

Use of chemical mixtures to differentiate mechanisms of endocrine action in a small fish model

Gerald T. Ankley*, Kathleen M. Jensen, Michael D. Kahl, Elizabeth J. Durhan, Elizabeth A. Makynen, Jenna E. Cavallin, Dalma Martinović, Leah C. Wehmas, Nathaniel D. Mueller, Daniel L. Villeneuve

U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804, USA

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ABSTRACT

Various assays with adult fish have been developed to identify potential endocrine-disrupting chemicals (EDCs) which may cause toxicity via alterations in the hypothalamic–pituitary–gonadal (HPG) axis. These assays can be sensitive and highly diagnostic for key mechanisms such as agonism of the estrogen and androgen receptors (ERs, ARs) and inhibition of steroid synthesis. However, most of the tests do not unambiguously identify AR antagonists. The purpose of this work was to explore the utility of a mixture test design with the fathead minnow (*Pimephales promelas*) for detecting different classes of EDCs including AR antagonists. Adults of both sexes were exposed via the water to EDCs with diverse mechanisms of action in the absence or presence of 17 β -trenbolone (TB), a potent AR agonist which masculinizes female fathead minnows. Similar to previous studies with the model AR antagonists flutamide and vinclozolin, exposure of females to the AR antagonist cyproterone acetate in the presence of TB decreased expression of an easily-observed masculinization response, nuptial tubercle formation. Mixture studies with TB and the model ER agonists, 17 α -ethinylestradiol and bisphenol A, also showed inhibition of tubercle formation in the females, but unlike the AR antagonists, the estrogens markedly induced synthesis of vitellogenin (VTG: egg yolk protein), particularly in males. The ER agonists also offset TB-induced depressions in plasma VTG concentrations in female fish. Additional mixture experiments were conducted with TB and triclocarban, an anti-microbial reported to enhance AR-mediated responses, or ammonia, a “negative control” with no known direct effects on HPG function. Neither chemical affected VTG status in males or females in the absence or presence of TB; however, both slightly enhanced TB-induced tubercle formation in females. Based on studies described herein and elsewhere with the fathead minnow, a TB co-exposure assay appears to be an effective approach for clearly identifying AR antagonists as well as potential EDCs with other relevant mechanisms of action.

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1. Introduction

Testing programs for endocrine-disrupting chemicals (EDCs) have been/are being developed by regulatory authorities throughout the world. These programs vary but, in general, employ a tiered framework to first identify (screen) chemicals with the potential to affect different mechanisms/pathways within the hypothalamic–pituitary–gonadal (HPG) axis, followed by more intensive testing, where warranted, to define actual risk of the chemicals in terms of adverse reproductive or developmental effects (e.g., U.S. Environmental Protection Agency, 1998; Organisation for Economic Cooperation and Development, 2009a). Specific HPG mechanisms of concern from the current regulatory

perspective include stimulation and antagonism of the estrogen and androgen receptors (ERs, ARs) and inhibition of sex steroid synthesis (U.S. Environmental Protection Agency, 1998; Organisation for Economic Cooperation and Development, 2009a). One of the challenges in screening for potential EDCs has been the development of a single assay capable of simultaneously discerning these multiple endocrine mechanisms.

Several fish species are being employed for both EDC screening and testing. For example, in the U.S. a 21-d fathead minnow (*Pimephales promelas*) assay has been developed for use in a congressionally mandated EDC screening program (U.S. Environmental Protection Agency, 1998, 2007). The assay includes assessment of reproductive success (egg production), in addition to determination of more mechanistic indicators of HPG axis disruption such as alterations in gonad histology, secondary sex characteristics, and plasma steroid and vitellogenin (VTG; egg yolk protein precursor) concentrations (Ankley et al., 2001). At an international

* Corresponding author.

E-mail address: ankley.gerald@epa.gov (G.T. Ankley).

level, the Organisation for Economic Cooperation and Development has published test guidelines for 21-d EDC screening assays with adults of three small fish models: Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and fathead minnow (Organisation for Economic Cooperation and Development, 2009b). Endpoints in these guidelines are limited to secondary sex characteristics and VTG.

Diagnostic utility of test endpoints in the 21-d fish EDC assays is far better for some endocrine pathways than others. For example, ER or AR agonists are effectively identified through relatively specific changes; ER agonists cause induction of VTG in males, while androgenic compounds cause females to develop male secondary sex characteristics. Chemicals that inhibit steroidogenesis consistently decrease VTG levels in female fish. However, the suite of responses produced by anti-androgenic chemicals in sexually mature fish can be somewhat ambiguous, making it difficult to clearly diagnose this important pathway (Jensen et al., 2004; Katsiadaki et al., 2006; Seki et al., 2006; Filby et al., 2007; Organisation for Economic Cooperation and Development, 2009b). A wide variety of known or potential environmental contaminants, including some dicarboximide fungicides (e.g., vinclozolin), conazole fungicides (e.g., prochloraz), organochlorine insecticides (e.g., p,p'-DDE), urea-based herbicides (e.g., linuron), polybrominated diphenyl ethers, and pharmaceuticals (e.g., flutamide) have been shown to bind to the AR, and cause anti-androgenic effects in vivo (Gray et al., 2006). Further, recent monitoring programs have highlighted the occurrence of anti-androgenic chemicals in complex environmental mixtures (Weiss et al., 2009; Hill et al., 2010).

Uncertainty as to clear identification of chemicals as AR antagonists using current test methods with fish led us to consider an indirect approach to achieve this. The basis of the approach lies in evaluating whether test chemicals block occurrence of an in vivo response mediated through the AR. Studies in our lab and elsewhere have shown that the synthetic androgen 17 β -trenbolone (TB) binds with high affinity to fish AR(s) and masculinizes female fish at concentrations in water in the range of 20–50 ng/L (Ankley et al., 2003; Wilson et al., 2004; Seki et al., 2006). In the fathead minnow, masculinization is manifested by a proliferation and thickening of the dorsal pad and the de novo development of craniofacial nuptial tubercles, external structures which can be visually detected and easily quantified (Jensen et al., 2001). Previously we showed that flutamide effectively blocked TB-induced production of nuptial tubercles in female fathead minnows, a finding consistent with its established mechanism of action (Ankley et al., 2004). Martinović et al. (2008) used a similar approach to help confirm that vinclozolin affects the reproductive endocrine system of fathead minnows through antagonism of the AR. Katsiadaki et al. (2006) have done analogous work with the stickleback (*Gasterosteus aculeatus*), a fish species in which the male produces an androgen-dependent protein, spiggin (nest-building “glue”), as part of its normal reproductive cycle. Exposure of female sticklebacks to androgens causes the abnormal production of spiggin, while co-exposures with flutamide and anti-androgenic pesticides block production of spiggin in females exposed to exogenous androgens.

