Effects of bisphenol A and triclocarban on brain-specific expression of aromatase in early zebrafish embryos

Eunah Chung, Maria C. Genco, Laura Megrels, and Joan V. Ruderman

Estrogen regulates numerous developmental and physiological processes. Most effects are mediated by estrogen receptors (ERs), which function as ligand-regulated transcription factors. Estrogen also regulates the activity of GPR30, a membrane-associated G protein-coupled receptor. Many different types of environmental contaminants can activate ERs; some can bind GPR30 as well. There is growing concern that exposure to some of these compounds, termed xenoestrogens, is interfering with the behavior and reproductive potential of numerous wildlife species, as well as affecting human health. Here, we investigated how two common, environmentally pervasive chemicals affect the in vivo expression of a known estrogen target gene in the brain of developing zebrafish embryos, aromatase AroB, which converts androgens to estrogens. We confirm that, like estrogen, the well-studied xenoestrogen bisphenol A (BPA, a plastics monomer), induces strong brain-specific overexpression of aromatase. Experiments using ER- and GPR30-selective modulators argue that this induction is largely through nuclear ERs. BPA induces dramatic overexpression of AroB RNA in the same subregions of the developing brain as estrogen. The antibacterial triclocarban (TCC) by itself stimulates AroB expression only slightly, but TCC strongly enhances the overexpression of AroB that is induced by exogenous estrogen. Thus, both BPA and TCC have the potential to elevate levels of aromatase and, thereby, levels of endogenous estrogens in the developing brain. In contrast to estrogen, BPA-induced AroB overexpression was suppressed by TCC. These results indicate that exposures to combinations of certain hormonally active pollutants can have outcomes that are not easily predicted from their individual effects.

Endocrine disruptors | Environmental estrogens

Estrogen plays important roles in many developmental and physiological processes (1). Best understood are its reproductive functions in females, where circulating estrogen produced by the ovaries regulates many of the events involved in sexual maturation and fertility. Estrogen also affects the development and maintenance of numerous organ systems in both sexes, including bone, the cardiovascular system, and the central nervous system. In the brain, for example, estrogen affects neuronal cell migration and survival, axonal outgrowth, synaptic plasticity, cognitive and social development, and memory (1, 2). Nuclear and membrane-associated estrogen receptors (ERs) are expressed in many regions of the brain, as is GPR30, a membrane-associated G protein-coupled estrogen receptor (2–6). Aromatase, the enzyme that synthesizes estrogen, is also expressed in the brains of both sexes, thus generating estrogen locally. Among the many different signals that can regulate aromatase gene expression is estrogen itself, providing opportunities for both positive and negative feedback regulation (1).

Over the past several decades, a steadily increasing number of synthetic chemicals used in agriculture, manufacturing, and consumer products have been identified as xenoestrogens (also called environmental estrogens), compounds that can mimic or interfere with the actions of endogenous estrogens and thus act as endocrine disruptors. Well-studied examples include many pesticides (7–9), surfactant derivatives (10, 11), and the plastics monomer bisphenol A (BPA) (12–14). Many xenoestrogens can directly regulate the transcriptional activities of ERs (7, 8, 15–17), but their effects are not always identical to those of natural estrogen (18). Xenoestrogens can also affect physiological and developmental programs through membrane-associated subsets of ERs and GPR30 (14, 19).

Most mammals contain a single aromatase gene (CYP19), the expression of which is controlled by multiple tissue-specific promoters that respond to estrogen or other physiological regulators (1). In contrast, many fish, including zebrafish, contain a pair of aromatase genes: cyp19a1a/cyp19a1b and cyp19a2/cyp19a2 (20), referred to here as AroA and AroB, respectively. The AroA gene, which is expressed most strongly in the ovary, lacks recognizable estrogen response elements and is not induced by estrogen. The AroB gene, which is expressed most strongly in the developing and adult brains of both sexes, contains estrogen response elements and is estrogen-responsive. Sawyer et al. (21) identified the period around 72 to 96 h postfertilization (hpf) as a time when the AroB gene is especially sensitive to further induction by exogenous estrogen. Thus, as noted in that study and others, the AroB gene has the potential to be useful in screening chemicals and environmental samples for estrogen-like activities and their effects on the CNS. Here, we have investigated the effects of two pervasive synthetic chemicals, BPA and triclocarban (TCC), on expression of AroB in developing zebrafish embryos.

