Exposure to 4-*tert*-Octylphenol Accelerates Sexual Differentiation and Disrupts Expression of Steroidogenic Factor 1 in Developing Bullfrogs

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Sex-specific gonadal steroidogenesis during development is critical to differentiation of the sexually dimorphic phenotype and reproductive function of adult organisms. Environmental contaminants may affect the process of sexual differentiation through disruption of steroid production and/or action. Control of the steroidogenic metabolic pathway is regulated partly by P450 cytochrome hydroxylases, and the expression of many of these enzymes is controlled by the orphan nuclear receptor, steroidogenic factor-1 (SF-1). In mammals, SF-1 expression is critical for development of the reproductive axis and adult reproductive function. In the bullfrog *Rana catesbeiana*, during sequential stages of development encompassing sexual differentiation, SF-1 protein expression becomes elevated in ovaries of sexually differentiating females, whereas expression in testes decreases. We exposed tadpoles to the industrial pollutant octylphenol (OP) for 24 hr before and during the critical stages of sexual differentiation to determine whether this known endocrine disruptor affects sex differentiation and SF-1 expression. We found that both females and males treated with an environmentally relevant low dose (10^{-9} M) of OP underwent early gonadal differentiation. Furthermore, OP exposure disrupted the sexually dimorphic expression of SF-1 that occurs during sexual differentiation. Our results suggest that OP exposure may affect developmental processes that could ultimately influence adult reproductive function and that these disruptive effects may be mediated in partly through disturbances in gene regulation by SF-1. *Key words:* amphibians, bullfrogs, octylphenol, sexual differentiation, steroidogenic factor 1. *Environ Health Perspect* 111:557–561 (2003). doi:10.1289/ehp.5304 available via *http://dx.doi.org/* [Online 3 December 2002]

Endocrine-disrupting compounds (EDCs) are ubiquitous in the environment and have been documented to have deleterious effects in many species including humans (*1*). In particular, if exposure during development disrupts endocrine function that is critical to organogenesis, organ function in the adult organism may be impaired. For this reason, an understanding of the effects of EDCs during development is critical.

In these studies, we used 4-*tert*-octylphenol (OP), a known EDC (*2*), which has been reported to disrupt endocrine function in fish (*3*), frogs (*4*), and rats (*5*). OP is an alkylphenol ethoxylate widely used in detergents, emulsifiers, solubilizers, wetting agents, and dispersants (*6,7*). OP makes up 15–20% of the total alkylphenol ethoxylate market. Furthermore, some alkylphenols are formed in anaerobic portions of sewer systems (*7*). OP has been reported in aquatic environments at levels of 0.084 ppb or 0.407×10^{-9} M (*8*). Exposure of wildlife and human populations to these levels of OP is therefore possible.

The expression of sexually dimorphic phenotypes during embryonic development requires that genetic sexual determination be translated to phenotype by a cascade of events that lead to the formation of a testis or ovary. Depending on the vertebrate group, the ovary or testis then produces steroid hormones that direct male or female sexual differentiation (*9*). Steroidogenesis is controlled by the expression of P450 cytochrome hydroxylases that are regulated by the nuclear transcription factor SF-1 (*10*). Because SF-1 is necessary for appropriate steroid hormone expression, and consequently sexual development (*10*), its disruption may lead to changes in sexual differentiation patterns and/or altered adult reproductive function.

Because amphibians have permeable skin that makes them sensitive to waterborne compounds, they have been used as models in studies on the effects of EDCs on reproduction and development (*4,11,12*). Studies in *Xenopus laevis* tadpoles have shown that treatment with OP skewed the sex ratio toward females (*4*). In another amphibian study, DDT congeners prematurely induced adult female coloration patterns in juvenile *Hyperolius argus* (*11*). Recently the herbicide atrazine was demonstrated to induce hermaphroditic gonad development in *X. laevis* (*12*). Taken together, these studies demonstrate that amphibians are sensitive to exposure to EDCs. Because of the large size of bullfrog tadpoles and their well-defined pattern of sexual differentiation (*13*), they provide an excellent model for determining the effects of EDC exposure on molecular changes that may affect sex differentiation processes.