In this paper, we describe several experiments focused on further evaluation of a mixture assay in the fathead minnow for identifying EDCs. First we evaluated whether a third widely studied model AR antagonist, the anti-neoplastic drug cyproterone acetate (CA; Sonneveld et al., 2005), would block TB-induced tubercle formation in fathead minnows in a manner similar to flutamide and vinclozolin. We also conducted mixture studies with TB and 17 α -ethinylestradiol (EE2) or bisphenol A (BPA) which are considered, respectively, strong and weak ER agonists (Kang et al., 2007; Caldwell et al., 2008). Although tubercle formation in fathead minnows is clearly an AR-mediated phenomenon, it is possible that ER agonists could affect development of tubercles in the fathead

minnow (Miles-Richardson et al., 1999). A comparatively recent HPG-related response of interest involves some common antimicrobial chemicals (triclocarban, triclosan) that seem to enhance responsiveness to AR agonists in rats and in vitro (Ahn et al., 2008; Chen et al., 2008; Blake et al., 2010). To assess cross-class generality of this phenomenon in vivo, we also conducted mixture studies with TB and triclocarban (TCC). Finally, mixture studies were performed with TB and ammonia (AM), a common environmental contaminant with no known HPG activity.

2. Materials and methods

2.1. Experimental design

Sexually mature (ca. 5–6 months old) fish used for the research were from an on-site facility. The fish came from a group culture setting that, although not conducive to active reproduction, resulted in animals that could be easily differentiated sexually. Fish were tested in glass tanks holding 10 L of clean Lake Superior water or test chemical dissolved in Lake Superior water, which were continuously renewed at a rate of 45 mL/min. General water quality characteristics measured in test tanks were: pH, 7.45; conductivity, 103 μ mhos; alkalinity, 44 mg/L as CaCO₃; hardness, 45 mg/L as CaCO₃; dissolved oxygen, 6.6 mg/L. The animals were held at 25 \pm 0.5 °C under a 16:8 L:D photoperiod, and were fed brine shrimp to satiation twice per day.

Five separate experiments were conducted with TB and CA, EE2, BPA, TCC or AM. Solvent-free solutions of TB (Sigma–Aldrich Chemical, St. Louis, MO, USA; >95% purity), CA (Sigma–Aldrich; >98% purity), EE2 (Sigma–Aldrich; >98% purity), BPA (Sigma–Aldrich; 99% purity), TCC (Sigma–Aldrich; 99% purity) and AM were prepared as concentrated, solvent-free stocks in Lake Superior water. The AM stock was an approximately 45:1 mixture of ammonium chloride (Fisher Scientific, Fair Lawn, NJ, USA; >99.5% purity) and ammonium hydroxide (Fisher; Certified ACS Plus Grade) designed to yield a neutral pH. To accommodate collection of additional data/samples related to other study objectives, experimental details (e.g., numbers of animals and replicates) varied somewhat from test-to-test (Table 1). However, the same basic experimental design was used for all five studies. Each study was comprised of six treatments: a control, a TB-only treatment (target concentration, 500 ng/L), low (L) and high (H) concentrations of each test chemical alone (Table 1), and the two test chemical treatments in conjunction with the 500 ng TB/L (i.e., TB + CAL, TB + CAH, TB + EE2L, TB + EE2H, etc.). With the exception of the TCC experiment (21 d), exposures were conducted for 14 d, a duration which, based on previous work with similar test concentrations of TB, should result in a high proportion of exposed females developing visually discernible nuptial tubercles (Ankley et al., 2003, 2004; Martinović et al., 2008).

2.2. Biological measurements

At conclusion of the exposures, the fish were anaesthetized with buffered tricaine methane sulfonate (MS-222), and their blood was sampled from the caudal artery/vein with a microhematocrit tube. Nuptial tubercles were enumerated and scored based on a combination of count data and a subjective assessment of size/expression using a scale of 1 (smallest) to 3 (Jensen et al., 2001; Martinović et al., 2008). Plasma concentrations of VTG in the fish were determined using an enzyme-linked immunosorbent assay (Korte et al., 2000).

2.3. Chemical measurements

Stock solution and tank water concentrations of all test materials were measured at least two times per week. Concentrations

Table 1
Experimental details for assays with five chemicals tested in conjunction with 17 β -trenbolone (500 μ g/L).

Test chemical	Mechanism of action ^a	Water concentrations (μ g/L)	Test duration (d)	Number of fish per replicate ^b	Number of replicates
Cyproterone acetate	AR antagonist	20, 200	14	4:4	2
17 α -Ethinylestradiol	ER agonist	0.001, 0.01	14	1:1	12
Bisphenol A	ER agonist	10, 100	14	4:4	4
Triclocarban	Androgen enhancer	5, 10	21	2:4	4
Ammonia	None known	1000, 5000	14	4:4	2

^a Presumptive mechanism of action within the hypothalamic–pituitary–gonadal axis. AR: androgen receptor, ER: estrogen receptor.

^b Males:females.

of TB were determined using direct injection, high-pressure liquid chromatography (HPLC) with fluorescence detection. Water samples (100–500 μ L) were injected onto a Synergi-Hydro RP-C18 column (3 \times 75 mm; Phenomenex, Torrance, CA, USA) and eluted isocratically with 70% methanol/water at a flow rate of 0.25 mL/min at 30 °C. Trenbolone was detected using excitation and emission wavelengths of 364 and 460 nm, respectively, and concentrations determined with an external standard method of quantification. Detection limit for the analysis typically was 50 ng/L. Lake Superior water (method) blanks, spiked-water samples, and duplicates from one or more of the exposure tanks were analyzed with each set of samples. No TB was detected in method blanks, control water, or treatment tanks containing only CA, EE2, BPA, TCC or AM. Mean recoveries of TB from spiked samples and agreement among duplicate TB analyses conducted over the course of the experiments were consistently greater than 73%, similar to previous studies we have conducted with the androgen (Ankley et al., 2003; Martinović et al., 2008).

A modification of the method described by Christiaens et al. (2003) was used for CA analyses. Water samples (200 μ L) were directly injected into an HPLC with diode array detection (280 nm), using a Synergi-Hydro RP 4 μ m column (2 \times 75 mm; Phenomenex). An isocratic elution, with a mobile phase of 70% acetonitrile and 30% phosphate buffer (0.015 M, pH 6.7), at a flow rate of 0.2 mL/min and column temperature of 25 °C was employed. An external standard method of quantification with a six-point calibration curve was used. No CA was detected (detection limit, 2 μ g/L) in the method blanks, control, or TB-only tanks. The mean (standard deviation; SD) percent recovery of CA from spiked-water samples was 92 (1.5, n = 5), and the percentage agreement among duplicate samples was 97 (4.8, n = 5).

