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REDUCTION OF VITELLOGENIN SYNTHESIS BY AN ARYL HYDROCARBON RECEPTOR AGONIST IN THE WHITE STURGEON (*ACIPENSER TRANSMONTAMUS*)

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Abstract

Migrating white sturgeon (*Acipenser transmontamus*) may be subject to agricultural, municipal, and industrial wastewater effluents that likely contain different classes of endocrine-disrupting contaminants. Concern is mounting about the negative effects of environmental estrogens on fish reproduction; however, in environmental mixtures, the affects from estrogenic compounds may be suppressed by aryl hydrocarbon receptor (AhR) ligands. Indeed, reductions in 17β-estradiol–induced (0.01 and 1 mg/kg) vitellogenin (VTG) levels were observed in white sturgeon coinjected with β-naphthoflavone (BNF; 50 mg/kg), a model for contaminants that activate the AhR. Variation in the time of injection was used to attempt to correlate VTG inhibition to ethoxyresorufin-*O*-deethylase activity. No evidence was found to suggest that the inhibition of VTG is a direct result of enhanced estrogen metabolism by BNF-induced enzymes. Results of the present study are relevant for monitoring programs that measure VTG, because these results show that AhR-active environmental contaminants can repress VTG synthesis, which commonly is used as an indicator of estrogen-mimicking contaminants. Furthermore, suppression of natural estrogen signaling by AhR agonists may have significant effects on fish reproduction.

Keywords

Vitellogenin; Aryl hydrocarbon receptor; Endocrine disruption; Sturgeon; Antiestrogen

INTRODUCTION

Approximately a decade ago, sewage effluents were found to have estrogenic properties, as demonstrated by elevated levels of the egg yolk protein vitellogenin (VTG) in caged fish [1]. Additionally, male fish with ovarian tissue in their gonads have been observed and correlated by proximity to municipal sewage effluents. Natural and synthetic estrogens, and other estrogen-mimicking contaminants in the effluents, are suspected to have feminized these fish [1,2]. These contaminants are examples of endocrine disruptors (EDs), a group of compounds having the potential to interfere with normal species reproduction by altering hormonal signaling. Endocrine disruptors vary in source, from industrial chemicals (e.g., surfactants, detergents, and pesticides) to natural and synthetic hormones to numerous

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compounds used in personal care products. Many end up in waterways, often through sewage effluents, where they likely have the greatest impact on aquatic wildlife.

Predicting the effects of EDs on wildlife is a complex problem, in part because a number of mechanisms exist by which EDs can alter hormone signaling. Focusing on estrogenic and androgenic mechanisms, these chemicals can be either agonists (positive regulators) or antagonists (negative regulators) of hormone receptor response. Accordingly, exposure of an organism to a complex mixture of EDs would produce a response based on all agonistic and antagonistic activities in the mixture rather than on the activity of a single chemical. These mixed-exposure scenarios are highly likely, and such interactions may confound the identification of EDs in effluents. Little effort, however, has been devoted to understanding mixtures of EDs with various mechanisms of action.

Proper estrogen signaling is required for normal growth, development, and reproduction. These events are regulated in part through ligand binding of estrogen to its receptor (ER). In this ligand-activated form, the ER can bind to a specific DNA sequence, the estrogen-response element, leading to coactivator recruitment and activation of the transcription of adjacent genes. In oviparous vertebrates, one of these genes is responsible for the synthesis of VTG, a protein precursor of egg yolk normally found in prespawning females and the expression and synthesis of which is estrogen-inducible. Exogenous estrogens or contaminants with estrogen-like activity can induce synthesis of VTG in males and juveniles, making this protein a useful indicator of chemicals that mimic estrogen [3].