BPA was among several orally active, nonsteroidal estrogenic compounds synthesized in the 1930s, but its activity was too low for clinical applications. In the 1950s, BPA found new use in the production of polycarbonate plastics and epoxy resins, which are now used in a wide variety of consumer products, including many food-packaging materials. Currently, BPA is one of the highest production volume chemicals worldwide (13, 22). BPA’s estrogenic properties were, in a way, accidentally rediscovered in the early 1990s as an activity that leached from polycarbonate laboratory flasks in quantities sufficient to stimulate ER-dependent gene transcription (12). Human exposures appear to come mainly from BPA that has leached from food containers lined with BPA-based resins (23). BPA is also a significant contaminant of wastewater and biosolids from sewage treatment plants, with potential for affecting wildlife at environmentally relevant concentrations (24–26). Experimental work with mammals shows that fetal, neonatal, and adolescent exposures can affect de-
veloping organ systems in ways that have adverse consequences in later life. For example, transient early exposures to BPA result in alterations in the morphology of developing mammary glands and increase the incidence of mutagen-induced breast cancer in adults (13, 27, 28). In the brain, early exposures to BPA can lead to permanent changes in brain morphology, disruptions in behavior that include increased levels of aggression and anxiety, and alterations in learning, memory, exploration, and emotional responsiveness (13, 29). In humans, the first prospective epidemiological study of prenatal BPA exposure and childhood behavior suggests that it may be associated with increases in hyperactivity and aggression in a subset of 2-y-olds (30).

TCC is an antimicrobial agent that is widely used in soaps and other personal care products, with some absorption occurring through the skin (31). TCC is one of the most frequently detected contaminants of wastewaters and biosolids derived from sewage treatment plants (32). Several studies have shown that TCC can bioaccumulate, raising concerns about potential effects on soil microorganisms, wildlife and—via application of biosolids to agricultural fields—humans (26). TCC by itself exhibits little or no agonist activity, but it increases hormonestimulated transcription of androgen receptor (AR) and ER reporter constructs in mammalian tissue culture cells by up to 30% (33). In vivo, TCC strongly amplifies effects of testosterone on male sex organs in castrated male rats (34, 35). In snails, TCC can stimulate an increase in embryo production, a response that has been used previously in that system to assay for xenosterogens (36). To date, no studies of TCC’s effects on transcription of known estrogen target genes have been reported.

Here, we confirm that BPA, like estrogen, induces strong overexpression of AroB in early zebrafish embryos. Experiments using ER- and GPR30-selective modulators argue that BPA induces AroB largely through ERs. BPA and estrogen induce strong overexpression of AroB RNA in the same discrete sub-regions of the developing brain. TCC alone does not stimulate any significant increase in AroB expression but strongly enhances the overexpression of AroB by exogenous estrogen. This result identifies a known estrogen target gene whose expression is amplified in vivo by TCC. These findings indicate that both BPA and TCC have the potential to elevate expression of AroB and, thus, the production of endogenous estrogens in the developing brain. In contrast to TCC’s enhancement of estrogen-induced AroB expression, TCC suppressed BPA-induced AroB overexpression. This result adds to growing concern (17) that responses to combinations of certain hormonally active pollutants can have effects that would not have been predicted from their individual effects.

Results

BPA Induces Overexpression of AroB in Zebrafish Embryos. Although AroB RNA levels can be quantified in single embryos, there is variation among individual embryos (37). Thus, to test effects of various compounds, we used groups of 30 embryos. In most experiments, embryos were treated from 72 to 96 hpf with indicated concentrations of estrogen (17β-estradiol) or other compounds. AroA and AroB RNA levels relative to β-actin RNA were quantified by qRT-PCR, as described previously (21).

As expected from previous work (21), exposures to estrogen ranging from 1 to 1,000 nM induced strong overexpression of AroB but had no effect on AroA (Fig. 1). In most experiments, treatment with 1 nM estrogen showed at least a 10-fold induction, and treatment with 10 nM estrogen led to 30- to 90-fold induction, close to the maximal induction achieved by exposure to 100 or 1,000 nM estrogen. However, occasional batches of embryos showed lower levels of induction despite the same standardized culture and exposure protocols. We were not able to link this difference to any identifiable factors. One possibility is that the difference is associated with unknown genetic variations among individual embryos that result in high and low expressers (37).