Water concentrations of EE2 were determined using LC–mass spectroscopy (LC/MS). Water samples (100 mL) were concentrated prior to LC/MS analysis using 200 mg StrataX (Phenomenex) solid phase extraction (SPE) columns. The SPE columns were activated with methanol and rinsed with de-ionized water, followed by sample application at 5 mL/min. The loaded columns were rinsed with de-ionized water then eluted with 100% methanol. The methanol fraction was brought to dryness using nitrogen and then re-suspended in 10% methanol/water for subsequent LC/MS analysis. An 1100 LC/MSD (Agilent Technologies, Wilmington, DE, USA) equipped with a quadrupole mass analyzer was used for the analyses. Each sample extract was injected onto a Zorbax SB C-18 2.1 \times 75 mm (Agilent) column at 30 °C. The mobile phase composition began at 50% methanol/water and a gradient method finished at 95% methanol/water at a flow rate of 200 μ L/min. Positive ion mass spectral data were acquired using a photoionization source with a toluene dopant. Quantifications were made using the 279 ion. The mean percent recovery (SD, n = 4) of EE2 in spiked matrix samples was 62 (0.15) and 70 (0.05), respectively, in the 1 and 10 ng/L treatments. The mean percent agreement (SD) between duplicate measurements was 93 (8.2, n = 8). No EE2 was detected in the method blanks, or control or TB-only tanks (detection limit, 1 ng/L).

Concentrations of BPA were measured using a modification of the method of Yoon et al. (2003), with HPLC and fluorescence detection. Water samples (35 μ L) were injected onto a Synergi-Hydro RP-C18 column (2 \times 50 mm; Phenomenex) and eluted isocratically with 40% acetonitrile/phosphate buffer (15 mM monobasic, pH 3.3) at a flow rate of 0.20 mL/min at 40 °C. The BPA was detected using excitation and emission wavelengths of 275 and 300 nm, respectively. An external standard method with a six-point calibration curve was used to quantify BPA. No BPA was detected in Lake Superior water method blanks (detection limit of 0.1 μ g BPA/L) but a small peak, which co-eluted with BPA, was detected in control and treatment tanks containing only TB. Subsequent MS work confirmed that the peak is not BPA, but at this time the peak remains unidentified (unpublished data). Mean (SD) percent recovery of BPA from spiked-water samples was 98 (2, n = 10), and percentage agreement among duplicate samples was 99 (1, n = 20).

Water concentrations of TCC were analyzed using a modification of the HPLC method described by Ying et al. (2007), with diode array detection at 265 nm. Water samples (100 μ L) were directly injected onto a Zorbax SB C18 column (2.1 \times 75 mm; Agilent) and eluted isocratically with 75% acetonitrile/25% water at a flow rate of 0.2 mL/min and column temperature of 21 °C. An external standard method of quantification with a six-point calibration curve was used. No TCC was detected (detection limit, 0.9 μ g/L) in the method blanks, control, or TB-only tanks. The mean (SD) percent recovery of TCC from spiked-water samples was 86 (3.2, n = 7), and the percentage agreement among duplicate samples was 89 (12.3, n = 20).

Concentrations of AM in water from the exposure tanks were determined according to manufacturer's recommendations using an automated ion analyzer (QuikChem 8000; Lachat Instruments, Loveland, CO, USA). No AM was detected (detection limit, 0.01 mg/L) in method blanks, control or TB-only tanks. Recoveries of AM from spiked samples, and agreement among duplicate analyses were always greater than 90%.

2.4. Data analysis

Analyses were performed using SYSTAT 11 (SYSTAT Software, San Jose, CA, USA). Analysis of variance followed by Bonferroni-adjusted multiple comparisons test was used to test for differences between treatments. When necessary, data were transformed to achieve normality and/or reduce variance heterogeneity. Results were considered significant at $p \leq 0.05$. All data are presented as mean (standard error; SE) of the replicate exposure units at each treatment.

3. Results

3.1. Cyproterone acetate

The measured concentration of TB in the CA study was similar in all tanks receiving the androgen, with a mean (SD, n = 30) value across the TB, TB + CAL, and TB + CAH treatments of 505 (84) ng/L.

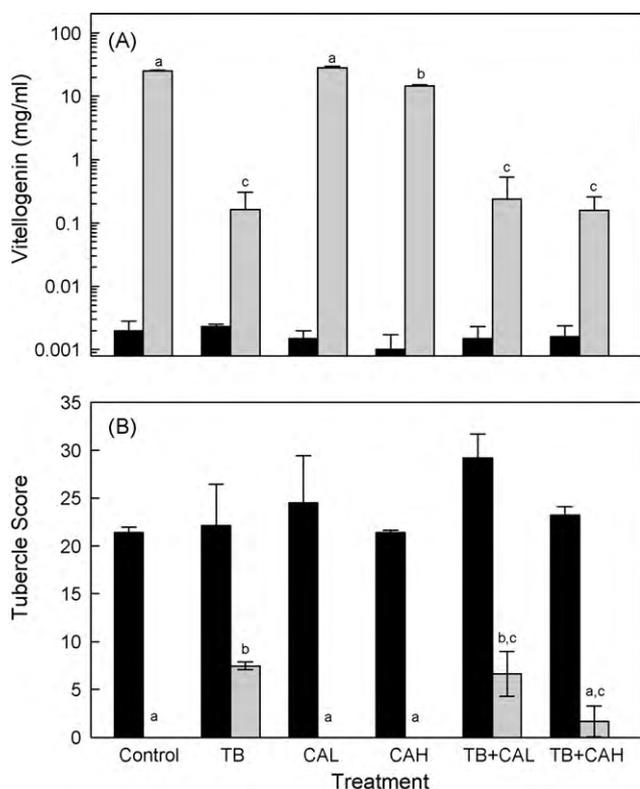


Fig. 1. Effects of 17 β -trenbolone (TB), and low (L) or high (H) cyproterone acetate (CA) exposure, singly or in combination, on (A) plasma vitellogenin concentrations, and (B) tubercle score in male (dark bars) and female (light bars) fathead minnows. Bars indicate the mean and standard error for two replicate tanks per treatment. Different letters indicate similarities and differences among the treatments, analyzed within sex.

