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INTERACTIVE EFFECTS OF *p,p′*-DICHLORODIPHENYLDICHLOROETHYLENE AND METHOXYCHLOR ON HORMONE SYNTHESIS IN LARGEMOUTH BASS OVARIAN CULTURES

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Abstract—*p,p′*-Dichlorodiphenyldichloroethylene (DDE) and methoxychlor were tested alone and in combination to assess the similarity of their actions on hormone synthesis in gonadal tissue from largemouth bass (*Micropterus salmoides floridanus*), a species whose reproductive fitness has relevance to ecosystem health in Florida (USA). Gonads were harvested from adult female bass (age, two to three years) during the peak reproductive season (January–May), minced, and incubated in culture medium with or without test agents for 48 h. Duplicates of each treatment were performed in each of three experiments using tissue from a different female. Both 17β-estradiol and testosterone were measured in aliquots of culture medium by validated radioimmunoassay procedures. Dose–response relationships of individual agents were characterized over a 6-log concentration range (1×10^{-2} to 1×10^{4} ppb). Both DDE and methoxychlor, tested individually, produced a dose-dependent decrease in testosterone levels. 17β-Estradiol levels were unaffected. Mixtures of the agents were tested at all concentration combinations of 0.01, 1, 100, and 10,000 ppb in culture medium. Statistical tests indicated that of 16 dose combinations tested, 15 were antagonistic, and only 1 was additive based on the Loewe additivity model of no interaction. These results imply that methoxychlor and DDE inhibit testosterone production by different mechanisms in bass ovaries.

Keywords—*p,p′*-Dichlorodiphenyldichloroethylene Methoxychlor *Micropterus salmoides* Dose additivity Sex steroids

INTRODUCTION

The ability to determine whether two chemicals share a similar mode of action has important implications for the regulation of pesticides and the risk assessment of carcinogens and chemical mixtures [1–3]. For assessing the toxicity of mixtures, both the Agency for Toxic Substances and Disease Registry and the U.S. Environmental Protection Agency recommend combining the toxicity of components that have similar modes of action. Although a number of different molecular and mechanistic features have been proposed as being important in determining mechanistic similarity, including similar molecular structures and toxicophores, shared toxicological intermediates, target molecules, biochemical pathways and target organs, and similar causal relationship between mechanistic steps, there would appear to be no universally agreed set of criteria that apply in all circumstances. Nonetheless, the need for chemicals to affect the same biochemical pathway in target tissues is generally acknowledged [3–5].

Both *p,p′*-dichlorodiphenyldichloroethylene (DDE) and methoxychlor (MCL) are chlorinated insecticides that have a similar mode of pesticidal action and similar molecular structures and that produce similar types of effects on endocrine function in laboratory test species [6–9]. Although mechanistic differences have been demonstrated for some endocrine effects of these agents in some systems [8], the exact mechanisms of toxicity have not been well established for either chemical. Field data collected in our laboratory (T.S. Gross, U.S. Geological Survey, Gainesville, FL, unpublished data) indicate that pesticides accumulate to higher concentrations in bass gonads than in other tissues, and altered steroid hormone production has been hypothesized as a mechanism of endocrine disruption. Therefore, we used sex-steroid hormone production as an endpoint to investigate whether DDE and MCL may operate through a similar mode of action in fish gonads. Mechanistic similarity was assessed by classical interaction experiments that compared the results produced by dose combinations of these agents with a no-interaction hypothesis based on Loewe additivity [10–12].

The Loewe additivity model, which is sometimes called dose addition or concentration addition, is based on the concept that an agent cannot interact with itself in producing a specific response. Dose combinations of a single chemical, which are mechanistically identical a priori, are defined as noninteracting. According to the model, dose combinations of different noninteracting chemicals should behave as if they were dilutions of one another, differing only in potency (for review, see [11] and original references therein). Thus, lack of conformity with the Loewe additivity model of noninteraction implies not only that two chemicals interact (either synergistically or antagonistically) in producing a specific response but also that the chemicals differ in the mechanism by which they produce the response. Testing a dose-additive no-interaction hypothesis is a classical method to probe how similarly drugs interact

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with a particular receptor. This method has been applied in ecotoxicology as well, including studies on osteolathyrogenic effects of chemicals in amphibian development [13,14].

The present study tested the hypothesis that two chlorinated chemicals—DDE and MCL—act via a similar mode of action in ovarian tissues of Florida largemouth bass (*Micropterus salmoides floridanus*), a species whose reproductive fitness has relevance to ecosystem health in Florida. To test our hypothesis, we measured the effects of DDE and MCL, both alone and in binary combinations, on 17β-estradiol and testosterone production in ovarian tissue harvested from female largemouth bass during the peak of their reproductive cycle in northern Florida.

MATERIALS AND METHODS

Fish

Reproductive-age female largemouth bass (age, two to three years) from hatchery ponds at the U.S. Geological Survey–Biological Resource Division–Center for Aquatic Resource Studies (Gainesville, FL, USA) were collected during the reproductive season (January–May) to maximize steroidogenic activity and available gonadal mass. Fish were transported to the laboratory and ovaries immediately collected by a sterile procedure. To prevent bacterial contamination, ovaries were removed carefully so as not to contact the outside of the fish carcass, then washed in sterile media containing antibiotics and stored in sterile media also containing antibiotics until processed for culture. Processing included rewashing the ovarian tissue with sterile media in a tissue-culture hood, placing the ovaries onto a sterile Petri dish, and using a sterile scalpel to remove the outer connective tissue and tease out oocytes.