In previous work where zebrafish were exposed to estrogen or BPA earlier (2–48 hpf embryos) or much later (17–20 d after fertilization), AroB induction was relatively low (38, 39). In contrast, when exposed during the more estrogen-sensitive window of 72 to 96 hpf used here (21), BPA was capable of much stronger induction. In Fig. 1B, for example, 5 μM BPA induced about 25-fold overexpression of AroB; this increase was nearly half of that induced by 10 nM estrogen. In Fig. 2A, 5 μM BPA induced AroB to about the same level (~80-fold) as 10 nM estrogen.

Fig. 1. Estrogen and BPA induce strong overexpression of AroB in zebrafish embryos. Groups of 30 zebrafish embryos were treated from 72 to 96 hpf with the indicated concentrations of 17β-estradiol (E2), BPA, or DMSO. Levels of AroA and AroB RNA, relative to β-actin RNA were determined by qRT-PCR. (A) AroA induction (Upper); AroB induction (Lower). (B) AroB induction.

Fig. 2. Induction of AroB by BPA is ER-dependent. Embryos were treated from 72 to 96 hpf with 1 nM or 10 nM estrogen or 5 μM BPA, either alone or in combination with the ER antagonist ICI 182,780 at concentrations (1–9.5 μM) as indicated at the bottom of each panel. AroB levels were determined by qRT-PCR. (A and B) Results from high and low expressers, respectively.
trogen. These results confirm and extend previous findings that BPA can induce overexpression of a developmentally relevant estrogen target gene in vivo.

**Induction of AroB by BPA Is Suppressed by the ER Inhibitor ICI 182,781.** Numerous cell-based reporter gene studies show that BPA can activate ERs; moreover, many BPA effects can be suppressed by the ER-antagonist ICI 182,781 (ICI) (14). Given that much of the increase in zebrafish AroB induced by estrogen is suppressed by ICI (40), it might be expected that BPA-induced overexpression of AroB also depends on conventional ERs. However, some xenoestrogens also affect the activities of membrane-associated ERs, including GPR30, which can lead to activation of transcriptional targets (3, 41). Thus, we used ICI, which inhibits ERα and ERβ but activates GPR30 (3, 41), to ask if ERs or GPR30 are involved in BPA’s effect on AroB.

In agreement with previous work (40), estrogen induction of AroB was significantly decreased in the presence of ICI. Two examples are provided in Fig. 2, one showing strong overall induction of AroB by estrogen and BPA (Fig. 2A) and the other where induction by each was lower (Fig. 2B). In both cases, ICI suppressed estrogen- and BPA-induced AroB overexpression with effectiveness comparable to that seen on other estrogen-induced responses in mammalian cells. However, given that some AroB induction was ICI-resistant and BPA has been reported to bind GPR30 (42), we asked whether GPR30 might also be involved.

**GPR30 Agonist G-1 Does Not Induce AroB Overexpression.** To test if GPR30 might contribute to some fraction of estrogen- or BPA-induced AroB overexpression in early zebrafish embryos, we treated embryos with the GPR30 agonist G-1 (43). As shown in Fig. 3A, up to 0.5 μM G-1 (the highest concentration that would stay in solution) induced little or no induction of AroB. Treatment with ICI alone (Fig. 3A) or 4-hydroxytamoxifen (4-OHT), which also activates GPR30 (3, 6), also failed to induce AroB.

As a positive control to check for the activity of G-1 and 4-OHT, we tested their ability to activate cytoplasmic signaling in human SKBr3 breast cancer cells, which lack both ERα and ERβ and in which estrogen activation of GPR30 has been extensively characterized (3, 6). Treatment of SKBr3 cells with estrogen, G-1, and 4-OHT each induced a rapid, transient activation of MAPK (Fig. 3B), just as described previously. The addition of EGF, which induces sustained activation of MAPK (3, 6), served as an additional positive control in this experiment. These results suggest that BPA-induced overexpression of AroB in zebrafish embryos is mediated largely, if not exclusively, by conventional ER-dependent transcription.