Other compounds have been found to affect estrogen signaling, but not by binding to the ER. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (hereafter referred to as dioxin) and related contaminants activate the aryl hydrocarbon receptor (AhR). These compounds display antiestrogenic activity that appears to occur though cross talk between the ER and AhR as well as effects on estrogen levels [4,5]. Dioxin-like chemicals include planar polychlorinated biphenyls, numerous polycyclic aromatic hydrocarbons, and polychlorinated dibenzofurans and dioxins, although recent evidence suggests that the AhR can bind and be activated by a structurally diverse array of chemicals [6,7]. β-Naphthoflavone (BNF), a relatively nontoxic AhR agonist, has inhibited VTG synthesis in vitro [8–11], and in vivo studies have demonstrated inhibitory and additive effects in rainbow trout (*Oncorhynchus mykiss*) [12] and mosquitofish (*Gambusia holbrooki*) [13]. These studies also suggested that BNF inhibits estrogen-induced VTG synthesis through a mechanism involving the AhR, but the exact mechanism remains unclear.

White sturgeon (*Acipenser transmontanus*) are prized for their caviar and meat, as an important commercial and sport fish, and scientifically, because evolutionarily, they are a very primitive species [14]. Unfortunately, their numbers have declined dramatically from historic levels because of overfishing, poaching, pollution, habitat destruction, and the presence of dams that bar access to their spawning sites [14,15]. In California, USA, sturgeon inhabit the Sacramento–San Joaquin River System as well as San Francisco Bay. These waters receive effluents from various industries and metropolitan sewage treatment plants, plus runoff from the extensively farmed Central Valley, all of which are typical sources of EDs. Sturgeon seldom have been the subject of ED studies, but a few investigators have suggested a link between contaminant levels and typical signs of endocrine disruption, such as the occurrence of intersex fish and gonad abnormalities, reduced gonad size, reduced plasma androgens, and elevated ethoxyresorufin-*O*-deethylase (EROD) activity [16–19]. Because these fish are among the oldest vertebrates still in existence, observations of similar contaminant effects in sturgeon compared to those in other modern teleost fish can attest to the widespread nature of ED effects on all fish and, perhaps, even all vertebrates.

The present study seeks to better characterize the interactions of estrogens and their antagonists in white sturgeon in vivo to provide guidance to scientists and regulatory agencies concerning how to consider antiestrogens when assessing the effects of EDs on fish and wildlife.

MATERIALS AND METHODS

Chemicals and reagents

Vitellogenin was purified from white sturgeon as detailed by Linares–Casenave et al. [20]. That reference also describes the anti-VTG antibody used for this assay, provided by S. Doroshov (University of California–Davis [UCD]). Bradford reagent, 17β-estradiol (E), BNF, goat anti-rabbit immunoglobulin G conjugate, nicotinamide adenine dinucleotide phosphate (NADPH), and bovine serum albumin were purchased from Sigma. The *p*-nonylphenol (NP) was from Fluka Chemika, and the MS-222 (tricaine methane sulfonate) was from Argent Chemical Laboratories.

Animals

Cultured juvenile white sturgeon (*A. transmontanus*), obtained gratis from Stolz Sea Farm, were maintained at the UCD Center for Aquatic Biology and Aquaculture in tanks (diameter, 2 m) supplied with ambient well water (19°C, 0.5‰ salinity) and fed Nelson and Sons Silver Cup™ Feed: Steelhead Formulation (sinking extruded style). Experiments took place over the course of 15 months. Fish weighed approximately 17 and 45 g (age, three to five months) for the E and NP dose– response experiments; 90, 150, and 300 g (age, six to nine months) for the three repeated 7-d BNF plus E experiments; and 500 to 700 g (age, nine to 17 months) for all other experiments. Gonads of sturgeon are not fully sexually differentiated at this size and age [21].

Sturgeon exposures

Exposures were conducted in the UCD Meyer Hall Animal Facility in a temperature-controlled room (19°C) with a 12: 12-h light:dark photoperiod. Fish were housed in recirculating, filtered, aerated tanks, with three fish per 200-L circular fiberglass tank (diameter, 1 m; depth, 0.3 m). Water was deionized, salted to 0.5‰ (Forty Fathoms®/Crystal Sea®, seawater salt mix), and dissolved oxygen (>80%), un-ionized ammonia (<0.05 ppm), temperature (18 – 20°C), and salinity (0.5–1.0‰) were monitored daily. Tanks were cleaned via siphoning, and 30% water changes were performed as needed to maintain water quality.

