchronous hermaphroditism, protandrous and protogynous hermaphroditism, to gonochorism (Yamamoto, 1969), it is now well known that the phenotypic sex in fish may depend on external factors (Chan and Yeung, 1983; Francis, 1992). Environmental factors, mainly temperature, can be critical in the sexual differentiation of gonochoristic fish (Baroiller

and Guiguen, 2001). In some lower vertebrates, steroids and the enzymes that regulate their production seem to play a crucial role in the process of gonadal sex differentiation (Bogart, 1987; Pieau, 1996). In gonochoristic fish, the role of aromatase (CYP19), the enzyme that converts testosterone (C19) to estradiol (C18), seems to be preponderant for ovarian differentiation. However, the ratio of 11-oxygenated androgens (11ß-hydroxyandrostenedione, 11ß-hydroxytestosterone, and 11-ketotestosterone) to estrogens could also be an important mechanism in directing sexual differentiation (Baroiller and Guiguen, 2001). Furthermore, recent molecular and immunohistochemical techniques have been able to localize differential expression of aromatase in brain regions of medaka (Melo and Ramsdell, 2001). These authors were able to show differences in aromatase expression and sexual dimorphism of male versus female brains after treatment with estradiol. An integration of nanoinjection exposures with immunohistochemical localization of critical enzymes during sensitive periods in organs including, but not limited to the brain, could be the next step in advancing the medaka model for evaluation of hormonally active compounds.

To conclude, by mimicking maternal early exposure resulting from oocyte uptake of persistent lipophilic contaminants such as DDE, this nanoinjection technique produced results related to those reported for some eggs of medaka or other fish species exposed to DDT and/or DDE, through similar or other conventional means. It is advantageous that these results can be produced in a much shorter experimental time when compared to species such as salmonids or other cyprinids like the fathead minnow.

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References

Abbot, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Ankley, G.T., Mihaich, E., Stahl, R., Tillitt, D.E., Colborn, T., McMaster, S., Miller, R., Bantle, J., Dickerson, R., Fry, M., Giesy, J.P., Gray, L.E., Guiney, P.D., Hutchinson, T., Kramer, V.J., LeBlanc, G.A., Mayes, M., Nimrod, A.C., Peterson, R.E., Purdy, R., Ringer, R.K., Thomas, P., Vander Kraak, G., Zacharewski, T., 1998. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environ. Toxicol. Chem. 17, 68–87.

Baroiller, J.F., Guiguen, Y., 2001. Endocrine and environmental aspects of sex differentiation in gonochoristic fish. In: Scherer, G., Schmid, M. (Eds.), Genes and mechanism in vertebrate sex determination. Birkhauser Verlag, Basel, Switzerland, pp. 178–201.

Bayley, M., Junge, M., Baatrup, E., 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat. Toxicol. 56, 227–239.

Blus, L.J., 1996. DDT, DDD, and DDE in birds. In: Beyer, W.N., Heinz, G.H., Redmon-Norwood, A.W. (Eds.), Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations. Lewis Publishers, Boca Raton, FL, USA, pp. 49–71.

Bogart, M.H., 1987. Sex determination: a hypothesis based on steroid ratios. J. Theor. Biol. 128, 349–357.

Carlson, D.B., Curtis, L.R., Williams, D.E., 2000. Salmonid sexual development is not consistently altered by embryonic exposure to endocrine-active chemicals. Environ. Health Perspect. 108, 249–255.

Chan, S.T.H., Yeung, W.S.B., 1983. Sex control and sex reversal in fish under natural conditions. In: Hoar, W.S., Randall, D.J., Donaldson, E.M. (Eds.), Fish Physiology, vol. IXB. Academic Press, New York, pp. 171–222.

Cheek, A.O., Brouwer, T.H., Carroll, S., Manning, S., McLachlan, J.A., Brouwer, M., 2001. Experimental evaluation of vitellogenin as a predictive biomarker for reproductive disruption. Environ. Health Perspect. 109, 681–690.