Binding studies in rats have demonstrated that OP binds the estrogen receptor (ER); however, the binding affinity is 1,000-fold less than that of 17β-estradiol (*2,14*). These ER binding studies provide evidence for potential estrogenic effects of OP. In studies with fetal rat testis, maternal treatment with OP reduced the expression of cytochrome P450 17α-hydroxylase/C17-20 lyase, an enzyme regulated by SF-1 (*15*). In subsequent experiments, maternal OP exposure reduced SF-1 mRNA expression in male offspring, but caused no significant change in expression in female offspring (*5*). These data indicate that SF-1 expression may be influenced by EDC exposure during development. However, it is unclear what the developmental consequences of OP exposure will be on differentiating gonads.

Using bullfrog tadpoles, we found a sexually dimorphic pattern of SF-1 protein expression that emerges at the time of gonadal sexual differentiation (*16*). In females, the expression of SF-1 increases just after the formation of ovaries, and in males SF-1 expression decreases as the testes develop. The purpose of this study was to determine whether there is a critical time during development when OP exerts a disruptive effect on amphibian larval sexual differentiation and whether OP disrupts the pattern of SF-1 expression that occurs concomitantly with the differentiation process.

Materials and Methods

Animals and tissue collection. We collected *Rana catesbeiana* tadpoles from the U.S. Fish and Wildlife Service Buenos Aires National Wildlife Refuge, Pima County, Arizona, in March and April 1999 in accordance with the "Guidelines for the Care and Use of Experimental Animals" of Northern Arizona University. Animals were transported to the laboratory in large containers (53 × 41 × 18 cm) in water from the collection site. Once in the laboratory, the animals were transferred to glass aquaria containing dechlorinated tap water. The animal density for each tank was

20 animals/10 L. They were fed commercial fish food (Tetra Min; Pfizer Inc., Morris Plains, NJ) twice a week. The fish-food ingredients did not contain any known EDCs per the manufacturer's product information. After a 2-week acclimation period, tadpoles were staged as described below and placed in individual glass containers containing 500 mL of dechlorinated tap water. Using a random number generator, we assigned animals an individual number which was placed on the individual glass container and recorded. For all studies, the room was maintained at 25 ± 4°C with 12 hr:12 hr light:dark cycle. The water temperature was 21.9 ± 3.5°C. The pH for all aquaria and individual containers was 6.0–6.5, monitored daily using pH test strips.

Testicular tissue collected from C57/BL6 adult mice (Harlan, St. Louis, MO) was snap-frozen in liquid nitrogen and stored at –80°C until protein extraction for use as reference standards in the Western blots.

Animal staging. Tadpoles were staged according to Gosner (*13*). Specifically, at stage 32, the hindlimb bud has begun to show the early signs of differentiation of one toe. By stage 36, all five toes are notable, but not fully developed. These stages match those described by Swingle (*17*) as the time before, during, and just after sex differentiation.

Treatment of staged animals with octylphenol. Twelve animals from each Gosner stage, 32–36, received 1 of 5 treatments of either 0, 10^{-9}, 10^{-8}, or 10^{-7} M OP, resulting in a total of 240 animals (12 animals/stage/ treatment). A 1-M stock solution of OP (Aldrich, Milwaukee, WI) in 100% ethanol (Fisher Scientific, Pittsburgh, PA) was prepared and stored at –20°C in brown glass until use. The control solution consisted of 100% ethanol stored in an identical manner. We prepared dilutions of the 1 M OP stock solution in ethanol daily, before treatment, and then delivered it into individual bowls with 500 mL dechlorinated tap water such that the final concentration of OP was as listed above. The final concentration of ethanol in all treatments including controls was 4.0×10^6 M. We selected a range of doses that include the dose at which feminization in males has been observed in the amphibian *X. laevis* (*4*). Because we were looking for subtle changes that may result from exposure to OP, we did not use any dose that had been reported to have toxic or lethal effects (*18*).