Before experiments, food was withheld, and fish were acclimated for at least 24 h. For all exposures, anesthetized (MS-222, 100 mg/L) sturgeon were injected intraperitoneally with compounds using 0.5 ml/kg of carrier (ethanol:saline or peanut oil). Blood was sampled from the caudal vasculature, just behind the anal fin, of anesthetized fish with heparin-rinsed syringes and then centrifuged at 2,500 rpm for 10 min, aliquoted, and frozen on dry ice. Fish were killed by overdose of MS-222 (500 mg/L), followed by severing of the notochord, and the liver was removed, weighed, and placed immediately in liquid N_2. Blood plasma and hepatic tissue samples were kept at −80°C until analysis.

The 7-d dose–repose relationships were characterized using E and NP (both in absolute ethanol:saline, 70:30; this proportion of ethanol was needed to dissolve NP, but less ethanol was used in later experiments, when the carrier was only used with E). For the BNF time-course experiment, fish were injected with 50 mg/kg of BNF (BNF is practically insoluble and was injected as slurry in peanut oil), and different fish were sampled after 1, 2, 3, and 7 d.

The first BNF and E mixture experiment lasted 7 d. All fish received two injections, with injection of the carrier when either active agent was not used. First was the BNF injection (peanut oil carrier), which was followed immediately by the E injection (ethanol:saline, 60:40, carrier). The six treatments with three fish each included vehicle control, BNF only (50 mg/kg), a low dose of E (0.01 mg/kg), a high dose of E (1 mg/kg), and both high and low E treatments with BNF added (50 mg/kg). This experiment was repeated three times to obtain nine replicates.

The second BNF and E mixture experiment explored the timing of the inhibition effect and involved treating fish with BNF before E. Three treatments were repeated from the first mixture experiment: Control, the high dose of E (1 mg/kg), and the mixture of the high dose of E and BNF (50 mg/kg; injected simultaneously and indicated as E+BNF-0). In three additional treatments using the same doses, BNF was injected 1 to 3 d before E (indicated as E+BNF-1, E+BNF-2, and E+BNF-3, respectively). In independently conducted experiments, fish were sampled 1 or 2 d after the E injection, as opposed to 7 d in the first mixture experiment. This shorter E exposure time was used to allow less influence of other biological processes to affect the VTG levels. As described above, these experiments were performed with three fish in each treatment and then repeated to achieve six to nine replicates. In these and later experiments, water quality was maintained under static conditions after injection of compounds to avoid contamination of the recirculation system with E.

A NP and BNF mixture experiment included three treatments with six fish each: Vehicle control (peanut oil), a dose of NP high enough to produce a VTG response in this short time period (50 mg/kg), and NP plus BNF (NP+BNF; 50 mg/kg).

Enzyme-linked immunosorbent assay

The procedure for the VTG enzyme-linked immunosorbent assay was based on that described by Linares–Casenave et al. [20]. Microtiter plates (96-well; Nunc, Maxisorb®; Fisher Scientific) were coated (18 h, 4°C) with 3 µg/ml of white sturgeon VTG diluted in carbonate–bicarbonate buffer (pH 9.6). Anti–white sturgeon VTG antibody was preincubated with an equal volume of sample or VTG standard (final antibody dilution, 1:60,000, in phosphate-buffered saline with 0.05% Tween-20 [PBST], pH 7.4, 18 h, room temperature). The following day, coating antigen was washed from the plate (five washes with 250 µl of PBST), and nonspecific binding was blocked (1% bovine serum albumin and 1% nonfat milk in carbonate–bicarbonate buffer, 100 µl, 1 h). After washing, the antibody–analyte mixture was pipetted into triplicate wells (100 µl, 1 h). The plate was washed, and bound antibody was detected with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate, diluted 1:5,500 in PBST (100 µl, 1 h), with a final wash followed by incubation of a solution of the chromogen (3,3′,5,5′-tetramethylbenzidine) and substrate (1% hydrogen peroxide in sodium citrate buffer, pH 5.5). The reaction was terminated with 2 M H_2SO_4, and the absorbance at 450 nm was measured in a Tecan Sunrise™ microplate reader (Phenix Research Products). A log–logit transformation, based on the four-parameter equation of Rodbard [22], was used to analyze the data.