Mean (SD, $n = 10$) measured water concentrations of CA were about 80% of target values, at 16 (1.6) and 160 (17.0) $\mu\text{g/L}$ in the CA-only tanks, and 16 (1.4) and 154 (15.2) $\mu\text{g/L}$ in the respective mixture tanks.

There was no treatment-related mortality of the fish in the CA experiment. Exposure to TB, alone or in combination with CA, caused marked reductions in plasma VTG concentrations in females (Fig. 1A). The highest concentration of CA alone reduced female VTG, but not to the extent caused by TB. None of the treatments in the CA experiment affected plasma VTG concentrations in male fathead minnows, which remained low to non-detectable (Fig. 1A).

There were no significant effects of TB or CA, alone or in combination, on male tubercle score (Fig. 1B). In the females there was no evidence of tubercle development in control animals or those exposed to CA alone, and TB caused a substantial induction of tubercles (Fig. 1B). The low concentration of CA in combination with TB (CAL + TB) decreased the female tubercle score slightly, but not significantly, while the CAH + TB treatment significantly decreased tubercle score in the females (Fig. 1B).

3.2. 17 α -Ethinylestradiol

The measured concentration of TB in the EE2 experiment was similar across all tanks receiving the androgen (TB, TB+EE2L, TB+EE2H), with a mean (SD, $n = 81$) value of 400 (91) ng/L. Mean (SD, $n = 12$) measured water concentrations of EE2 were close to target values at 1.6 (0.42) and 7.8 (1.3) ng/L in the two EE2-only treatments, and 2.2 (0.76) and 8.9 (2.1) ng/L in the respective TB mixture tanks.

There was no treatment-related mortality in the EE2 experiment. The estrogen caused a substantial, concentration-dependent

induction of plasma VTG concentrations in males that was not modulated by the TB co-exposure (Fig. 2A). In females TB alone caused a reduction in plasma VTG concentration, while EE2H caused a significant elevation over controls (Fig. 2A). Co-treatment of the females with EE2 offset the TB-induced reduction in VTG, resulting in concentrations of the lipoprotein similar to (TB + EE2L) or greater than (TB + EE2H) those observed in controls (Fig. 2A).

Exposure to EE2 did not affect male tubercle status (Fig. 2B). In a relatively rare occurrence (at least in our lab), one control female exhibited a slight amount of tubercle development, as did one female from the EE2H treatment (Fig. 2B). Exposure to TB caused a significant increase in tubercle development in the females, which was not affected by co-treatment with the lower EE2 concentration; however, EE2H caused a significant decrease in TB-induced tubercle production in the females (Fig. 2B).

3.3. Bisphenol A

In the BPA study, the measured concentration of TB was similar in all tanks receiving the androgen, with a mean (SD, $n = 60$) value across the TB, TB + BPAL, and TB + BPAH of 517 (47) ng/L. Mean (SD, $n = 25$) measured water concentrations of BPA were close to target values, at 11.2 (2.0) and 102 (17) $\mu\text{g/L}$ in the BPA-only tanks, and 12.6 (1.2) and 94 (14) $\mu\text{g/L}$ in the respective TB + BPA mixture tanks.

There was no treatment-related mortality in the BPA study. The high test concentration of BPA caused a significant induction of plasma VTG in male fathead minnows (Fig. 3A). Co-exposure with TB reduced this response, albeit to VTG levels still well above controls. Trenbolone alone decreased plasma VTG in females, and the high concentration of BPA significantly increased concentrations of

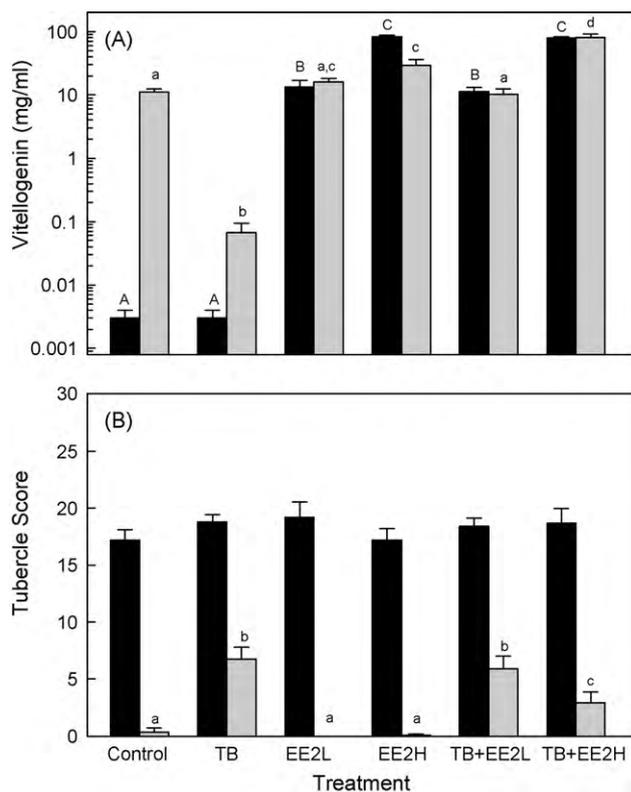


Fig. 2. Effects of 17 β -trenbolone (TB), and low (L) or high (H) 17 α -ethinylestradiol (EE2) exposure, singly or in combination, on (A) plasma vitellogenin concentrations, and (B) tubercle score in male (dark bars) and female (light bars) fathead minnows. Bars indicate the mean and standard error for 12 replicate chambers per treatment. Different letters (upper case, males; lower case, females) indicate similarities and differences among the treatments, analyzed within sex.

the lipoprotein in the females (Fig. 3A). Co-treatment of TB-exposed females with BPAH significantly offset the reduction in plasma VTG caused by the androgen (Fig. 3A).

The high test concentration of BPA alone caused a significant reduction in basal male tubercle expression (Fig. 3B). No tubercles were observed in females from the control or BPAL groups, while one female in the BPAH treatment had a slight amount of tubercle development (Fig. 3B). Co-treatment of TB-exposed females with the high concentration of BPA reduced expression of tubercles caused by the androgen (Fig. 3B).

3.4. Triclocarban

Concentrations of TB in the TCC study were similar across all tanks receiving the androgen (TB, TB+TCCL, TB+TCCH), with a mean (SD, $n=90$) value of 416 (60) ng/L. Mean (SD, $n=28$) measured water concentrations of TCC were close to target values at 6.1 (1.8) and 9.8 (2.2) $\mu\text{g/L}$ in the TCC-only tanks, and 5.5 (1.0) and 10.5 (1.5) $\mu\text{g/L}$ in the respective TB+TCC mixture tanks.