We treated animals in groups of 12/hr, 3 groups per day, to maintain constancy of treatment exposure across all treatment groups. To minimize the effects of circadian rhythms, each group of 12 was started on the hour beginning at 0800 hr. Therefore, the last of the three groups each day was initiated at 1000 hr. Before treatment, animals were selected at random and staged as described previously, placed individually in one of the five treatment solutions selected at random, with treatment and time recorded for each individual. The glass bowls were placed randomly on the animal rack. All animals were dosed by immersion in the test solution for 24 hr and then killed by immersion in methanesulfonate-222 (0.1%). No animals died during the course of OP dosing. Once killed, animals were then staged, weighed, and one gonad from each animal was harvested for histological analysis, weighed, and placed in tissue-freezing medium (Fisher Scientific). The contralateral gonad was harvested, weighed, and snap-frozen in liquid nitrogen for protein extraction and SF-1 protein quantification. During the 24-hr treatment period we observed no change in the Gosner stage of development for any individual.

Histology. We sectioned embedded frozen tissue at 18 μm in a cryostat and thaw-mounted the tissue to Superfrost slides (Fisher Scientific). Slides were fixed in cold acetone for 30 sec, washed in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), and stained with Mayer's hematoxylin and counterstained in 1% eosin (*19*). We examined individual sections of gonadal tissue to establish sexual phenotype based on morphological criteria (*17*). Animals were determined to be female if there was an observable ovarian vesicle and if large follicles and oocytes were distinguishable. We identified males by the presence of a large medullary region and no distinguishable cortical area and by the densely packed cells. Undifferentiated gonads were characterized by a solid structure with a visible cortex and medulla and no other female or male characteristics. Sexes were determined without the observer knowing the treatment of the individual. In some treatment groups, some of the 12 gonadal tissues did not provide clear enough histology to determine the sex of the individual. We dropped these animals from the overall analysis for both the Western blot SF-1 determination and sex ratios. Therefore, the final *n* value/treatment is sometimes less than 12.

Immunoprecipitation and Western blots. Frozen tissue was lysed in an ice-cold buffer containing 1% Triton-X-100, 150 mM NaCl, 10% glycerol, 0.1% SDS (Fisher Scientific), 50 mM 2 hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES; Biowhittaker, Walkersville, MD), 50 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM activated sodium orthovanadate (Sigma, St. Louis, MO). Samples were homogenized for 30 sec in 500 μL lysis buffer per gonad and incubated at 4°C for 30 min. Samples were then centrifuged at $10,000 \times g$ for 15 min at 4°C. Supernatants were removed, spun again for 15 min at 4°C at $10,000 \times g$, and the final supernatants were stored at –20°C. We determined total protein using the commercial CBQCA protein assay (Molecular Probes, Temecula, CA) using bovine serum albumin as the standard.

The entire sample volume was then diluted to a total protein concentration of 1 mg/mL in phosphate-buffered saline and incubated with 35 μL of a 50% slurry of protein A agarose beads (Pierce, Rockford, IL) in phosphate-buffered saline and rocked at 4°C for 10 min to preclear the samples of endogenous immunoglobulin. Samples were then spun at $14,000 \times g$ for 10 min at 4°C. Supernatants were removed and brought up to 500 μL with the tissue lysis buffer and incubated rocking overnight at 4°C with 5 μg rabbit anti-mouse SF-1 (Upstate Biotech, Lake Placid, NY) per 0.5–1.0 mg sample total protein. Immunocomplexes were bound to phosphate-buffered saline–washed protein A agarose beads at 4°C for 10 min, pulse spun, supernatant was removed, and the complex was resuspended in 60 μL Laemmli sample buffer (nonreducing; 125 mM Tris pH 6.8, 4% SDS, 20% glycerol, and 0.0025% bromophenol blue). We used nonreducing conditions in the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to ensure there was no antibody heavy-chain contamination from the immunoprecipitation step. Complexes were then boiled for 5 min and the beads removed by pulse centrifugation per manufacturer's directions (Pierce). We again determined total protein as previously described, and 20 μg of protein from each supernatant sample was separated on SDS-PAGE, using a 10% gel at 240 V and 40 mA of constant current for 105 min and transferred electrophoretically to nitrocellulose membranes. Immunoblots were probed with rabbit anti-mouse SF-1 at a dilution of 1:2,500 (Upstate Biotech, NY). The first antibody was detected with an alkaline-phosphatase conjugated goat anti-rabbit antibody at 1:10,000 (Pierce) and visualized with
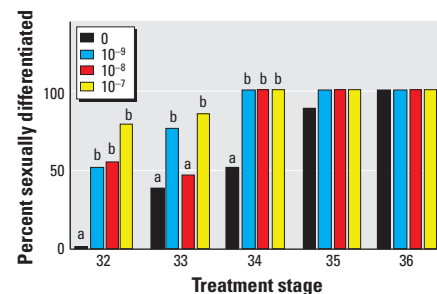
Figure 1. Effects of OP treatment on sexual differentiation by stage was determined by histological analysis (*n* = 9–12 per group). The total percentage of differentiation includes both male and female individuals. Groups with different letters denote statistically significant differences ($p < 0.05$).