Chevre, N., Becker, v., Tarradellas, J., Brazzale, A.R., Behra, R., Guettinger, H., 2002. Effects of dinoseb on the life cycle of Daphnia magna: modeling survival time and a proposal for an alternative to the no-observed-effect concentration. Environ. Toxicol. Chem. 21, 828–833.

Coldham, N.G., Dave, M., Sivapathasundaram, S., McDonnell, D.P., Connor, C., Sauer, M.J., 1997. Evaluation of a recombinant yeast cell estrogen screening assay. Environ. Health Perspect. 105, 734–742.

Crosby, D.G., 1998. Environmental Toxicology and Chemistry. Oxford University Press, Oxford.

Dacre, J.C., Scott, D., 1971. Possible DDT mortality in young rainbow trout. New Zeal. J. Mar. Fresh. Res. 5, 58–65.

Dethlefsen, V., 1977. The influence of DDT and DDE on the embryogenesis and the mortality of larvae of cod (*Gadus morhua*). Meeresforschung 25, 115–148.

Donohoe, R., Curtis, L.R., 1996. Estrogenic activity of chlordecone, *o,p'*-DDT and *o,p'*-DDE in juvenile rainbow trout: induction of vitellogenesis and interaction with hepatic estrogen binding sites. Aquat. Toxicol. 36, 31–52.

Edmunds, J.S., McCarthy, R.A., Ramsdell, J.S., 2000. Permanent and functional male-to-female sex reversal in d-rR strain medaka (*Oryzias latipes*) following egg microinjection of *o,p'*-DDT. Environ. Health Perspect. 108, 219–224.

Elonen, G.E., Spehar, R.L., Holcombe, G.W., Johnson, R.D., Fernandez, J.D., Erickson, R.J., Tietge, J.E., Cook, P.M., 1998. Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to seven freshwater fish species during early life stage development. Environ. Toxicol. Chem. 17, 472–483.

Francis, R.C., 1992. Sexual lability in teleost: development factors. Quart. Rev. Biol. 67, 1–17.

Giesy, J.P., Newsted, J.L., Garling, D.L., 1986. Relationships between chlorinated hydrocarbon concentrations and rearing mortality of Chinook salmon (*Oncorhynchus tshawytscha*) eggs from Lake Michigan. J. Great Lakes Res. 12, 82–98.

Greenlee, A.R., Quail, C.A., Berg, R.L., 1999. Developmental alterations in murine embryos exposed in vitro to an estrogenic pesticide, *o,p'*-DDT. Reprod. Toxicol. 13, 555–565.

Hopkins, C.L., Solly, S.R.B., Ritchie, A.R., 1969. DDT in trout and its possible effect on reproductive potential. New Zeal. J. Mar. Fresh. Res. 3, 220–229.

Johnson, H.E., Pecor, C., 1969. Coho salmon mortality and DDT in Lake Michigan.

Kannan, K., Tanabe, S., Tatsukawa, R., 1995. Geographical distribution and accumulation features of an organochlorine residues in fish in tropical Asia and Oceania. Environ. Sci. Technol. 29, 2673–2683.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop. Environ. Health Perspect. 104 (Suppl. 4), 715–740.

Kitzman, J.V., 1993. Specific ion and toxicologic analysis. In: Stoskopf, M.K. (Ed.), Fish Medicine. Saunders, Philadelphia, p. 205.

Melo, A.C., Ramsdell, J.S., 2001. Sexual dimorphism of brain aromatase activity in medaka: induction of a female phenotype by estradiol. Environ. Health Perspect. 109, 257 264.

Metcalfe, T.L., Metcalfe, C.D., Kiparissis, Y., Niimi, A.J., Foran, C.M., Benson, W.H., 2000. Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to *o,p'*-DDT in water or through maternal transfer. Environ. Toxicol. Chem. 19, 1893–1900.

Papoulias, D.M., Noltie, D.B., Tillitt, D.E., 2000. An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethinyl estradiol. Aquat. Toxicol. 48, 37–50.