Fish exposed to the high TCC concentration (10 $\mu\text{g/L}$) exhibited signs of overt toxicity, particularly in males. Three fish (two males) died in the TCCH and TB+TCCH treatments, and several more males ceased feeding and were relatively inactive. Exposure to TCC, either alone or in combination with TB, did not induce VTG concentrations in male fathead minnows (Fig. 4A). Exposure to TB depressed VTG in females; treatment with TCC alone or in conjunction with the androgen did not affect VTG status (Fig. 4A).

Exposure to TCC, alone or with TB, did not affect tubercle status in male fish (Fig. 4B). In females, the high TCC concentration significantly enhanced the induction of tubercles by TB (Fig. 4B).

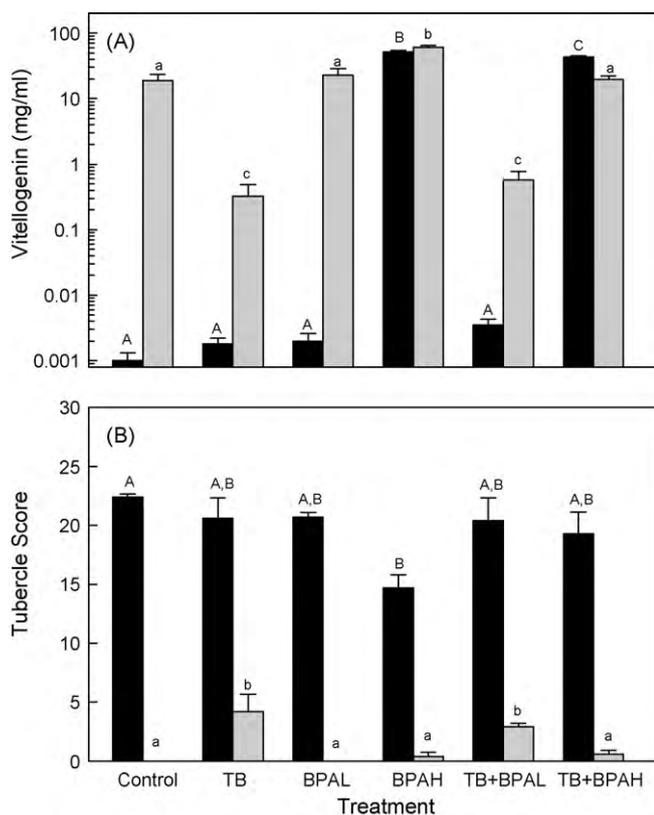


Fig. 3. Effects of 17 β -trenbolone (TB), and low (L) or high (H) bisphenol A (BPA) exposure, singly or in combination, on (A) plasma vitellogenin concentrations, and (B) tubercle score in male (dark bars) and female (light bars) fathead minnows. Bars indicate the mean and standard error for four replicate tanks per treatment. Different letters (upper case, males; lower case, females) indicate similarities and differences among the treatments, analyzed within sex.

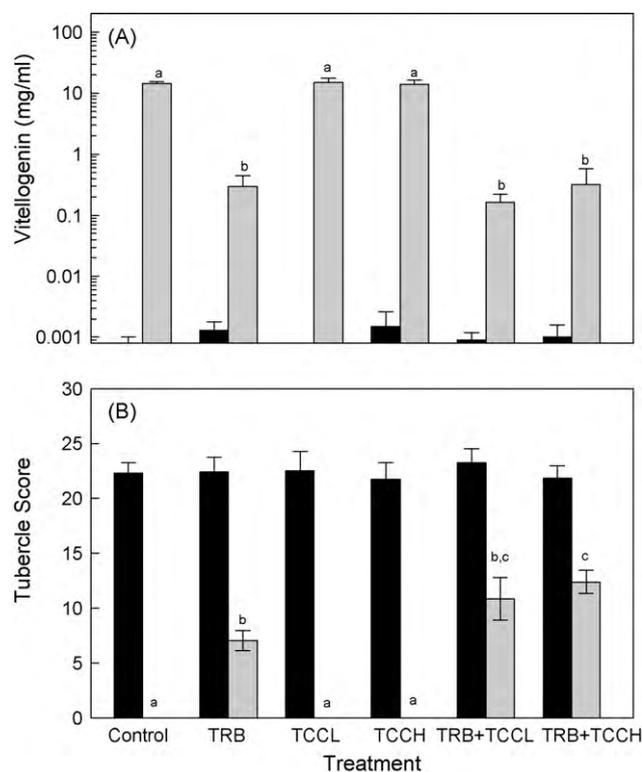


Fig. 4. Effects of 17 β -trenbolone (TB), and low (L) or high (H) triclocarban (TCC) exposure, singly or in combination, on (A) plasma vitellogenin concentrations, and (B) tubercle score in male (dark bars) and female (light bars) fathead minnows. Bars indicate the mean and standard error for four replicate tanks per treatment. Different letters indicate similarities and differences among the treatments, analyzed within sex.

3.5. Ammonia

In the AM study, the concentration of TB was similar in the three treatments receiving the androgen (TB, TB+AML, TB+AMH), with a mean (SD) value of 465 (48, $n=24$) ng/L. Mean (SD, $n=8$) measured water concentrations of AM were consistently within 15% of target values, at 0.96 (0.08) and 5.41 (0.41) mg/L in the AM-only tanks, and 0.84 (0.13) and 5.54 (0.40) mg/L in the respective mixture treatments.

There was no treatment-related mortality of the fish in the AM study. Exposure to TB, alone or in combination with AM, caused marked reductions in plasma VTG concentrations in females (Fig. 5A). Ammonia alone did not affect plasma concentrations of VTG in females. None of the treatments induced VTG in male fathead minnows (Fig. 5A).

Neither TB nor AM (alone) affected normal male tubercle status (Fig. 5B). Neither control nor AM-exposed females developed tubercles (Fig. 5B). Exposure to AM did not decrease TB-induced tubercle development in females; in fact, tubercle score in the TB+AMH treatment was significantly higher than in the TB or TB+AML groups (Fig. 5B).