Supersignal, a chemiluminescent substrate (Pierce). We prepared mouse testis reference protein the same as frog tissue, and one lane on each gel contained 20 μg of mouse testis reference protein.

Analysis and quantification of SF-1. To quantitate SF-1 protein expression in the developing frog gonads, we first determined the minimum amount of protein necessary to detect SF-1 in the smallest gonadal sample (*16*). Densitometric analysis for each film image of the individual immunoblots was performed using the Eagle Eye II computerized digital camera and Eagle Sight 3.0 software (Stratagene, La Jolla, CA). We determined individual intensity values used to reflect SF-1 expression by using a ratio of the sample intensity to an intensity from the mouse testis reference standard. All samples were then normalized to total gonadal protein for each sample as reported in arbitrary integrated density units (IDU). We analyzed 240 samples on 18 separate gels. The mouse testis standard was randomly placed on each gel to avoid any lane effect; the intergel variability was 8.8%.

Statistical analysis. To determine statistically significant differences among treatment groups compared to controls for the percentage of the total population that had sexually differentiated, we constructed contingency tables and used Fisher's exact test to make comparisons between individual treatments and controls. In the analyses of sex, we compared the observed percentages of females to males in each treatment group at each stage to the expected percentages as observed in controls for the given stage. To determine significant differences in sex differentiation by stage with treatment, we used a binomial test. Before testing for any significant differences in SF-1 expression among treatments by stage, we tested for homogeneity of variance with an F_{max} test with $p = 0.01$. For those groups where the variances were not heterogeneous, we transformed the data using a $\log_{10}$ transformation. SF-1 expression data were tested for normality and homoscedasticity. Differences in SF-1 expression within sex across stages were determined by separate one-way analysis of variance (ANOVA) for each sex. When data showed a significant effect, we further

analyzed data using a Tukey-Kramer post hoc pairwise analysis with the significance for the test set at $p = 0.05$. To determine differences by treatment for each sex within stages, we used a two-way ANOVA with sex and treatment as the response variables. Analysis of the effects of treatment on total animal weight for each stage was performed using a one-way ANOVA for each individual stage.

Results

Effect of OP on timing and outcome of sexual differentiation. We previously observed almost complete sexual differentiation of the gonads in both sexes in bullfrogs by Gosner stage 35 (*13,16*). Here we examined tissues from stages 32–36 for sexually distinct gonadal morphology (Figure 1). For control animals, primary sexual differentiation was not observed at stage 32 ($n = 9$), but the percentage of animals differentiated in controls increased until all animals had either distinct ovaries or testes by stage 36 (Figure 1).

For all OP treatment groups in stages 32–34, a greater percentage of individuals underwent gonadal differentiation than did controls (Figure 1). For animals at stages 35 and 36 differentiation was essentially complete for control and OP-treated animals (Figure 1). These results suggest that OP advanced the stage at which the bipotential gonad differentiates into a morphologically distinct ovary or testis.