**BPA Induces Overexpression of AroB in the Same Regions of the Developing Zebrafish Brain as Estrogen.** AroB is most strongly expressed in several discrete regions of the brain of developing zebrafish embryos, including the telencephalon, hypothalamus, and preoptic areas, and estrogen increases AroB RNA levels in these regions (44–47). To ask if BPA induces AroB in similar or different regions, or elsewhere in the embryo, we first compared expression patterns in embryos exposed at 72 to 96 hpf. This exposure window is the most estrogen-sensitive period in the first 5 d after fertilization (21) and the one for which we have considerable quantitative expression data. Whole-mount in situ hybridizations showed estrogen and BPA induced similar patterns of AroB overexpression in the brain during this period. However, 96-hpf embryos are very fragile (48), making it difficult to compile effective sets of comparisons. Thus, we used embryos that had been exposed from 48 to 72 hpf, when expression is also sensitive to estrogen (21).

As shown in Fig. 4, exposure to 10 nM estrogen induced dramatic overexpression of AroB in regions that closely resembled those previously identified to be sites of estrogen-induced AroB expression (44–47). Ten micromolars of BPA induced strong expression of AroB in virtually identical regions as those induced by estrogen, including the telencephalon, preoptic area, and hypothalamus (Fig. 4). Exposure to 1 μM BPA induced

---

**Fig. 3.** Induction of AroB by BPA is GPR30-independent. (A) Embryos were treated from 72 to 96 hpf with 10 nM estrogen or 5 μM BPA, either alone or with the indicated concentrations of ICI, or with the GPR30 agonist G-1. AroB levels were determined by qRT-PCR. (B) SKBr3 human breast cancer cells were treated with estrogen, 4-OHT, G-1, or EGF at the indicated concentrations. At the designated times, cells were collected and then processed for immunoblotting to detect phosphorylated MAPK and total MAPK. **(Upper)** Both bands represent phosphorylated versions of MAPK.

**Fig. 4.** BPA induces AroB in the same brain regions as E2 in early zebrafish development. Embryos were treated as indicated from 48 to 72 hpf and analyzed by whole-mount in situ hybridization. MBH, mediobasal hypothalamus; POA, preoptic area; Tel, Telencephalon.
lower levels in very similar regions (Fig. 4). No obvious sites of strong expression were seen elsewhere in the embryo, although small changes would not have been detected. These results demonstrate that BPA strongly up-regulates AroB expression in the same regions of the embryo as does estrogen, and argue that exposure to BPA has the potential to stimulate endogenous estrogen production in the developing brain.

**TCC Enhances Overexpression of AroB Induced by Estrogen but Suppresses Overexpression Induced by BPA.** The antimicrobial TCC, which does not appear to bind to ERs, can enhance estrogen-stimulated-transcription of an ER-responsive reporter construct in tissue culture cells in vitro by about 30% to 40% (33). To ask whether TCC can affect the transcription of a known estrogen target gene in vivo, we exposed embryos to TCC, either alone or in combination with estrogen, and compared AroB expression by qRT-PCR. Treatment with TCC alone resulted in only a very small (two- to threefold) effect on AroB RNA levels; in contrast, TCC strongly amplified the effects of exposure to exogenous estrogen (Fig. 5). For example, 1 nM estrogen induced AroB eightfold but the combination of 1 nM estrogen plus 0.25 μM TCC resulted in an 18-fold induction. The ability of TCC to augment estrogen-stimulated transcription was also seen at higher concentrations of estrogen (10 and 100 nM), giving increases of 50% and 65%, respectively. These results confirm and extend previous findings that TCC can enhance effects of estrogen, and now provide a specific example of TCC increasing transcription of a known, physiologically relevant estrogen target gene in vivo.

BPA can substitute for estrogen in numerous settings (13, 14, 49). Thus, it might be expected that TCC would also amplify BPA-stimulated transcription of AroB; however, this was not the case. TCC consistently and substantially suppressed BPA-mediated transcription of AroB (Fig. 5B). Given that the mechanisms by which TCC affects hormone-dependent processes are unknown at this time, it is difficult to offer ideas about TCC’s opposite effects on estrogen vs. BPA-stimulated AroB transcription. Nevertheless, this result adds to growing concern (17) that responses to combinations of certain hormonally active pollutants can have effects, either synergistic or antagonistic, that would not have been predicted from their individual effects.