Chemicals

Chemicals were obtained from Aldrich Chemical (Milwaukee, WI, USA). The MCL (1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane) had a purity of 98.9%. The DDE (2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene) had a purity of 99.0%.

Histology

Ovarian tissue samples were collected from each fish and preserved in 10% buffered formalin for histological evaluation. Tissues were cut transversally, embedded in paraffin, sectioned (thickness, 5 μm), mounted on glass slides, air-dried, and stained with Mayer's hematoxylin and eosin. Ovaries were classified into five stages of sexual maturation: Undeveloped (stage 1; mostly perinucleolar oocytes at various stages of previtellogenic growth), previtellogenic (stage 2; perinucleolar oocytes and cortical alveoli oocytes, no vitellogenic oocytes), early vitellogenic (stage 3; some vitellogenic oocytes of different sizes, with few to moderate amounts of vitelline granules and few to no fully developed eggs), late vitellogenic (stage 4; most of the oocytes contained numerous vitelline granules), and postvitellogenic/atretic (stage 5; mix of oocytes with numerous vitellogenic granules and atretic follicles, potentially postovulatory).

Tissue culture

In vitro steroid production was assessed using the following protocol. Follicular tissue, approximately 100 mg/well, was incubated in 24-well incubation plates containing sterile minimum essential media with 0.1% bovine serum albumin and 0.01% penicillin and streptomycin. Culture plates were incubated for 48 h in an atmosphere of 4% CO_2 at 26.5°C. Before beginning the incubations, half the control wells received 100 μl of hCG (50 injection units/ml; a potent gonadotropic hormone agonist in fish) as a positive control for a final incubation volume of 1.5 ml. For each fish, follicles were incubated with and without (basal) hCG (three replicates). Negative controls (wells containing medium with and without hCG but no tissue) were included on each plate. At the end of the incubations, medium was collected, centrifuged at 3,000 rpm for 10 min, and stored at −80°C before measurement of testosterone or 17β-estradiol by radioimmunoassay.

Hormone analysis

Media samples from cultures of follicular tissue from largemouth bass were analyzed for 17β-estradiol and testosterone using a direct radioimmunoassay procedure. Each sample (50 μl) was analyzed in duplicate. Standard curves were prepared in media with known amounts of radioinert 17β-estradiol or testosterone (1, 5, 10, 25, 50, 100, 250, 500, and 1,000 pg). The minimum concentration distinguishable from zero was 6.4 pg/ml for 17β-estradiol and 9.4 pg/ml for testosterone. Cross-reactivities of the 17β-estradiol antiserum with other steroids were 11.2% for estrone, 1.7% for estriol, less than 1.0% for estradiol-17α and androstenedione, and less than 0.1% for all other steroids examined. Cross-reactivities of the testosterone antiserum with other steroids were 8.42% for dihydrotestosterone, 2.1% for androstenedione, less than 1.0 % for testosterone, and less than 0.1% for all other steroids examined. A pooled sample of tissue conditioned media (~275 pg 17β-estradiol/ml, 312 pg testosterone/ml) was assayed serially in 10-, 20-, 30-, 40-, and 50-μl volumes (final volume, 50 μl with media). The resulting inhibition curves were parallel to the respective standard curve, with the tests for homogeneity of regression indicating that the curves did not differ. Further characterization of the assays involved measurement of known amounts (1, 2, 5, 10, 25, 50, 100, 250, and 500 pg) of 17β-estradiol or testosterone in 50 μl of tissue-conditioned media (for 17β-estradiol: $y = 21.36 + 0.92x$, $r^2 = 0.9291$; for testosterone: $y = 15.4 + 1.03x$, $r^2 = 0.9252$; y = amount of 17β-estradiol or testosterone measured [pg]; x = amount of 17β-estradiol or testosterone added [pg]). Inter- and intraassay coefficients of variation were 8.3% and 9.1%, respectively, for media 17β-estradiol and 10.4% and 8.7%, respectively, for media testosterone. Results were corrected for tissue mass per well and listed as pg/g tissue weight.

Test design

For dose–response estimation, ovaries from three female bass were separated into individual culture wells and exposed in triplicate to control, seven DDE concentrations (0.01, 0.1, 1.0, 10, 100, 1,000, and 10,000 ppb), and seven MCL concentrations (0.01, 0.1, 1.0, 10, 100, 1,000, and 10,000 ppb). To assess mixture effects, 16 combinations of DDE and MCL were tested in triplicate with each chemical at four concentrations (0.01, 1, 100, and 10,000 ppb). Ninety treatment and 16 control (i.e., tissue-only) wells were used per bass for each of three bass, for a total of 318 culture wells used in the analysis.

Statistical analysis

We used the Hill model to represent dose–response curves (DRCs) for the effects of DDE and MCL on testosterone and estradiol production. The Hill model, which is displayed in Equation 1, has three parameters and is sufficiently flexible to

capture a broad range of dose–response patterns [10]. It allows inflection points to be identified, and it is applicable to both increasing and decreasing dose–response relationships. The parameters in the model have direct interpretations, as indicated below:

$$E[R] = \frac{(E_{CON} - B)\cdot\left(\frac{\text{DOSE}}{C}\right)^{A}}{1 + \left(\frac{\text{DOSE}}{C}\right)^{A}} + B \quad (1)$$

where $E[R]$ is the expected value of the testosterone measurement (i.e., either testosterone or 17β-estradiol), A is the slope parameter, B is the background response (i.e., media blank; response caused by the assay alone), C is the median effective dose (approximately the median inhibitory concentration), DOSE is the applied dose, and E_{CON} is the control response (tissue control, DOSE = 0). A normal probability model, $N\{E[R], \sigma^2\}$, was used for the response at each dose. The σ^2 is the variance of the response.