Liver microsome isolation and EROD assay

Livers were defrosted on ice, weighed, minced, and homogenized 1:5 (w/v) in ice-cold buffer (0.02 M Tris and 1.15% KCl, pH 7.4) using Teflon® glass homogenizers (six strokes). The mixture was centrifuged at 9,000 *g* for 20 min at 2°C, and the supernatant was centrifuged at 100,000 *g* for 1 h at 4°C. To wash the microsomal pellet, the supernatant was decanted and the microsomal pellet resuspended in half the original volume of buffer, then recentrifuged at 100,000 *g* as described before. The supernatant was again decanted, and

approximately 1 ml of glycerol/ethylenediaminetetra-acetic acid [EDTA]/Tris buffer (GET; 0.2 g/ml of glycerol, 1 mM EDTA, and 0.01 M Tris-Cl, pH 7.4) was added per gram of original tissue. The microsomes were resuspended with a small Teflon glass hand homogenizer, aliquoted, and stored at −80°C until analysis.

Microsomal protein was measured with the Bradford Assay (Bio-Rad Laboratories), and solutions were diluted with GET buffer to a concentration of 1 mg/ml. Cytochrome P450 (CYP) 1A1/1B1 activities were assessed by measuring EROD activity. Assays were performed in black, clear-bottomed, 96-well plates (Costar®; Fisher Scientific) using a modification of procedures described in detail elsewhere [23,24]. Each well contained 40 µg of microsomal protein (in 40 µl of GET buffer) and a final concentration of 3.85 µM 7-ethoxyresorufin, 0.6 mM NADPH, and 0.5% Tween-20 in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) buffer (pH 7.8) in 130 µl. The fluorescence wavelengths used were 544 and 590 nm for excitation and emission, respectively, and the enzyme activites were measured kinetically every 90 s for 25 min, with a Fluorostar fluorescence plate reader (BMG Labtechnologies). The linear portion of the curve was used for quantification by comparison to a 5.3 to 42.5 pmol of resorufin standard curve.

Statistical analysis

Because the VTG responses often extended over two orders of magnitude with proportional variability, making the variance unequal across treatment groups, the VTG data were log-transformed to provide a normal distribution before analysis of variance (ANOVA). In the second mixture experiment, the VTG responses (omitting control) were not transformed, because they did not have such a wide range with proportional variability but, rather, conformed to a normal distribution (Shapiro–Wilk: $W > 0.95$). A two-way ANOVA of the data from both the first and second mixture experiments showed that differences in the individual experiments (e.g., from fish growing or aging in between the three replicate experiments) were negligible ($p = 0.53$ and 0.62) and that these replicates could be pooled for analysis. All statistical analyses were performed using SAS® software (SAS Institute).

RESULTS

Sturgeon exhibited a dose-dependent plasma VTG response to both E and NP (Fig. 1) 7 d after being injected once with control solvents or with a range of either compound. The response to NP plateaued at the highest doses, although the maximal VTG response to NP was one-tenth the amount of VTG at the highest E dose tested.

To examine the effect of BNF on E-induced plasma VTG levels, fish were exposed to E alone and in combination with BNF (50 mg/kg), using a high (1 mg/kg) and a low (0.01 mg/kg) dose of E for 7 d. The E-only treatments induced plasma VTG proportional to the dose. A factorial ANOVA on the four treatments of E or E+BNF revealed a significant overall effect from the BNF (Fig. 2A) ($p = 0.0038$). Comparing the high and low doses of E individually to their E+BNF counterpart, BNF significantly reduced VTG in the high-E treatment ($p = 0.0016$), whereas the reduction was not quite statistically significant in the low-E treatment ($p = 0.0518$). The BNF-treated fish exhibited significantly induced EROD activity (Fig. 2B), and this activity was unaffected by E concentration. On dissection, a large portion of solid BNF always was found in the intraperitoneal cavity of treated animals, indicating that much of the BNF was not absorbed.