**Discussion**

This study confirms and extends previous work showing that in developing zebrafish embryos AroB expression is confined almost exclusively to the brain and that exposure to estrogen or certain xenoestrogens, such as BPA, can induce significant overexpression of AroB RNA. Importantly, this study shows that BPA induces strong overexpression of AroB in the same specific subregions of the developing brain as those shown previously to be affected by estrogen, including the telencephalon, hypothalamus, and preoptic areas. Given that BPA was able to induce AroB expression by as much as 60- to 80-fold in our experiments, it would not be surprising if BPA exposures also led to significantly elevated levels of overall aromatase activity in the brain and, thus, elevated levels of locally produced endogenous estrogen.

These effects of BPA on expression of AroB add to growing evidence showing the ability of BPA to affect brain development at molecular, cellular, organ, and functional levels. For example, studies using rodents show that in utero or perinatal exposures to BPA can lead to permanent disruptions in behavior, including increased levels of aggression and anxiety, and alterations in learning, memory, exploration, and emotional responsiveness (see for example, refs. 29 and 50–57). In humans, a recent prospective epidemiological study suggests that prenatal exposure to BPA may be associated with increases in hyperactivity and aggression in a subset of young children (30).

The 5-μM concentration of BPA used in many experiments in this study was chosen because it allowed us to test easily for reproducible effects of BPA on both overall and tissue-specific expression of a well-established estrogen target gene in an in vivo developmental setting. Although this concentration is too high to be environmentally relevant, given that the BPA concentrations in human serum are typically in the low nanomolar range, concentrations as high as 60 nM have been reported in some populations (58, 59). Urinary concentrations of excreted BPA in occupationally exposed workers suggest much higher serum levels in those individuals (60). Moreover, the lower concentrations used in our experiments are only slightly more than one order of magnitude above the current United States “safe” reference dose for humans of 50 μg/kg body weight per day (58). Acute exposure to reference dose levels of BPA could result in overexpression of aromatase or other genes that, even as low as twofold, could have significant effects if it occurs during critical developmental windows (61). Dramatic reproductive and behavioral effects of chronic, low-level, environmentally relevant exposures of fish and birds to the pharmaceutical estrogen 17α-ethinylestradiol and BPA, respectively, have already been documented (25, 62). Moreover, humans and other organisms are routinely exposed to combinations of xenoestrogens and other endocrine disruptors. Although exposure to a low level of any single compound may not have significant effects, responses to particular combinations of endocrine disruptors have the potential to be additive or synergistic (16).

In contrast to BPA, whose estrogenic effects were recognized more than 70-y ago, TCC’s ability to influence processes regulated by steroid hormones was discovered only recently. Ahn et al. (33) screened TCC, triclosan, and related antimicrobials for biological activity toward estrogen, androgen, arylhydrocarbon, and ryanodine receptors. One micromolar of TCC by itself had little agonist activity toward any of these receptors but enhanced hormone-dependent induction of ER- and AR-dependent transcription of reporter constructs in cell-based assays. Enhancement was typically in the range of 20% to 40%, a finding confirmed in subsequent studies (34, 35, 63). In vivo studies using castrated male rats found that exposure to TCC and testosterone together significantly increased the weights of several male sex accessory organs and reproductive tract tissues relative to exposure to testosterone alone (34, 35).

![Fig. 5. The antimicrobial TCC enhances the expression of AroB by E2, but suppresses expression induced by BPA. Embryos were treated from 72 to 96 hpf with estrogen (A) or BPA (B), alone or in combination with TCC. Relative amounts of AroB mRNA were determined by qRT-PCR.](image)
Our work using zebrafish embryos confirms that TCC has enhancing effects on estrogen-stimulated gene transcription and provides a demonstration that TCC can enhance expression of a well-established, physiologically relevant estrogen target gene in vivo. Furthermore, TCC enhancement of estrogen-induced AroB overexpression was readily apparent at 0.25 μM, lower than the 1-μM exposures used in mammalian cell studies, indicating that the in vivo zebrafish AroB expression assay used here is at least as sensitive as mammalian cells used in previous work. Taking advantage of this sensitivity, we examined how TCC affected BPA-induced AroB overexpression. In contrast to estrogen, BPA’s effects were strongly suppressed by TCC. Even in the absence of a mechanistic explanation for this difference, this finding adds to growing appreciation that responses to combinations of endocrine disruptors cannot be predicted simply from responses to individual compounds but, instead, must be directly tested. Taken together, our results indicate that the zebrafish assay has the potential to provide a way to test easily for in vivo effects of TCC and related antimicrobials, alone and in combination with other endocrine disruptors, on numerous other genes and processes regulated by steroid hormones.