4. Discussion

Alterations in secondary sex characteristics of sexually dimorphic vertebrates can be relatively unambiguous and easily detected indicators of exposure to endocrine-active chemicals. Almost 35 years ago, Smith (1974) described the induction of male-type dorsal pads and nuptial tubercles in adult female fathead minnows exposed to the synthetic androgen methyltestosterone. More recent studies have better characterized this response with

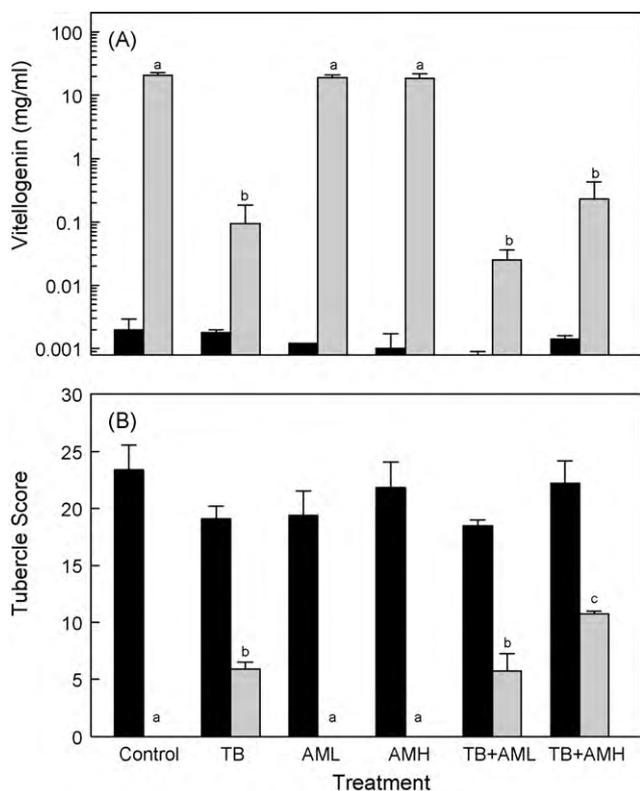


Fig. 5. Effects of 17β-trenbolone (TB), and low (L) or high (H) ammonia (AM) exposure, singly or in combination, on plasma (A) vitellogenin concentrations, and (B) tubercle score in male (dark bars) and female (light bars) fathead minnows. Bars indicate the mean and standard error for two replicate tanks per treatment. Different letters indicate similarities and differences among the treatments, analyzed within sex.

additional chemicals (such as TB) both *in vivo* and *in vitro*, showing that the masculinization is indeed AR-mediated (Ankley et al., 2003; Wilson et al., 2004). This indicates the feasibility of detecting AR antagonists through their ability to block the *in vivo* induction of male secondary sex characteristics by androgens in female fathead minnows. We first examined the approach in studies with flutamide, a pharmaceutical used to treat AR-dependent prostate cancer. Flutamide effectively blocked TB-induced tubercle development in females (Ankley et al., 2004). The fungicide vinclozolin (or, more specifically, one or more of its metabolites) is another established AR antagonist in mammals (Gray et al., 2006). The anti-androgenic nature of vinclozolin in fish also was demonstrable through the ability of the fungicide to block tubercle development in female fathead minnows exposed to TB (Martinović et al., 2008). The present work builds on our prior studies with flutamide or vinclozolin and TB, and shows that another model AR antagonist, CA, also blocks tubercle production in female fathead minnows exposed to TB.

While inhibition of TB-induced tubercle formation in female fathead minnows is a technically reasonable approach to identifying AR antagonists, our studies also showed that two ER agonists produced a similar response. Both EE2 and BPA suppressed tubercle production in females exposed to TB. Based on data from *in vitro* work with the human AR, it appears that BPA may have sufficient affinity for the fathead minnow AR to act as a direct antagonist to TB (Vickie Wilson, U.S. Environmental Protection Agency, RTP, NC, USA, personal communication). Recent studies with kidney cell cultures from female sticklebacks also suggest that at relatively high concentrations BPA may act as an AR antagonist in fish (Jolly et al., 2009). Contributing to the plausibility of this hypothesis, the

water concentration of BPA which reduced tubercle expression in females in the present study was about 200-fold higher than TB. However, the possibility that EE2 reduced the masculinizing effect of TB in female fathead minnows through antagonism of the AR seems less likely than for BPA, especially given that test concentrations of the synthetic estrogen were 50- to 500-fold lower than those of TB.

In male fathead minnows BPA, but not EE2, decreased basal expression of tubercles in sexually mature fish. Given that EE2 produced a level of induction of VTG comparable to BPA in the males, we postulate that de-masculinization of the BPA-exposed males was not modulated through activation of the ER. Again, this effect could be due to antagonism of the AR by BPA. Previous studies have shown that—at least on occasion—AR antagonists such as flutamide or vinclozolin can depress basal tubercle expression in male fathead minnows (Panter et al., 2002; Martinović et al., 2008).

Although EE2 and BPA both inhibited TB-induced masculinization of female fathead minnows and BPA also decreased tubercle expression in males, the chemicals nonetheless were unequivocally identified as ER agonists in our study. Both xenoestrogens caused significant induction of plasma VTG concentrations in males and females. Because VTG induction in males is an abnormal physiological process, occurring against a very low (often non-detectable) background, the response is considered an excellent indicator of exposure to exogenous estrogens (Wheeler et al., 2005). Previous work with several fish species, including the fathead minnow, has documented induction of VTG in male and immature animals by EE2 at water concentrations comparable to those used in this study (for reviews, see U.S. Environmental Protection Agency, 2008; Caldwell et al., 2008). Kang et al. (2007) reviewed the effects of BPA on aquatic organisms, and reported induction of VTG in several fish species, including the fathead minnow, at water concentrations similar to or greater than that producing effects in the present study (100 μg/L).

An interesting observation from the present study involved interactions between TB and the two ER agonists on VTG status in females. Specifically, both EE2 and BPA appeared to offset TB-induced depression in plasma VTG concentrations. Earlier studies from our lab showed that TB reduces VTG concentrations in female fish coincident with a depression in plasma concentrations of testosterone and 17β-estradiol (Ankley et al., 2003). We hypothesized that exposure to the AR agonist resulted in feedback inhibition of steroid synthesis which reduced activation of the ER by estradiol and, hence, VTG production. Data from the present study support this hypothesis. Since both BPA and EE2 activate the ER directly, this would obviate the effects of TB on VTG production through depressed steroid synthesis.

In addition to studies with the AR antagonist CA and the model estrogens EE2 and BPA, we conducted mixture experiments with TB and TCC or AM. Ammonia, a common environmental contaminant, is not known to exert direct effects on the HPG axis, and did not inhibit TB-induced masculinization of female fathead minnows at concentrations that exceeded the chronic AM water quality criterion for fish (at the incoming Lake Superior water pH of 7.8) by more than a factor of three (U.S. Environmental Protection Agency, 1985). Unexpectedly, exposure to the higher test concentration of AM enhanced TB-induced tubercle production in female fathead minnows.