We determined whether both females and males had accelerated sexual differentiation as a result of OP treatment and whether OP treatment induced a change in the expected 50:50 sex ratio (Figure 2A). At stage 32, at each OP dose there was a significant increase in the percentage of female tadpoles ($n = 9–12$, $p < 0.05$) compared to control tadpoles. At stage 32, there was also a significant increase in percentage of male sexual differentiation relative to controls ($n = 9–12$, $p < 0.05$) for each dose. At stage 33 (Figure 2B), only male differentiation was significantly different from controls ($n = 11–12$, $p < 0.05$). However, by stage 33 many females in the controls had already differentiated. Similar results are seen for animals treated at stage 34 (Figure 2C; $n = 10–11$, $p < 0.05$). At stages

35 and 36 (data not shown), all control and treatment animals were differentiated. Sex ratios of OP-treated animals did not differ from controls at any dose (data not shown).

Changes in SF-1 expression as a result of OP treatment in differentiating gonads of bullfrog tadpoles. During sexual differentiation in the bullfrog there is a sexually dimorphic expression of SF-1. Relative to undifferentiated bullfrogs there are elevated levels in females but significantly lower levels in males starting at stage 34 (*16*). We investigated the effect of OP treatment on SF-1 gonadal expression during development. In the control groups we found the same expression pattern of SF-1 that we observed previously: SF-1 protein levels were significantly different between females and males beginning at stage 34 (Figure 3; between males and females at the 0 dose across all graphs; ANOVA, $n = 9–11$, $p = 0.0192$, $F = 6.56$). At stage 32, there were no significant differences in SF-1 expression among the OP treatment groups (Figure 3A). Because there was no observable sexual differentiation in the controls, the data labeled "males + undifferentiated" represents 100% of the sample size. At stage 33 there was a significant increase in SF-1 expression in the ovaries of 10^{-8}-M–treated females relative to the males (Figure 3B; ANOVA, $n = 9$, $p = 0.0473$, $F = 5.16$). In the male treatment groups of stage 34, there was a significant difference in the high dose (10^{-7} M) group compared to other treatment groups (Figure 3C; ANOVA, $n = 12$, $p = 0.044$, $F = 4.31$). In stages 35 and 36 there were no significant differences between sexes among treatment groups within each stage (Figure 3D,E). For all OP treatment groups at all stages, there was no difference in SF-1 protein levels between sexes. For each of the OP treatment groups and for all stages, there was no animal loss, nor was there a significant effect of OP treatment on whole animal weight (data not shown).

Discussion

The purpose of this study was to determine the effects of the endocrine disrupter OP on gonadal differentiation in bullfrogs and to determine whether there was any detectable disruption in the gonadal expression of SF-1 during this
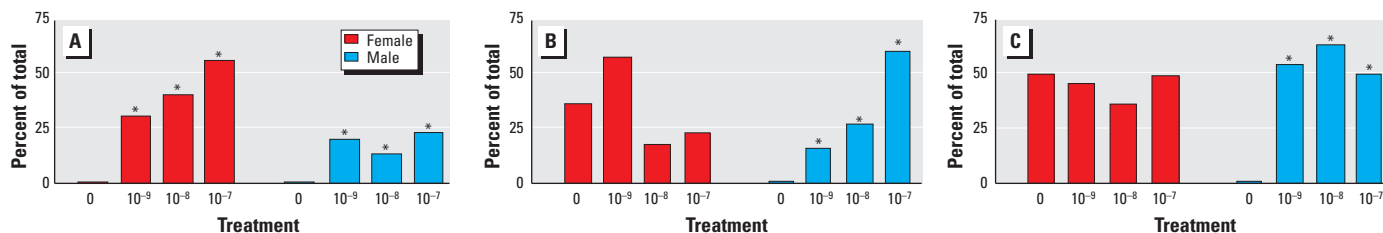
Figure 2. The effects of OP treatment on differentiating females and/or males was determined by histological analysis. Gonads from 0, 10^{-9}, 10^{-8}, or 10^{-7} M OP-treated tadpoles were analyzed to determine sex of differentiated individuals in Gosner stages (*A*) 32, (*B*) 33, and (*C*) 34, where percentage of sexual differentiation was significantly different from controls.
*Groups that are significantly different from controls within each sex by stage ($n = 9–12$ per group, $p < 0.05$).

period of development. Consistent with our previous work, in control groups we saw stage-dependent gonadal differentiation. This is also consistent with previous studies in bullfrog in our laboratory (*16*) and in laboratories of other investigators (*20*). These data indicate that there is a potential critical period (stage 32–34) when gonadal differentiation occurs and may be sensitive to disruption by OP.