The DRCs for each of the three animals were estimated individually. Numerical values for the Hill model parameters (E_{CON}, C, A, and σ^2) were obtained using maximum-likelihood estimation [15], a statistical procedure that estimates the most likely model parameters that would produce the collected data. The value of B was estimated separately as the average of two background measurements of the media alone (i.e., media blanks; no tissue and no test agents).

We tested for interaction effects by employing Loewe additivity as our null hypothesis of no interaction. For Loewe additivity, the additive response (R_{ADD}) for a particular mixture (x_1, x_2) is the value of R satisfying

$$1 = \frac{x_1}{g_1^{-1}(R)} + \frac{x_2}{g_2^{-1}(R)} \quad (2)$$

where $\{g_i\}$ are DRCs for the individual chemicals and $\{x_i\}$ are doses of each chemical in the dose combination [16].

We solved Equation 2 for R, denoted as R_{ADD}, using the nonlinear equations module of the GAUSS software package [17], which effectively solves complex systems of nonlinear equations. Results for R_{ADD} were compared to the average measured response, R_{AVE}, for the mixture (x_1, x_2). The comparison was based on a statistical test using the Z-statistic defined in Equation 3, where R_{AVE_i} is the average response based on two measurements for the i-th bass, R_{ADD_i} is the estimated expected additive response for the i-th bass, $\sigma^2_{AVE_i}$ is the variance of R_{AVE} for the i-th bass, $\sigma^2_{ADD_i}$ is the variance of R_{ADD} for the i-th bass, and the sum, Σ, is across all bass tested with the mixture (x_1, x_2).

The Z-statistic was calculated separately for each of the 16 mixture combinations:

$$Z = \frac{\sum (R_{AVE_i} - R_{ADD_i})}{\sqrt{\sum (\sigma^2_{AVE_i} + \sigma^2_{ADD_i})}} \quad (3)$$

We assumed that Z is approximated by the standard normal probability distribution. Basing our statistical interpretation on a significance level of 0.05, $Z > 1.96$ was an indication of antagonism, and $Z < -1.96$ was an indication of synergism.

Both R_{AVE} and its variance, σ^2_{AVE}, were estimated directly from the assay data. The value of R_{ADD} was calculated from Equation 2. The variance of R_{ADD}, σ^2_{ADD}, was estimated by Monte Carlo simulation. This was accomplished by generating Monte Carlo replicates of the dose–response data for every bass × agent × hormone combination. Specifically, for each particular bass × agent × hormone combination, 100 normally distributed, random values were generated for each dose. These data had the same response mean and standard deviation as the experimental data for the dose in question. Response values less than zero were truncated at zero. We therefore produced 100 dose–response datasets similar to the experimental data. Each of these 100 sets of simulated dose–response data were used to fit a DRC by the same methods employed for the experimental data. The DRCs for DDE and MCL were paired, and R_{ADD} was calculated using Equation 2 from each of the 100 pairs for each of the 16 dose combinations of interest. The 100 values of R_{ADD} were then used to calculate an estimate of σ^2_{ADD}, which in turn was used to calculate Z.

RESULTS

Fish used in this experiment were harvested during their peak reproductive season; therefore, gonadal steroid production was likely also at its peak. We examined the possibility of insufficient gonadotropic stimulation in vitro by adding hCG to half the replicates. Whereas hCG-treated wells demonstrated greater overall steroid production, the magnitude and trend of the response above background did not differ from those of non-hCG-treated wells.

We attempted to estimate DRCs for the effects of DDE and MCL on both testosterone and 17β-estradiol production. The data indicated no relationship between dose and response for either DDE or MCL and estradiol (Fig. 1). Estimates of the DRC parameters for testosterone are displayed in Table 1. Plots of the dose–response data and curve fits are shown in Figure 2. These figures, scaled identically, demonstrate the advantages of estimating a DRC for each animal separately. The DRCs fit to the data pooled across all animals exhibited much less precision (data not shown).

The data for fish 1 did not support a statistically significant dose–response relationship for testosterone versus DDE (the DRC slope, A, was not statistically distinguishable from zero) (Table 1 and Fig. 2a). In addition, histologic analysis of the ovarian tissue revealed that fish 2 and 3 were at stage 4 of sexual maturation (preovulatory), as expected for the time of year, whereas fish 1 was at stage 5 (postovulatory/atretic). Endocrine status is significantly different between stages 4 and 5, with ovarian steroidogenesis significantly reduced after ovulation [18]. Therefore, fish 1 was excluded from subsequent statistical analyses of DDE and MCL mixtures.

The average measured responses for the mixture combinations and the expected additive responses are displayed in Table 2. The statistical results for testing additivity (i.e., comparisons of R_{AVE} with R_{ADD}) are summarized in Table 3. Using a significance level (α) of 0.05, 15 of 16 dose combinations indicated antagonism between DDE and MCL (i.e., $Z > 1.96$). Only one dose combination (DDE = 1 ppb, MCL = 100 ppb) indicated additivity ($|Z| < 1.96$) and no combinations indicated synergism. A graphical summary of the interaction test results is shown in Figure 3.