The CYP activity from a single BNF injection (50 mg/kg) was monitored over several days and found to reach maximal activity 3 d after injection (Fig. 3). To explore the affect of CYP activity on VTG inhibition, a second mixture experiment was conducted, which focused on

the high dose of E (1 mg/kg) from the first mixture experiment and its mixture with BNF (50 mg/kg). Plus, additional treatments were included, with the BNF injected 1 to 3 d before E. Fish were sampled much sooner after E treatment than in the first experiment. In fish sampled 1 d after E treatment (Fig. 4A, white bars), plasma VTG was elevated in the five treatments compared to control, whereas BNF did not affect the VTG response. In the samples taken another 24 h later, which was 2 d after E treatment (Fig. 4B, black bars), the VTG levels were much higher. Specifically, in the E-only treatment, the VTG response after 2 d was more than 10-fold greater than the response after 1 d. Furthermore, in the simultaneously administered mixture (E+BNF-0, black bars in Fig. 4A), VTG was reduced by approximately 50% compared with exposure to E alone, similar to the first 7-d experiment (Fig. 2A). Dunnett's test showed that the plasma VTG concentrations in the E-only treatment were significantly different than the levels in the E+BNF-0 (simultaneous injection), E+BNF-1, and E+BNF-3 treatments, but not the VTG level in the E+BNF-2 treatment. Tukey pairwise comparisons of the four E+BNF treatments suggested that injecting BNF 1 to 3 d before injecting E did not alter the VTG response ($p > 0.10$). The VTG responses in the last treatments displayed much higher variability than in the pervious experiments, which could not be related to pooling data from three separate experiments (see *Statistical Analysis*). The three replicate experiments for the E+BNF-2 and E+BNF-3 treatment groups had an average coefficient of variation of 48%, compared with a coefficient of variation of 20% in the individual replicate experiments that made up the E, E +BNF-0, and E+BNF-1 treatments. Ethoxyresorufin-*O*-deethylase activity was measured in these same fish (Fig. 4B). In fish sampled after 1 d (white bars), maximum EROD activity is first reached in the E+BNF-2 treatment, which was exposed to BNF for a total of 3 d. Fish sampled after 2 d (black bars) exhibited no statistically significant maximum but followed the general trend of higher enzyme activity after 2 to 5 d of exposure to BNF compared to control.

To examine the effect of the AhR agonist on NP-induced plasma VTG levels, fish were injected with control solvents, NP, or NP+BNF (both 50 mg/kg), and plasma VTG was determined after 2 d. The NP treatment appeared to produce higher plasma VTG levels, but the resulting VTG levels were highly variable. Additionally, no differences were statistically significant. The response in the NP+BNF treatment was not different than that in the NP-alone treatment. Plasma VTG concentrations were 1.4 ± 1.0 μg/ml (mean ± standard deviation, $n = 6$) in control fish, 5.2 ± 6.9 μg/ml in NP-treated fish, and 5.1 ± 6.2 μg/ml in the NP+BNF-treated fish.

DISCUSSION

The present study confirms that an AhR agonist can reduce VTG synthesis in fish. In two different experiments, the antiestrogenic effect of BNF in white sturgeon was observed after 2 d (Fig. 4A, black bars, E+BNF-0) and 7 d (Fig. 2A). In the longer experiment, two concentrations of E were used: The high concentration was used to represent a fish undergoing vitellogenesis, whereas the low concentration was used to exemplify a smaller elevation in VTG to mimic an estrogenic contaminant. The lack of effect from the BNF in fish sampled after 1 d in the second mixture experiment (Fig. 4A, white bars) could result from the fact that at this time, VTG was accumulating very quickly, and any slight differences in sampling could have affected the results. High variability may have hidden any effect of BNF on VTG synthesis in the experiment with NP (see *Results* for data). Because NP is much less potent than E, a longer experiment may have better captured the effect.