Treatment with OP accelerated bullfrog gonadal differentiation in both females and males. We observed acceleration at every Gosner stage and at every OP dose, 10^{-9} M, 10^{-8} M, and 10^{-7} M. The lowest OP dose, 10^{-9} M, is an environmentally relevant concentration that is similar to that detected in aquatic environments of 0.4×10^{-9} M (*8*). As we had observed previously, gonadal differentiation in control animals occurs at an earlier stage in females than males (*16*). OP treatment accelerated gonadal differentiation in both females and males such that each differentiated at earlier developmental stages. OP-mediated male acceleration resulted in gonadal differentiation

that occurred three stages earlier than is observed in controls. Female OP-mediated acceleration of gonadal differentiation occurred at least one stage earlier. Because we did not treat animals before stage 32 of development, we do not know what accelerating effects OP treatment may have during these earlier developmental periods. Although this is the first report of acceleration of gonadal differentiation in any species due to OP treatment, other studies have demonstrated that in rats OP treatment *in utero* accelerates vaginal opening (*21*).

There was no change in sex ratio, as the expected 50:50 distribution of females to males occurred with and without OP treatment. Kloas et al. (*4*) found in another amphibian species, *X. laevis*, that 10^{-8} M OP significantly skews the sex ratio toward females. However, in that study, animals were treated for 12 weeks instead of 1 day as in our study, suggesting that longer-term exposure to OP in *R. catesbeiana* tadpoles may result in not only accelerated differentiation (not

examined in the Kloas et al. study), but also gonadal feminization.

OP has estrogen-like activity, as evidenced by estrogen receptor-binding studies in rats (*14*) and frogs (*22*). OP treatment may have accelerated female bullfrog gonadal differentiation in our studies due to its estrogenic activity. Female bullfrog ovaries are secreting estradiol by stage 35 (*23*), and sexual development of amphibians is sensitive to estrogen; Treatments with estradiol can shift sex ratios to 100% female in *X. laevis* (*4,24,25*), but treatment with estradiol masculinizes already differentiated female tadpole ovaries (*24*). Furthermore, blocking 3-β hydroxysteroid dehydrogenase activity with cyptoterone throughout the differentiation process leads to masculinazation of the gonad (*26*). However, there are no reports of estradiol or other steroid treatment accelerating bullfrog gonadal differentiation as we found here, and we do not yet know if the change in SF-1 expression we found plays a role in this acceleration. Future studies in our laboratory will begin to sort out the interactions among OP, the estrogen receptor, and SF-1 expression. It is unclear whether OP is accelerating differentiation via a pathway that involves binding to estrogen receptors or through a different molecular mechanism.