DISCUSSION

Both MCL and DDE have been reported to produce reproductive and developmental toxicity in rodents and wildlife [8] by disrupting endocrine function through estrogenic, antiestrogenic, or antiandrogenic mechanisms. Each of these mechanisms may alter steroid hormone production. Furthermore,

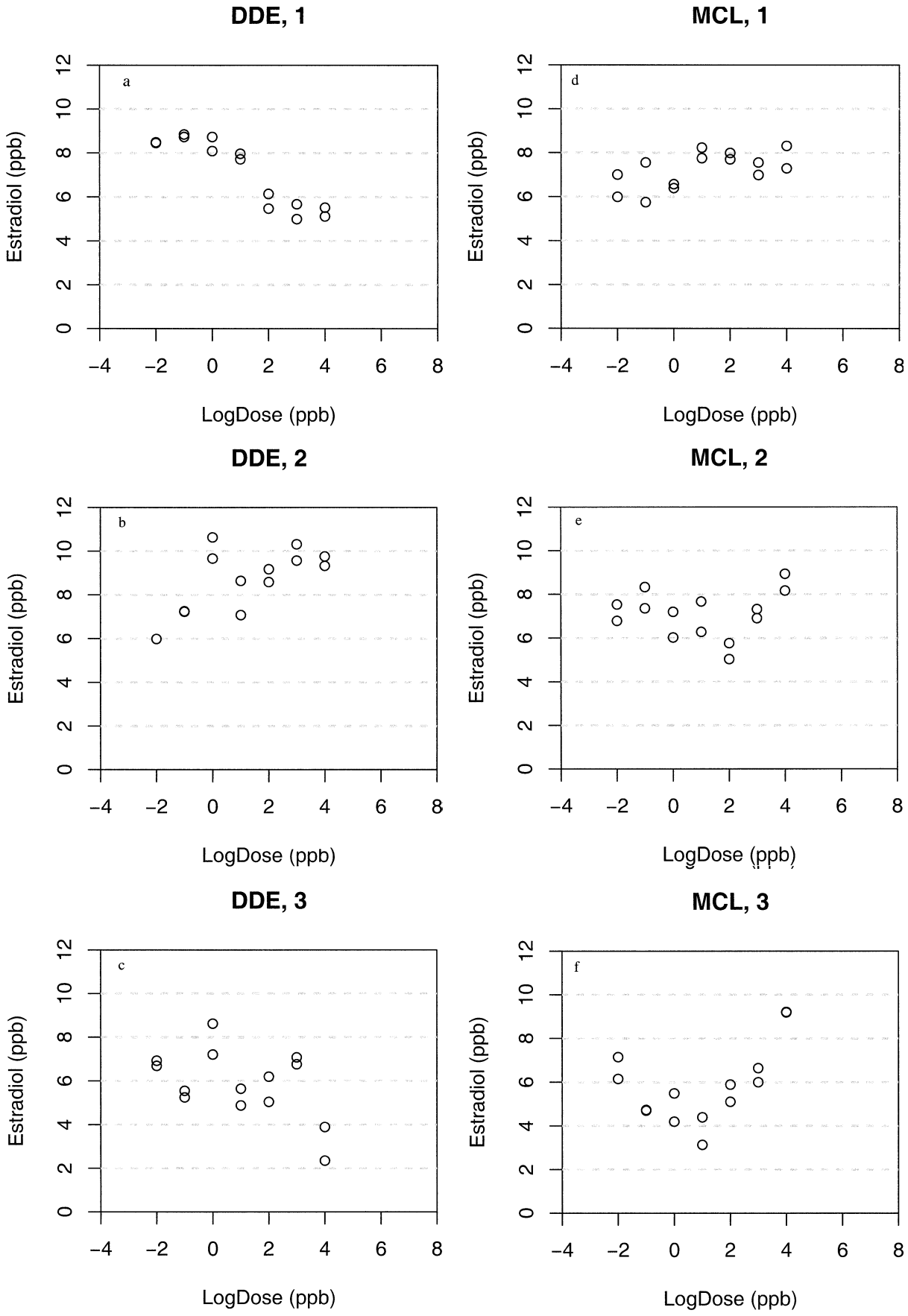

Fig. 1. Plots of estradiol concentration versus the log dose of *p,p′*-dichlorodiphenyldichloroethylene (DDE) or methoxychlor (MCL). Estradiol concentrations do not change systematically with increasing concentrations of DDE or MCL. Headings indicate chemical treatment and fish number.

Table 1. Estimated dose–response curve parameters for *p,p′*-dichlorodiphenyldichloroethylene (DDE) and methoxychlor (MCL) for each individual largemouth bass (in duplicate, $n = 14$)[a]

Chemical	Animal	*n*	*A*	*C* (ppb)	S^2	E_{CON} (ppb)	*B* (ppb)
DDE	1	30	−0.0129	0.0000	0.4683	4.0601	0.403
			0.0151	*NA*	*0.1203*	*0.1695*	*0.261*
	2	30	−0.0652	45.7317	0.3274	5.1107	0.223
			0.0282	*102.9009*	*0.0845*	*0.1431*	*0.154*
	3	30	−0.0796	126.3134	0.2942	5.4171	0.192
			0.0251	*223.2376*	*0.0760*	*0.1354*	*0.172*
MCL	1	30	−0.1712	0.0260	0.3565	4.0716	0.403
			0.0597	*0.0482*	*0.0920*	*0.1493*	*0.261*
	2	30	−0.2669	163.2252	0.3396	5.1532	0.223
			0.0480	*116.1115*	*0.0877*	*0.1412*	*0.154*
	3	30	−0.1141	4.1351	0.2784	5.4131	0.192
			0.0268	*4.6467*	*0.0719*	*0.1318*	*0.172*

[a] *A* represents the slope parameter, *C* the median effective dose (ppb), and S^2 the response variance. The control response (E_{CON}) was estimated using the 16 control samples from each bass ($n = 16$). The background response (*B*) was estimated separately as the average of two background measurements. The standard error for each term is listed in italic. NA = not available.

the synthesis of steroid hormones involves up to five different hydroxylases, two dehydrogenases, a reductase, and an aromatase [19], all of which could possibly be induced or inhibited in the presence of exogenous chemicals. Gonadal steroid production is stimulated by seasonal increases in pituitary gonadotropin secretion and can be modulated by negative-feedback loops to the hypothalamus or pituitary or even within the gonad itself [20–22], demonstrating several potential pathways that could be targeted by MCL or DDE. In the present study, hCG treatment increased overall steroid hormone production, but it did not affect the magnitude and trend of the response in treated or control cultures, indicating that the bass were in a reproductive state and sensitive to gonadotropic stimulation in vivo.