TCC is widely used as an antimicrobial agent in bar and liquid soaps, usually present as 1% to 5% by weight. TCC is also added to other personal care products, including cosmetics and deodorants (31). Absorption through the skin appears to be the major route of human exposure; a recent study estimated that an average of 0.1 mg TCC is absorbed during a typical shower using a TCC-containing bar soap (64). Use of antimicrobial soaps remains high despite evidence that washing with TCC-containing soaps is not any more effective than washing with soaps lacking TCC (65). Furthermore, numerous studies show that TCC is one of the most widespread contaminants of surface waters, where it has the potential to affect the development of a wide range of aquatic species (66, 67). TCC is also a common contaminant of treated municipal sewage sludge that is applied to agricultural fields, where it is subject to bioaccumulation in both plants and animals (66, 68). Thus, new information identifying TCC as a novel type of endocrine disruptor—including data from this study—should be taken into account when evaluating TCC’s safety.

**Methods**

**Embryo Treatments and RNA Analysis.** Zebrafish embryos were collected after natural spawnsings, incubated in egg water at 28 °C, and kept on a 14-h light, 10-h dark cycle, as previously described (38). Trichlorocarbanilide (Sigma; #E2757), BPA (Aldrich; #239565), ICI 182,780 (Sigma; #44290), and the GPR30 agonist, G1 (Calbiochem; #371705) were prepared in DMSO (Sigma; #D8418), and diluted in egg water at indicated concentrations. Groups of 30 embryos were incubated in 20 μL at each concentration from 72 to 96 hpf, harvested into Eppendorf tubes, and frozen in liquid nitrogen. RNA was extracted using TRIzol (Invitrogen) and qRT-PCR reactions were carried out using AroA and AroB primers, as described previously (21).

**Whole-Mount in Situ Hybridization.** If necessary, embryos were manually dechorionated. All dechorionated embryos were exposed to test chemicals from 48 to 72 hpf and then processed for in situ hybridization, as previously described (48). An AroB antisense probe was synthesized from a plasmid containing a 1,606-bp fragment of the coding region of AroB (45), (gift of Christopher Lasilitter, Roanoke College, Salem, VA). The construct was linearized with Apal and transcribed in vitro with RNA polymerase (5′, Roche; #101801274001) and DIG-11-UTP RNA mix (Roche; #11277073910) and treated for 20 min with DNase I (Roche; #04716725001). The probe was purified on a QuickSpin G50 column (Roche; #1274015) and stored at −80 °C.

**SKBR3 Cell Culture and Analysis.** Human SKBR3 breast cancer cells (gift of Edward Filardo, Rhode Island Hospital, Providence, RI) were cultured as described previously (66, 67). A zebrafish model of quiescent (38) embryos in fresh phenol-red-free, serum-free medium using 17α-estradiol (Sigma; #E2757), BPA, 4-hydroxytamoxifen (Sigma; #H7904), EGF (Peprotech; #100–15), or GPR30 agonist G1 (Calbiochem; #371705) as indicated. Following treatment, cells were lysed and analyzed by SDS-PAGE (70) followed by immunoblotting. Following transfer to nitrocellulose, membranes were blocked for 1 h in 3% milk in TBST (50 mM Tris pH7.4, 150 mM NaCl, 1% Tween-20), briefly rinsed in TBST, and probed overnight at 4 °C with α-phospho-MAPK antibody (Cell Signaling; #9101) diluted 1:5,000 in 2% BSA in TBST. The membranes were washed in TBST, then incubated in secondary antibody donkey-α-rabbit IgG, HRP-linked (Amer sham; # NA 934) diluted 1:5,000 in TBST for 1 h at room temperature. Phospho-MAPK bands were detected by chemiluminescence (SuperSignal West Pico Chemilumi nescent Substrate; Thermo Scientific). To visualize total MAPK, the same membrane was stripped using Re-Blot Plus-Mild solution (Millipore), blocked with 3% milk, reprobed at 4 °C with α-ERK1/2 total (Cell Signaling; #9102) diluted 1:1,000 in 2% BSA in TBST, and processed further as described above.