Vitellogenin is a commonly used biomarker for estrogen-mimicking contaminants. The AhR-activating contaminants likely are present along with estrogen mimics and, as seen

here, can suppress VTG synthesis. These apparent antiestrogenic effects of AhR ligands may mask VTG responses where estrogenic compounds are present. The AhR ligands also may confound field studies that attempt to relate specific compounds from the environment with estrogenic responses in animals. For instance, two environmental samples with similar profiles of estrogen mimics may have different in vivo responses based on the presence or absence of AhR ligands, making it appear as though one sample contains additional estrogen mimics. Furthermore, many studies acknowledge that VTG does not correlate well with the occurrence of intersex [2]. Evidence that VTG responses are not exclusively dependent on estrogen mimics alone adds another possible explanation for this incongruity.

The mechanism by which this antiestrogenic effect occurs remains unclear. β-Naphthoflavone has little affinity for the ER as determined in an estradiol-binding assay [25,26], making it unlikely that the effect results primarily from BNF competitively inhibiting estrogen binding to the ER. The earliest report of the inhibitory effect of AhR agonists on estrogen-induced VTG synthesis involved rainbow trout liver cells exposed to three different AhR ligands: Dioxin, BNF, or 2,3,4,7,8-pentachlorodibenzofuran [8]. It revealed that BNF did not alter either synthesis of other proteins (e.g., albumin) or general cell function and was consistent with a mechanism involving the AhR. From studies using cultured mammalian cells, evidence exits for several mechanisms of cross talk between the AhR and the ER that could explain such antiestrogenic effects: Induction of CYP isoforms, some of which metabolize estrogen to an ER-inactive form [27]; inhibition of the estrogen-ER complex binding to its DNA recognition site, the estrogen-response element [28]; downregulation of ER via a repressor site in the promoter region of the ER gene [29]; enhanced degradation of ER through proteasome activation [30] or a ubiquitin ligase complex [31]; inhibition of an ER coactivator [32]; or competition between the AhR and ER for binding to limited levels of nuclear coactivators [33]. Other evidence shows that new proteins made in response to AhR-dependent gene expression were critical, because a general protein synthesis inhibitor was shown to prevent the antiestrogenic effects of the AhR ligand [5]. Thus, there appear to be many mechanisms by which AhR activation can lead to a reduction in E-dependent gene expression and cellular responses.

As mentioned first in the list above, CYP1A1, CYP1A2, and CYP1B1 metabolize E in both humans and animals, which would decrease the amount of estrogen available to produce biological effects. In the initial experiment, BNF and E were injected simultaneously. As such, E was injected when the activity of AhR-inducible related CYPs was low. If these enzymes were primarily responsible for the mechanism, then injecting E at the time when enzyme levels are elevated (1–3 d post-BNF treatment) (Fig. 4) should increase the metabolism of E and result in even lower levels of VTG. This hypothesis was tested in the second mixture experiment. As mentioned above, fish sampled 1 d after E injection exhibited no VTG inhibition from BNF (Fig. 4A, white bars), whereas fish sampled 2 d after E injection exhibited less plasma VTG when also treated with BNF (Fig. 4A, black bars, E +BNF-0). No evidence suggests that injecting BNF before estrogen further reduced VTG (Fig. 4A, black bars, last four treatments), providing no indication that CYP is directly responsible for VTG inhibition. Then again, the variability in these treatments, especially in the last treatment (E+BNF-3), is high enough to obscure differences. An increase in stress to the fish may have confounded the data shown in Figure 4, because most of these fish were injected on two separate days. The E+BNF-2 group, however, was not statistically different from the E-only treatment, and if anything, the change in the VTG response, especially in the E+BNF-2 group, seemed to be in the opposite direction of what would be expected if CYPs were the direct mechanism of the antiestrogenic effect of BNF.