The endocrine-disruptive effects of chlorinated pesticides have been studied most extensively in rodent models. In rodents, MCL is thought to act primarily as an estrogen [23]. Estrogenic activity is markedly enhanced by hepatic oxidative biotransformation of MCL to hydroxyphenyl trichloroethane (HPTE) in rat liver [24]. However, the estrogenic actions of MCL are complex. Hall et al. [25] showed that MCL alters normal preimplantation embryonic development in the mouse, acting as an estrogen agonist in the uterus and oviduct but as an antiestrogen in the ovary. Gaido et al. [26] showed that in human hepatoma cells, HPTE displays potent estrogen receptor (ER) α–agonist activity but has minimal agonist activity with either human or rat ERβ; those authors also showed that HPTE acts as a potent ERβ antagonist with several estrogen-responsive promoters. Raloxifene blocks uterotrophic responses to DDT and MCL but not to 17β-estradiol, suggesting that these xenoestrogens exert their activity via a different site on the ER or through a different mechanism than that of 17β-estradiol [27].

In contrast, DDE is most strongly associated with antiandrogenic actions [28] and may not interact directly with the ER [29]. In rats, central actions appear to be more important than peripheral actions in mediating the antiandrogenic effects [30]. You et al. [31] found that oral administration of DDE greatly increased hepatic aromatase protein and enzyme activity but did not alter serum 17β-estradiol concentrations. Using HepG2 cells transiently transfected with human androgen receptors, Maness et al. [32] confirmed the antiandrogenic activity of DDE and demonstrated the antiandrogenic potential of MCL and its metabolite, HPTE. These results, together with those of previously published reports concerning the estrogenic activity of DDT isomers and HPTE, raise the possibility that some environmental chemicals can interact with more than one steroid receptor.

Chedrese and Feyles [33] investigated the effects of DDE (96% *p,p′*-DDE, 4% *o,p′*-DDE) and MCL on steroidogenesis and follicle-stimulating hormone (FSH) responsiveness in ovarian cells in vitro. Both DDE and estradiol, but not MCL, increased proliferation in primary cultures of porcine granulosa cells. The DDE decreased FSH-stimulated cyclic adenosine monophosphate (cAMP) synthesis in both granulosa cells and Chinese hamster ovary cells stably transfected with an FSH receptor–reporter construct and decreased progesterone synthesis in granulosa cells. The MCL did not affect cAMP generation in either type of cell, but it did inhibit both unstimulated and 17β-estradiol–stimulated progesterone synthesis in the granulosa cells. Those authors concluded that DDE primarily inhibits cAMP generation, whereas MCL suppresses a step in progesterone synthesis downstream of cAMP generation.

In rodents and humans, antiandrogenic effects may occur via binding and blockade of the androgen receptor in androgen-responsive tissues [34], by alteration of adrenal steroid hormone synthesis, or by induction of steroid hormone catabolism [35]. The DDE and, to a lesser extent, MCL inhibit androgen receptor–dependent transcriptional activity at 10^{-6} M and exhibit weak androgen-agonist activity at 10^{-5} M in vitro in HepG2 cells transiently transfected with the human androgen receptor and androgen-responsive reporter gene [32]. In addition to complex mechanisms involving steroid hormone action, other pathways have been proposed for MCL and DDE toxicity. For example, Latchoumycandane and Mathur [36] showed that MCL elicited a dose-dependent depletion of antioxidant enzymes with a concomitant increase in levels of hydrogen peroxide and lipid peroxidation in mitochondrial and microsome-rich fractions of rat testis. They suggest that adverse effects of MCL on the male reproductive system may be caused by induction of oxidative stress.

The endocrine activity of DDE and MCL has been characterized less thoroughly in aquatic species. Mills et al. [37] found that DDE did not alter heptasomatic or gonadosomatic indices, plasma steroid hormone levels, vitellogenin production, or gonadal development in juvenile summer flounder (*Paralichthys dentatus*). No useful indicator of exposure to