SF-1 is required for gonadal development in mice (*27*), but the role of SF-1 in bullfrog sexual differentiation has not been delineated. In bullfrogs, SF-1 expression is dimorphic during sexual differentiation (*16*). As bullfrogs develop, SF-1 expression increases in females after they differentiate, but it decreases in males just before differentiation. These results suggests that a decline in SF-1 may be necessary for male testicular differentiation, a finding that merits further investigation. However, if SF-1 were directly involved in the control and/or regulation of sexual differentiation, we might predict that in the groups where OP treatment accelerated sexual differentiation, SF-1 levels should have decreased in males at earlier stages than in controls. Instead, although OP induced testicular development in some males by stage 33, SF-1 levels remained comparable to controls. The same result is seen in males that were treated at any stage. These results suggest that SF-1 may not play a direct role in testicular differentiation in this species. We also found that within stages, the sexually dimorphic expression of SF-1 in stage 34–36 control animals was disrupted by OP treatment. Although the outcome of the gonadal sex did not appear to be affected by these relatively rapid changes in SF-1 protein expression, the long-term affects of this disrupted pattern of SF-1 availability might lead to later anomalies in amphibian reproductive development. Interestingly, exposure to OP in mice also leads to disrupted SF-1 expression (*28*), and
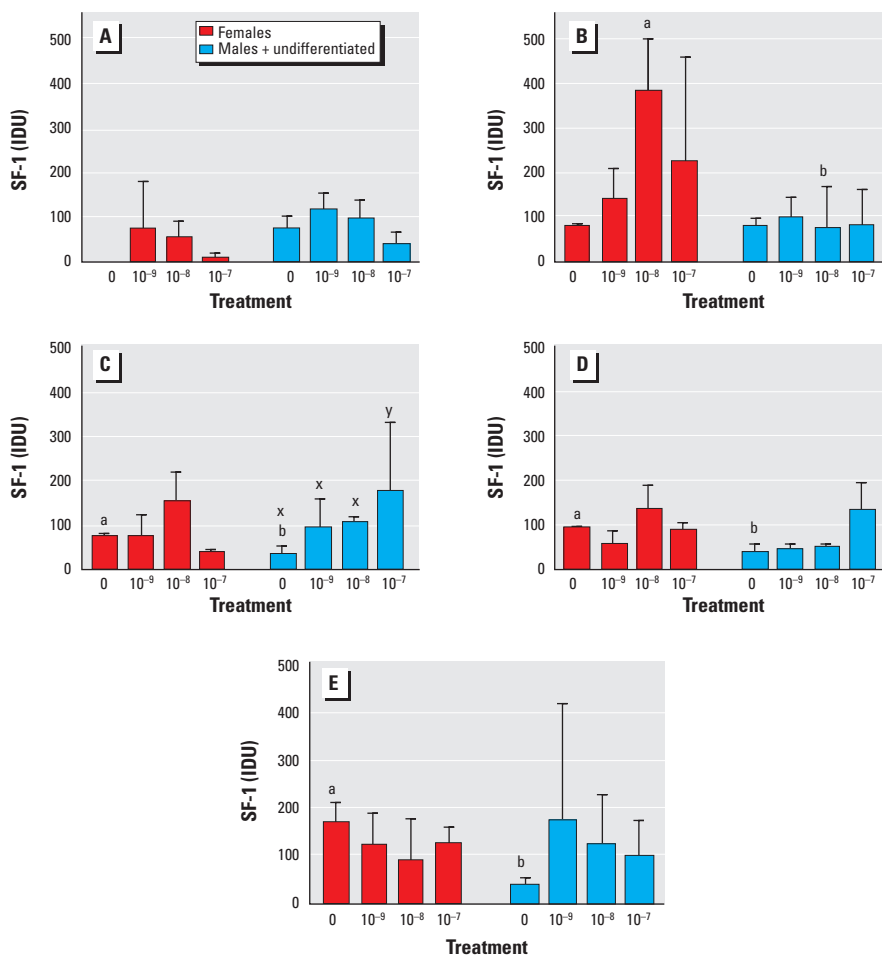
Figure 3. Effects of OP treatment on gonadal SF-1 expression (IDU) was determined for each treatment group. (*A*) Stage 32; (*B*) stage 33; (*C*) stage 34; (*D*) stage 35; (*E*) stage 36. Means with different letters denote statistical significance. Within a stage group, across all samples, a and b denote statistical significance (two-way ANOVA main effect, $n = 9$–11 per stage, $p = 0.0192$, $F = 6.56$); within a stage by sex. Within a sex, across treatment groups, x and y denote statistical significance (two-way ANOVA main effect, $n = 5$–7 per group, $p = 0.0436$, $F = 4.31$).

in rats, offspring of females exposed to OP exhibit changes in gonadal morphology and adult reproductive behavior function (*29*).

Our studies have shown that low-level doses of OP are sufficient to accelerate the onset of sexual differentiation. Also, OP treatment disrupted the sexually dimorphic expression of SF-1 without affecting the outcome of gonadal differentiation with regard to sex. Together, the OP-mediated alterations of gonadal differentiation may have significant effects on bullfrog reproduction. An abnormality in the timing of sexual differentiation and/or SF-1 expression could have a detrimental effect on sexual maturation and, ultimately, on the success of adult reproduction.

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