**ACKNOWLEDGMENTS.** We thank Gloria Callard and Lucinda Burnam, Sean Megason, Leonard Zon, Ramil Noche, Christopher Lawrence, and Emily Huang for help and advice; all members of the J.V.R.’s laboratory for their interest and encouragement; and especially Sara Wylie and Angelo Kloepfer for their initial efforts in this area. This work was supported by the Harvard University Center for the Environment, the Radcliffe Institute, and the Marine Biological Laboratory Zebrafish Course (supported by National Institutes of Health Grant 2R13NS037791, in which J.V.R. was a student).
22. Vogel SA (2009) The politics of plastics: The making and unmaking of bisphenol A "safety". Am J Public Health 99(suppl 3):5553-5566.

23. von Goetz N, Wormuth M, Scherringer M, Hungerbühler K (2010) Bisphenol A: How the most relevant exposure sources contribute to total consumer exposure. Risk Anal 30:473-487.

24. Crain DA, et al. (2007) An ecological assessment of bisphenol-A: Evidence from comparative biology. Reprod Toxicol 24:225-239.

25. Markman S, et al. (2008) Pollutants increase song complexity and the volume of the brain area HVC in a songbird. PLoS One 3:e1674.

26. Clarke BG, Smith SR (2011) Review of ‘emerging’ organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ Int 37:226-247.

27. Soto AM, Vandenberg LN, Maffini MV, Sonnenschein C (2008) Does breast cancer start in the womb? Basic Clin Pharmacol Toxicol 102:125-133.

28. Soto AM, Sonnenschein C (2010) Environmental causes of cancer: Endocrine disruptors as carcinogens. Nat Rev Endocrinol 6:363-370.

29. Wolstenholme JT, Rissman EF, Connelly JJ (2011) The role of Bisphenol A in shaping the brain, epigenome and behavior. Harm Behav 59:296-305.

30. Braun JM, et al. (2009) Prenatal bisphenol A exposure and early childhood behavior. Environ Health Perspect 117:1945-1952.

31. EPA (2002) Robust Summaries and Test Plans: Triclocarban. http://www.epa.gov/hpw/pubsummaries/triclocarban14186sp.pdf. Accessed January 22, 2011.

32. McClellan K, Halden RU (2010) Pharmaceuticals and personal care products in archived U.S. biosolids from the 2001 EPA National Sewage Sludge Survey. Water Res 44:658-668.

33. Ahn KC, et al. (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.

34. Chen J, et al. (2008) Triclocarban enhances testosterone action: A new type of endocrine disruptor? Endocrinology 149:1173-1179.

35. Duleba AJ, et al. (2011) Effects of triclocarban on intact immature male rat: Augmentation of androgen action. Reprod Sci 18:119-127.

36. Giudice BD, Young TM (2010) The antimicrobial triclocarban stimulates embryo production in the freshwater mudsnail Potamopyrgus antipodarum. Environ Toxicol Chem 29:966-970.

37. Kallivretaki E, Eggen RI, Neuhauss SC, Kah O, Segner H (2007) The zebra finch brain aromatase gene. Comp Biochem Physiol B Biochem Mol Biol 148:43-50.

38. Kishida M, McLellan K, Miranda JA, Callard GV (2001) Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol 129:261-268.

39. Suteri C, M, Ingraham HA (2008) The herbicide atrazine activates endocrine gene networks via non-steroidal NRSA nuclear receptors in fish and mammalian cells. PLoS One 3:e2117.

40. Kishida M, Callard GV (2001) Distinct cytochrome P450 aromatase isoforms in zebrafish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology 142:740-750.

41. Watson CS, Jeng YJ, Kochukov MY (2010) Nongenomic signaling pathways of estrogen toxicity. Toxicol Sci 115:1-11.

42. Thomas P, Dong J (2006) Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of estrogen disruption. J Steroid Biochem Mol Biol 102:175-179.