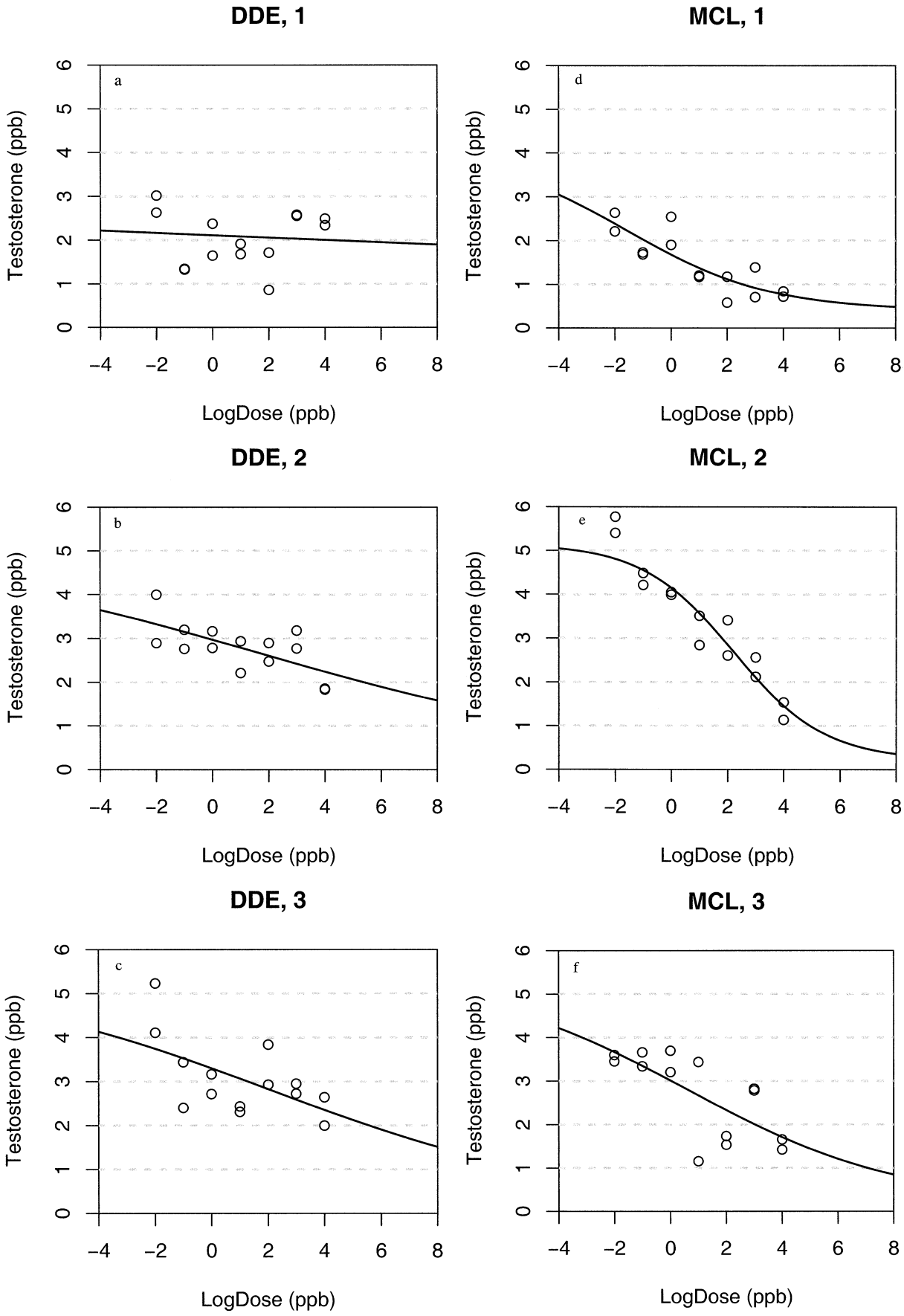

Fig. 2. Plots of testosterone concentration versus the log dose of *p,p′*-dichlorodiphenyldichloroethylene (DDE) or methoxychlor (MCL). Plots of each dose–response curve show that the doses tested span approximately the full range of responses for testosterone. Headings indicate chemical treatment and fish number.

Table 2. Binary dose combinations of *p,p′*-dichlorodiphenyldichloroethylene (DDE) and methoxychlor (MCL) with the resulting average response (R_{AVE}), estimated additive response (R_{ADD}), and their respective variances (σ_{AVE}^2 and σ_{ADD}^2)

Doses			Responses	
DDE	MCL	Animal	R_{AVE} ($\pm$ σ_{AVE}^2)	R_{ADD} ($\pm$ σ_{ADD}^2)
0.01	0.01	2	5.22l6 (0.147)	3.322 (0.243)
0.01	0.01	3	4.810 (1.162)	3.623 (0.159)
0.01	1	2	5.157 (0.224)	3.319 (0.238)
0.01	1	3	4.711 (0.224)	3.013 (0.158)
0.01	100	2	4.692 (0.307)	2.848 (0.127)
0.01	100	3	5.057 (0.187)	2.333 (0.161)
0.01	10,000	2	6.356 (0.218)	1.456 (0.143)
0.01	10,000	3	6.227 (0.115)	1.713 (0.150)
1	0.01	2	5.084 (0.521)	2.970 (0.015)
1	0.01	3	4.724 (0.417)	3.294 (0.157)
1	1	2	4.938 (0.408)	2.969 (0.148)
1	1	3	4.764 (0.057)	3.005 (0.135)
1	100	2	2.740 (0.352)	2.805 (0.109)
1	100	3	3.110 (0.254)	2.333 (0.160)
1	10,000	2	2.930 (0.143)	1.456 (0.143)
1	10,000	3	3.436 (0.077)	1.713 (0.150)
100	0.01	2	4.903 (0.186)	2.605 (0.085)
100	0.01	3	4.574 (0.316)	2.829 (0.118)
100	1	2	5.178 (0.089)	2.604 (0.085)
100	1	3	4.849 (0.086)	2.801 (0.100)
100	100	2	4.410 (0.314)	2.562 (0.079)
100	100	3	4.914 (0.088)	2.332 (0.154)
100	10,000	2	2.894 (0.203)	1.456 (0.143)
100	10,000	3	1.971 (0.014)	1.713 (0.150)
10,000	0.01	2	5.105 (0.296)	2.242 (0.141)
10,000	0.01	3	5.275 (0.502)	2.355 (0.183)
10,000	1	2	4.876 (0.661)	2.242 (0.141)
10,000	1	3	4.511 (0.057)	2.353 (0.178)
10,000	100	2	3.109 (0.182)	2.229 (0.139)
10,000	100	3	3.411 (0.155)	2.261 (0.137)
10,000	10,000	2	2.938 (0.205)	1.456 (0.142)
10,000	10,000	3	2.136 (0.078)	1.713 (0.147)