Triclocarban, an anti-microbial chemical commonly found in aquatic systems (Sapkota et al., 2007), recently was reported to enhance AR-mediated responses to testosterone both in mammalian cell cultures and rats *in vivo* (Ahn et al., 2008; Chen et al., 2008; Blake et al., 2010). The mechanism whereby this effect occurs is not known (Ahn et al., 2008; Chen et al., 2008), but our basic mixture study design provided a unique opportunity to assess whether TCC could enhance AR-mediated responses in fish. Triclocarban

did not affect basal tubercle expression in male fathead minnows, but exposure of fathead minnow females to TB in the presence of 10 µg TCC/L slightly, but significantly, enhanced tubercle production. This is consistent with the effects of TCC in mammalian systems (Ahn et al., 2008; Chen et al., 2008), suggesting that the phenomenon is neither class-specific nor related only to testosterone. An interesting question raised by our studies, however, is whether the ability of TCC to enhance activation of the AR receptor is related to a direct interaction of the chemical with the HPG axis, versus some other more generalized mechanism. Specifically, AM also enhanced TB-induced production of tubercles in female fathead minnows. Further work is required to ascertain the mechanistic basis of interactions between TCC and androgens, as well as the significance of this mechanism to human health or ecological risk.

Short-term (typically 21-d) assays with adult fish are being used by regulatory authorities throughout the world as a basis for identifying EDCs (Seki et al., 2006; U.S. Environmental Protection Agency, 2007; Organisation for Economic Cooperation and Development, 2009b). While some endocrine mechanisms are reliably detected in these assays, anti-androgens are more difficult to identify in an unambiguous fashion (Seki et al., 2006; Katsiadaki et al., 2006; Filby et al., 2007; U.S. Environmental Protection Agency, 2007; Organisation for Economic Cooperation and Development, 2009b). Based on experiments described herein and elsewhere (Ankley et al., 2004; Martinović et al., 2008), it appears that a 14-d test with the fathead minnow that considers the effects of an unknown test chemical on TB-induced alterations in tubercle production in females can effectively identify chemicals as AR antagonists. While in the present study two model ER agonists also inhibited tubercle production in TB-treated females, the estrogenic nature of these chemicals was clearly differentiated from that of model anti-androgens (flutamide, vinclozolin, CA) through measurement of plasma VTG titers in male fish from the same exposure.

Previous studies in our lab have shown that chemicals that affect HPG function through depression of steroid synthesis (including aromatase inhibitors) can be detected in relatively short-term (e.g., 8-d) exposures through depression of basal VTG in adult female fathead minnows (Villeneuve et al., 2009; Ankley et al., 2009). Those studies, in conjunction with work with ER- and AR-active chemicals reported herein and elsewhere (Ankley et al., 2004; Martinović et al., 2008), suggest that it should be possible to detect all the HPG mechanisms of current regulatory concern: ER agonists and antagonists, AR agonists and antagonists, and inhibitors of steroidogenesis (and in addition, perhaps, enhancement of AR-mediated responses), using a single 14-d assay similar to that described in this paper. The proposed test could identify chemicals with different endocrine mechanisms through their effects on two easily measured endpoints in adult fathead minnows, occurrence of tubercles and VTG (Table 2). Estrogens induce VTG in males and, occasionally females, and depress TB-induced tubercle production in females; AR agonists cause tubercle production and VTG depression in females; AR antagonists block TB-induced tubercle production in females; and steroidogenesis inhibitors (as well as ER antagonists; Panter et al., 2002) depress VTG in females, but do not affect tubercle status. This proposed assay is conceptually similar to a longer (21-d) test with pre-spawning adult fathead minnows suggested by Panter et al. (2002); however, our use of an androgen co-exposure design increases specificity of the system. While such an assay would not capture ecologically important, integrative responses in fish such as egg production, a single 14-d test capable of discerning multiple, biologically conserved endocrine mechanisms could reduce the number of assays/resources needed for EDC screening, thereby increasing the number of chemicals that could be considered. Testing with additional chemicals in our lab is ongoing to further explore feasibility of the test.

Table 2

Contingency table for effects of endocrine-active chemicals with differing mechanisms of action on vitellogenin (VTG) concentrations and nuptial tubercles (TUB) in fathead minnows. Arrows indicate direction of change. Responses in parentheses indicate a change that can occur, albeit not consistently.

Mechanism of action ^a	Male		Female	
	VTG	TUB	VTG	TUB ^b
ER agonist	↑	(↓)	(↑)	↓
ER antagonist	—	—	↓	— ^c
AR agonist	—	(↑)	↓	↑
AR antagonist	—	(↓)	—	↓
Steroidogenesis inhibitor	—	—	↓	— ^c
Androgen enhancer	—	—	—	↑

^a Refers to primary mechanism within the hypothalamic–pituitary–gonadal axis. ER: estrogen receptor, AR: androgen receptor.

^b Response occurring in conjunction with exposure to 17β-trenbolone.

^c Anticipated response, but co-exposure design not yet conducted with chemical(s) with this mechanism.

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References

- Ahn, K.C., Zhao, B., Chen, J., Cherednichenko, G., Sanmarti, E., Denison, M.S., Lasley, B., Pessah, I.N., Kultz, D., Chang, D.P., Gee, S.J., Hammock, B.D., 2008. *In vitro* biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. *Environ. Health Perspect.* 116, 1203–1210.
- Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 20, 1276–1290.
- Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., Gray, L.E., 2003. Effects of the androgenic growth promoter 17β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ. Toxicol. Chem.* 22, 1350–1360.
- Ankley, G.T., DeFoe, D.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Miracle, A., Hartig, P., Gray, L.E., Cardon, M., Wilson, V., 2004. Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.* 38, 6322–6327.
- Ankley, G.T., Bencic, D.C., Cavallin, J.E., Jensen, K.M., Kahl, M.D., Makynen, E.A., Martinovic, D., Mueller, N.D., Wehmas, L.C., Villeneuve, D.L., 2009. Dynamic nature of alterations in the endocrine system of fathead minnows exposed to the fungicide prochloraz. *Toxicol. Sci.* 112, 344–353.
- Blake, L.S., Martinovic, D., Gray, L.E., Wilson, V.S., Regal, R.R., Villeneuve, D.L., Ankley, G.T., 2010. Characterization of the androgen-responsive MDA-kb2 call line for assessing complex environmental mixtures. *Environ. Toxicol. Chem.* 29, 1367–1376.
- Caldwell, D.J., Mastrocco, F., Hutchinson, T.H., Länge, R., Heijerick, D., Janssen, C., Anderson, P.D., Sumpter, J.P., 2008. Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17 α-ethinyl estradiol. *Environ. Sci. Technol.* 42, 7046–7054.
- Chen, J., Ahn, K.C., Gee, N.A., Ahmed, M.I., Duleba, A.J., Zhao, L., Gee, S.J., Hammock, B.D., Lasley, B.L., 2008. Triclocarban enhances testosterone action: a new type of endocrine disruptor? *Endocrinology* 149, 1173–1179.
- Christiaens, B., Chiap, P., Rbeida, O., Cello, D., Crommen, J., Hubert, P., 2003. Fully automated method for the liquid chromatographic determination of cyproterone acetate in plasma using restricted access material for sample pretreatment. *J. Chromatogr. B* 795, 73–82.
- Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquat. Toxicol.* 81, 219–231.
- Gray, L.E., Wilson, V.S., Stoker, T., Lambright, C., Furr, J., Noriega, N., Howdeshell, K., Ankley, G.T., Guillette, L., 2006. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. *Int. J. Androl.* 29, 96–104.