43. Bologa CG, et al. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat Chem Biol 2:207-212.

44. Menuet A, et al. (2005) Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. J Comp Neurol 485:304-320.

45. Lassiter CS, Linney E (2007) Embryonic expression and steroid regulation of brain aromatase cyp19a1b in zebrafish (Danio rerio). Zebrafish 4:29-57.

46. Tong SK, et al. (2009) A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial gial cells. Genesis 47:67-73.

47. Moutrie K, et al. (2009) Androgens upregulate cyp19a1b (aromatase B) gene expression in the brain of zebrafish (Danio rerio) through estrogen receptors. Biol Reprod 80:889-896.

48. Thiese C, Thiese B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 3:59-69.

49. Toda K, Miyaura C, Okada T, Shizuta Y (2002) Dietary bisphenol A prevents ovarian degeneration and bone loss in female mice lacking the aromatase gene (Cyp19). Eur J Biochem 269:2214-2222.

50. Farabollini F, Porrini S, Della Setta D, Bianchi F, Desi-Fulgieri F (2002) Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ Health Perspect 110(suppl 3):409-414.

51. Rubin BS, et al. (2006) Evidence of altered brain sexual differentiation in mice exposed perinatally to bisphenol A. Endocrinology 147:3681-3691.

52. Miyatake M, Miyagawa K, Mizuo K, Narita M, Suzuki T (2006) Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neureones and astrocytes. J Neuroendocrinol 18:434-444.

53. Kawai K, et al. (2007) Changes in estrogen receptors alpha and beta expression in the brain of mice exposed perinatally to bisphenol A. Regul Toxicol Pharmacol 47:166-170.

54. Palanza P, Gioiosa L, vom Saal FS, Parmigiani S (2008) Effects of developmental exposure to bisphenol A on brain and behavior in mice. Environ Res 108:150-157.

55. Tian YH, Baek JH, Lee SY, Jang CG (2010) Prenatal and perinatal exposure to bisphenol A induces anxiety behaviors and cognitive deficits in mice. Synapse 64:432-439.

56. Xu X, Tian D, Hong X, Chen L, Xie L (2011) Sex-specific influence of exposure to bisphenol-A between adolescence and young adulthood on mouse behaviors. Neuropharmacology 61:556-573.

57. Galea LA, Barha CK (2011) Maternal bisphenol A (BPA) decreases attractiveness of male offspring. Proc Natl Acad Sci USA 108:11305-11306.

58. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007) Human exposure to bisphenol A (BPA). Reprod Toxicol 24:139-177.

59. Lee YJ, et al. (2008) Maternal and fetal exposure to bisphenol A in Korea. Reprod Toxicol 25:413-419.

60. Li DK, et al. (2010) Relationship between urine bisphenol-A level and declining male sexual function. J Androl 31:500-506.

61. vom Saal FS, Hughes C (2005) An extensive new literature considering low-dose effects of bisphenol A shows the need for a new risk assessment. Environ Health Perspect 113:926-933.

62. Kidd KA, et al. (2007) Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci USA 104:8897-8901.

63. Christen V, Crettaz P, Oberli-Schrämmli A, Fett K (2010) Some flame retardants and the antimicrobials triclocarban and triclosan enhance the androgenic activity in vitro. Chemosphere 81:1245-1252.

64. Scheib NH, et al. (2011) Investigation of human exposure to triclocarban after showering and preliminary evaluation of its biological effects. Environ Sci Technol 45:11398-11405.

65. Luby SP, et al. (2005) Effect of handwashing on child health: A randomised controlled trial. Lancet 366:225-233.

66. Chalow TE, Halden RU (2009) Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. J Am Water Works Assoc 95:4-13.

67. Snyder SA, Benotti MJ (2010) Endocrine disruptors and pharmaceuticals: Implications for water sustainability. Water Sci Technol 61:145-154.

68. Snyder EH, O’Connor GA, McAvoy DC (2011) Toxicity and bioaccumulation of bio-solids-borne triclocarban (TCC) in terrestrial organisms. Chemosphere 82:460-467.

69. Fialdo EJ, Quinn JA, Bland KJ, Frackelton AR, Jr. (2000) Estradiol-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14:1649-1660.

70. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.