Table 3. The *Z*-statistic for testing the no-interaction hypothesis for testosterone[a]

DDE dose (ppb)	MCL dose (ppb)	*Z*
0.01	0.01	2.562
0.01	1	8.297
0.01	100	11.050
0.01	10,000	29.263
1	0.01	5.172
1	1	8.145
1	100	1.497
1	10,000	12.158
100	0.01	10.256
100	1	25.624
100	100	12.013
100	10,000	5.845
10,000	0.01	9.231
10,000	1	6.833
10,000	100	6.580
10,000	10,000	6.360

[a] The distribution of *Z* is approximated by the standard normal distribution. Using a significance level (α) of 0.05, 15 of 16 dose combinations indicate antagonism between *p,p′*-dichlorodiphenyldichloroethylene (DDE) and methoxychlor (MCL) (i.e., $Z > 1.96$). Only one dose combination (DDE = 1 ppb, MCL = 100 ppb) indicates additivity for these chemicals (i.e., $|Z| \leq 1.96$). No dose combinations indicated synergism for these chemicals (i.e., $Z < -1.96$).

DDE was found, despite at least two useful markers—gonadosomatic index and depressed plasma testosterone concentrations—of exposure to estrogenic chemicals being identified. Both DDE and MCL have also been shown to bind the androgen receptor and competitively displace testosterone from the receptor in goldfish testis but not in ovaries [38]. The MCL treatment in channel catfish (*Ictalurus punctatus*) increased serum estradiol and vitellogenin levels, demonstrating estrogenic activity [39].

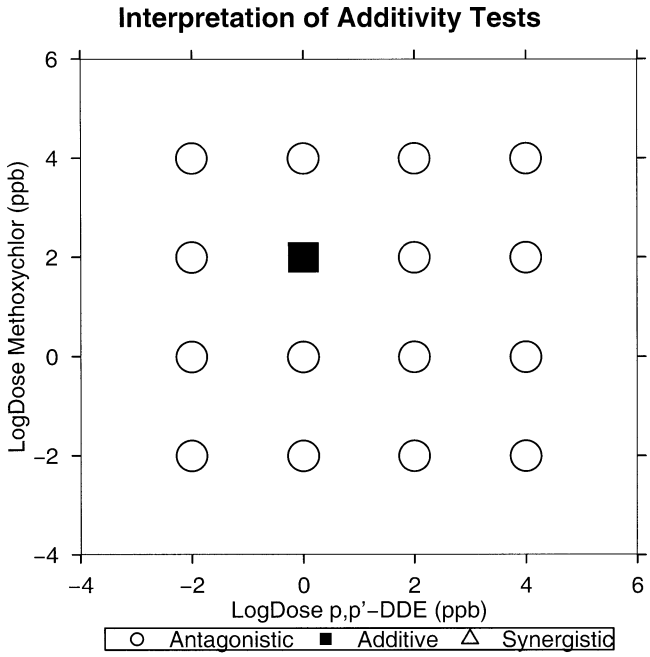

Fig. 3. Results of statistical tests of no interaction for dose combinations of *p,p′*-dichlorodiphenyldichloroethylene (DDE) and methoxychlor (MCL) are plotted on a log-dose by log-dose scale. Fifteen of 16 dose combinations indicated an antagonistic interaction; that is, the testosterone levels were significantly greater than predicted by the Loewe additivity no-interaction hypothesis (significance level [α] of 0.05). Only one dose combination (DDE = 1 ppb, MCL = 100 ppb) was consistent with Loewe additivity. Synergism was not indicated for any dose combination. Numerical results for the statistical tests are listed in Table 3.

Pickford and Morris [40] showed that frogs (*Xenopus laevis*) exposed to MCL at concentrations sufficient to induce systemic toxicity also delayed gonadotropin-induced oviposition, reduced egg output, increased the gonadosomatic index, elevated estradiol to progesterone and estradiol to testosterone ratios, and reduced plasma vitellogenin levels. Ex vivo progesterone synthesis was significantly reduced in ovarian explants from MCL-treated frogs, suggesting that the alteration of sex-steroid ratios was caused by a reduction in progesterone synthesis. It is unclear how these effects of MCL at high doses relate to those that occur at environmentally relevant levels of exposure.

To our knowledge, the present study is the first to investigate the effects of DDE and MCL on steroid hormone production in ovarian cell cultures from largemouth bass. The results indicate that both chemicals reduced testosterone production in a dose-dependent fashion. Neither substance systematically affected levels of 17β-estradiol in the cultures, suggesting that the reduction in testosterone was not caused by aromatization of androgens to estrogens. However, because estradiol concentrations in these ovarian cultures are approximately 10-fold greater than testosterone levels, a slight increase in aromatase activity could produce a substantial decrease in testosterone levels without a statistically significant increase in 17β-estadiol. That is, the study may be more sensitive to an effect on testosterone levels than to an effect on estradiol levels.

The results reported here demonstrate that certain combinations of MCL and DDE are antagonistic based on the fact that dose combinations of the two agents inhibited testosterone production less than predicted by the Loewe additivity model of noninteraction. The mechanisms underlying the antagonism are unclear, however, and numerous possibilities exist, including noncompetitive inhibitory binding to the same site on a single enzyme in the pathway of ovarian androgen production, inhibition of different enzymes involved in androgen synthesis, or interference with androgen or estrogen catabolism. Although the exact pathways and enzymes involved are uncertain, MCL and DDE appear to reduce testosterone production in bass ovarian tissue by different mechanisms based on rejection of the null hypothesis (Loewe additivity) at a majority of the dose combinations tested in the present study.