- Hill, E.M., Evans, K.L., Horwood, J., Rostkowski, P., Oladapo, F.O., Gibson, R., Shears, J.A., Tyler, C.R., 2010. Profiles and some initial identifications of (anti)androgenic compounds in fish exposed to wastewater treatment works effluents. *Environ. Sci. Technol.* 44, 1137–1143.
- Jensen, K.M., Korte, J.J., Kahl, M.D., Ankley, G.T., 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comp. Biochem. Physiol. C* 128, 127–141.
- Jensen, K.M., Kahl, M.D., Makynen, E.A., Korte, J.J., Leino, R.L., Butterworth, B.C., Ankley, G.T., 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. *Aquat. Toxicol.* 70, 99–110.
- Jolly, C., Katsiadaki, I., Morris, S., Le Belle, N., Dufour, S., Mayer, I., Pottinger, T.G., Scott, A.P., 2009. Detection of the anti-androgenic effect of endocrine disrupting environmental contaminants using *in vivo* and *in vitro* assays in the three-spined stickleback. *Aquat. Toxicol.* 92, 228–239.
- Kang, J.-H., Aasi, D., Katayama, Y., 2007. Bisphenol A in the aquatic environment and its endocrine disruptive effects on aquatic organisms. *Crit. Rev. Toxicol.* 37, 607–625.
- Katsiadaki, I., Morris, S., Squires, C., Hurst, M.R., James, J.D., Scott, A.P., 2006. Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive *in vivo* test for detection of environmental anti-androgens. *Environ. Health Perspect.* 114 (Suppl. 1), 115–121.
- Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlanc, G.A., Ankley, G.T., 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17 β -estradiol treatment. *Environ. Toxicol. Chem.* 19, 972–981.
- Martinović, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2008. Reproductive toxicity of vinclozolin in the fathead minnow: confirming an anti-androgenic mode of action. *Environ. Toxicol. Chem.* 27, 478–488.
- Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J., Giesy, J.P., 1999. Effects of waterborne exposure of 17 β -estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat. Toxicol.* 47, 129–145.
- Organisation for Economic Cooperation and Development, 2009a. Workshop report on OECD countries activities regarding testing, assessment and management of endocrine disrupters. Paris, France.
- Organisation for Economic Cooperation and Development, 2009b. 21-Day fish assay: a short-term screening for oestrogenic and androgenic activity, and aromatase inhibition. TG 230. Paris, France.
- Panther, G.H., Hutchinson, T.H., Hurd, K.S., Sherren, A., Stanley, R.D., Tyler, C.R., 2002. Successful detection of (anti-)androgenic and aromatase inhibitors in pre-spawning adult fathead minnows (*Pimephales promelas*) using easily-measured endpoints of sexual development. *Aquat. Toxicol.* 70, 11–21.
- Sapkota, A., Heidler, J., Halden, R.U., 2007. Detection of triclocarban and two co-contaminating chlorocarbanilides in US aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry. *Environ. Res.* 103, 21–29.
- Seki, M., Fujishima, S., Nozaka, T., Maeda, M., Kobayashi, K., 2006. Comparison of response to 17 β -estradiol and 17 β -trenbolone among three small fish species. *Environ. Toxicol. Chem.* 25, 2742–2752.
- Smith, R.J.F., 1974. Effects of 17 α -methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). *Can. J. Zool.* 52, 1031–1038.
- Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., van der Burg, B., 2005. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicol. Sci.* 83, 136–148.
- U.S. Environmental Protection Agency, 1985. Ambient water quality criteria for ammonia. EPA-440/05-85/001. Washington, DC.
- U.S. Environmental Protection Agency, 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) report. Office of Prevention, Pesticides and Toxic Substances, Washington, DC.
- U.S. Environmental Protection Agency, 2007. Fish screening assay integrated summary validation report. Endocrine Disruptor Screening Program, Office of Science Coordination and Policy, Office of Prevention, Pesticides and Toxic Substances, Washington, DC.
- U.S. Environmental Protection Agency, 2008. Aquatic life criteria for contaminants of emerging concern. Part 2: Illustration of recommendations using data for 17 α -ethinylestradiol (EE2). Office of Water, Washington, DC.
- Villeneuve, D.L., Mueller, N.D., Martinovic, D., Makynen, E.A., Kahl, M.D., Jensen, K.M., Durhan, E.J., Cavallin, J.E., Bencic, D., Ankley, G.T., 2009. Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. *Environ. Health Perspect.* 117, 624–631.
- Weiss, J., Hamers, T., Thomas, K., van der Linden, S., Leonards, P., Lamoree, M., 2009. Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. *Anal. Bioanal. Chem.* 394, 1385–1397.
- Wheeler, J.R., Gimeno, S., Crane, M., Lopez-Juez, E., Morrirt, D., 2005. Vitellogenin: a review of analytical methods to detect (anti) estrogenic activity in fish. *Toxicol. Mech. Methods* 15, 293–306.
- Wilson, V.S., Cardon, M.C., Thornton, J., Korte, J.J., Ankley, G.T., Welch, J., Gray, L.E., Hartig, P.C., 2004. Cloning and *in vitro* expression and characterization of the androgen receptor and isolation of estrogen receptor α from the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.* 38, 6314–6321.
- Ying, G.-G., Yu, X.-Y., Kookana, R.S., 2007. Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modeling. *Environ. Pollut.* 150, 300–305.
- Yoon, Y., Westerhoff, P., Snyder, S.A., Esparza, M., 2003. HPLC-fluorescence detection and adsorption of bisphenol A, 17 β -estradiol, and 17 α -ethinyl estradiol on powdered activated carbon. *Water Res.* 37, 3530–3537.