Papoulias, D.M., Villalobos, S.A., Meadows, J.C., Noltie, D.B., Giesy, J.P., Tillitt, D.E., 2003. In ovo exposure to *o,p'*-DDE affects sexual development but not sexual differentiation in Japanese medaka (*Oryzias latipes*). Environ. Health Perspect. 111, 29–32.

Pereira, W.E., Hostettler, F.D., Rapp, J.B., 1996. Distributions and fate of chlorinated pesticides, biomarkers and polycyclic aromatic hydrocarbons in sediments along a contamination gradient from a point-source in San Francisco Bay, California. Mar. Environ. Res. 41, 299–314.

Pieau, C., 1996. Temperature variation and sex determination in reptiles. Bioassays 18, 19 26.

PlotIT Software ver 3.2, 1994. Scientific Programming Enterprises. Haslett, MI.

Rapaport, R.A., Uban, N.R., Capel, P.D., Baker, J.E., Looney, B.B., Eisenreich, S.J., Gorham, E., 1985. "New" DDT inputs to North America: atmospheric deposition. Chemosphere 14, 1167–1173.

Schmitt, C.J., Zajicek, J.L., Peterman, P.H., 1990. National contaminant biomonitoring program—residues of organochlorine chemicals in U.S. freshwater fish, 1976–1984. Arch. Environ. Contam. Toxicol. 19, 748–781.

Senthilkumar, K., Kannan, K., Sinha, K., Tanabe, S., Giesy, J.P., 1999. Bioaccumulation profiles of polychlorinated biphenyl congeners and organochlorine pesticides in Ganges river dolphins. Environ. Toxicol. Chem. 18, 1511–1520.

Smith, R.M., Cole, C.F., 1973. Effects of egg concentrations of DDT and dieldrin on development in winter flounder (*Pseudopleuronectes americanus*). J. Fish Res. Board Can. 30, 1894–1898.

Spies, R.B., Thomas, P., 1997. Reproductive and endocrine status of female kelp bass from a contaminated site in the Southern California bight and estrogen receptor binding of DDTs. In: Poland, R.M., Gilbertson, M., Peterson, R.E. (Eds.), Proceedings from a session at the 1995 Wingspread Conference. SETAC Press, Pensacola, pp. 113–133.

Tanabe, S., Tatsukawa, R., Kawano, M., Hidaka, H., 1982. Global distribution and atmospheric transport of chlorinated hydrocarbons: HCH (BCH) isomers and DDT compounds in the western Pacific, eastern India, and Antarctic oceans. J. Oceanogr. Soc. Jpn. 38, 137–148.

ToxCalc Program. 1984. Developed by Giesy, J.P. Orvis, East Lansing, MI.

Ungerer, J.R., Thomas, P., 1996. Role of very low density lipoproteins in the accumulation of *o,p'*-DDT in fish ovaries during gonadal recrudescence. Aquat. Toxicol. 35, 183–195.

Van den Belt, K., Wester, P.W., van der Ven, L.T., Verheyen, R., Witters, H., 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility. Environ. Toxicol. Chem. 21, 767–775.

Villalobos, S.A., Papoulias, D.M., Meadows, J.C., Blankenship, A.L., Pastva, S.D., Kannan, K., Tillitt, D.E., Giesy, J.P., 2000. Toxic responses of medaka (d-rR) strain to polychlorinated naphthalene mixtures after embryonic exposure by in ovo nanoinjection: a partial life cycle assessment. Environ. Toxicol. Chem. 19, 432–440.

Von Westernhagen, H., 1988. Sublethal effects of pollutants on fish eggs and larvae. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, San Diego, CA, USA, pp. 253–346.

Weis, J.S., Perlmutter, A., 1967. Technique for microinjecting materials into the eggs of fishes. Trans. Am. Fish. Soc. 96, 57–60.

Yamamoto, T., 1969. Sex differentiation. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, New York, pp. 117–175.