Currently, great interest exists in identifying mixture effects among endocrine-active chemicals. In light of this interest, one might interpret the results presented here as further evidence that endocrine-active agents interact in producing effects on organisms. We caution against such an overinterpretation for a number of reasons. The first and most critical reason is the importance of assessing interactions at relevant levels of biologic organization [11]. In vitro assays, such as those used in the present study, may identify interactions at the biochemical or physiologic level that manifest no effects at the whole-organism level. One of two conditions would have to be demonstrated for the studies presented here to constitute a relevant level of biologic organization for assessing interactions between chemicals: Either reduced gonadal testosterone production would be shown to be the mechanism by which the effects of these agents are manifested in the whole organism, or interactions between MCL and DDE would be demonstrated in androgen-dependent processes in largemouth bass. To our knowledge, neither of these conditions has been demonstrated as yet.

The second reason for caution against overinterpretation of these results as evidence of relevant physiologic interactions is the sheer number of candidate mechanisms of action for these agents, as has been observed in mammalian species [8]. In fact, it may be difficult to associate a particular effect in the whole organism with a single mechanism of action. Gaido et al. [26] have discussed the difficulties inherent in associating specific mechanisms of action with specific effects on an organism for agents that operate via multiple endocrine mechanisms. As such, the present study is useful for probing the similarity of mechanisms by which DDE and MCL affect ovarian hormone production and for generating additional hypotheses regarding the relevance of the effects. However, such studies are not predictive of interactions occurring between these or other endocrine-active agents in fish or other species.

Finally, we note the apparent large interfish variability (Figs. 1 and 2) as an additional reason for caution against interpreting our results as evidence that endocrine-active agents interact in producing effects on organisms. The 17β-estradiol responses for the three fish appear to be nonuniform, possibly because of the relatively high estradiol concentrations in these ovarian cultures, as discussed above. Figure 1 shows the absence of a monotonic dose–response relationship between 17β-estradiol and either DDE or MCL. When the DRC for either DDE or MCL is flat (i.e., response does not change with increasing dose), R_{ADD}, the expected response under Loewe additivity, does not exist. For testosterone, when statistically significant DRCs existed, we maximized the effect of interfish variability in our statistical analysis by estimating a DRC for each fish before summarizing results rather than aggregating data across fish before estimating a DRC. Developing individual DRCs for each fish allowed calculation of an expected additive response for each fish. Therefore, the *Z*-statistic used to test for an interaction effect implicitly accounts for interfish variability. In that respect, our result is stronger than if the data for the three fish were combined to produce a single DRC for each agent.

One consequence of our approach was the necessity to omit fish 1 from the testosterone analysis, because the data did not support a monotonic DRC for DDE. A DRC failure in one from among only three fish in the present study weakens our conclusions, but we have no way at present of determining its quantitative impact. We believe that the DRC failure in one fish was caused by the different reproductive stage of that fish, as evidenced by the different histologic appearance of its gonad. Nonetheless, evaluating the DRC for testosterone versus DDE in more fish would help to establish a dose–response relationship between DDE and testosterone.

Finally, it is worth considering how our study design controls for the multiple sources of variability inherent to an endocrine response. Responses in this in vitro model were tested for significance only in light of intraindividual variability. In this way, variability caused by temporal, population-based, or interindividual shifts in a baseline response is normalized. Consequently, the sensitivity of the study design for each dose combination of DDE and MCL is improved, and fewer duplicate analyses within an individual animal need to be performed. The economy of this study design conserves enough follicular material and practical resources such that the number of replicate individuals analyzed for each dose combination may be increased slightly, the range of the DRCs for the individual treatments can be expanded to seven orders of magnitude, and the number of binary dose combinations of DDE and MCL tested can also be expanded to six orders of mag-

nitude. The net effect of this design is a significant expansion of the number of replicates conducted per assay endpoint, coupled with complete coverage of the range of responses.

Even though the number of treatments and replicate wells tested were large, the conclusions to be drawn from this factorial analysis are strictly localized to the particular dose combinations tested. The statistical power and significance level of the study design are conditioned on this constraint. In this sense, the study design is intended to cover a wide range of possible dose combinations without drawing global conclusions regarding the interactivity of DDE and MCL. Likewise, whereas population and temporal variability are important considerations for extrapolating test results to real-world impacts, population-based assessment is beyond the scope of the present analysis.

Risk assessment methodologies for chemical mixtures commonly assume a noninteraction model based on additivity for mixtures of chemicals with similar modes of toxicity [1–3]. Questions have been raised, however, regarding the degree of mechanistic detail that must be known to characterize a chemical's mode of action sufficiently to accurately predict combined action in a mixture [11]. Rather than a few mechanistic features (mode of action) being used to predict the combined action of chemicals in mixtures, considerable mechanistic detail may be required. Although DDE and MCL might be categorized as having a similar mechanism of action according to some proposed criteria [4], the results shown here call into question the applicability of criteria that would lead to an assumption that the effects of DDE and MCL can be modeled by dose additivity.

CONCLUSION

Both MCL and DDE appear to inhibit testosterone production in cultured bass ovarian tissue when administered either singly or in combination. The mechanism of inhibition is unknown, but it appears to be dissimilar for these two chemicals based on nonadditive combined action. Antagonism was inferred in 15 of 16 dose combinations tested, spanning a broad range of concentrations and implying that the mechanistic differences are independent of dose. These results suggest risk models based on Loewe additivity (i.e., toxic equivalents) may be inappropriate for endocrine effects of DDE and MCL produced by alterations in hormone synthesis.

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