The EROD activity in these same fish (Fig. 4B) was measured at the end of the exposure for the corresponding VTG measurement. However, EROD activity at the moment of E

injection would be a better comparison with the VTG data. To consider EROD activity for the E+BNF-2 treatment, the E+BNF-0 treatment could be used to estimate enzyme activity after 2 d, which is the same length of time that the E + BNF-2 fish were exposed to BNF when E was injected. On the other hand, Figure 3 more clearly shows enzyme activity on a particular day. Overall, the data shown in Figure 4B agree with those shown in Figure 3 in that the EROD activity increased over a few days compared to control, whereas Figure 4A provides no indication that VTG inhibition was enhanced by injection of E when EROD activity was higher. Although the data in Figure 4 are limited, the lack of evidence for the involvement of CYPs concurs with the results of other studies of fish or fish tissues that suggest the antiestrogenic mechanism of BNF is not primarily a result of CYP-dependent metabolism [9,10,13]. Taken together, reason exists to investigate alternative mechanisms. Another in vivo study reported anti- and proestrogenic behavior of BNF and showed that BNF cotreatment increased and decreased the availability of estrogen-binding sites, corresponding to the pro- and antiestrogenic effects observed, suggesting that it may be the ER that is affected [12]. The mechanism responsible for the antiestrogenic effect of AhR ligands remains to be determined.

Although AhR agonists may reduce VTG, it should not be assumed that organisms therefore will be unaffected by endocrine disruption. Fish exposed to both estrogenic compounds and AhR ligands that do not exhibit VTG likely suffer from suppressed estrogen signaling. Indeed, a variety of AhR ligands have been found to affect reproductive fitness in fish. A significant decrease in plasma E was found in flounder (*Platichthys flesus*) exposed to polycyclic aromatic hydrocarbons [34]. Chronic dietary exposure to dioxin was found to decrease egg production, spawning success, serum estradiol concentrations, and serum VTG in zebrafish (*Danio rerio*) [35]. Both Aroclor 1254 and benzo[*a*]pyrene have been found to reduce plasma VTG levels in rainbow trout [36] and croaker (*Micropogonias undulates*) [37]. Reproductive effects have been recorded in wild minnows (*Pimephales promelas*) living in waters receiving pulp mill effluent since the mid to late 1980s, including reduced spawning events, fewer eggs produced, ovipositor development in males, and development of male secondary sex characteristics in females [38]. Sea bass (*Dicentrarchus laorybrax*) fed a BNF-enriched diet for six months had lower plasma VTG concentrations at the middle of the spawning period, when VTG levels normally peak [39]. Meanwhile, plasma estradiol and luteinizing hormone levels were higher than those in the untreated bass, indicating an interference with the hypothalamus–pituitary–gonadal axis.

Compared to other species, sturgeon may be more susceptible to EDs because of their long life history [18]. It takes from 1.5 to 2 years for most sturgeon species to differentiate sexually and from 10 to 20 years to mature [21], making these processes vulnerable to the influence of endocrine-disrupting contaminants for a much longer time. Disruption of vitellogenesis and other estrogen-related events and behaviors might place spawning out-of-synch with males or proper environmental conditions. Even small chemical disturbances could be significant in species like sturgeon that spawn only once every couple of years, especially as their habitats have been reduced by impoundments and traditional spawning areas have become inaccessible.

Sturgeon also are an interesting model for endocrine disruption, because they are unlike most other fish. Sturgeon-like fish hold an important place in evolution as a link between tetrapods and fish. The dose-dependent increase of white sturgeon VTG to E and NP confirms that these primitive fish respond to E and a well-known estrogen mimic, similar to modern teleost species. Additionally, as shown here, an AhR agonist also can act as an antiestrogen in vivo in white sturgeon. The comparable response to NP, a nonnative ligand for the ER, and the antiestrogen effects of the AhR agonist in this species, other fish, and

mammalian cells suggest that all vertebrates likely are affected by these kinds of endocrine-disrupting contaminants.

Vitellogenin is both a popular and a valuable marker of estrogen-mimicking contaminants in fish. Reduction of VTG by contaminants with antiestrogenic properties is an important consideration for those using VTG for biomonitoring purposes, because these compounds may mask or confound VTG responses. Although CYP induction may be useful to indicate AhR compounds, these enzymes probably are not the cause of the antiestrogenic effect. The reduction of VTG should not be interpreted as being less of a threat to wildlife. Animals exposed to both types of agents, but exhibiting no estrogenic response, would still suffer the effects of the antiestrogen. Chronic suppression of estrogen signaling by AhR-acting agents could loom as a larger problem than that of the estrogen mimics and be a harder issue to study.

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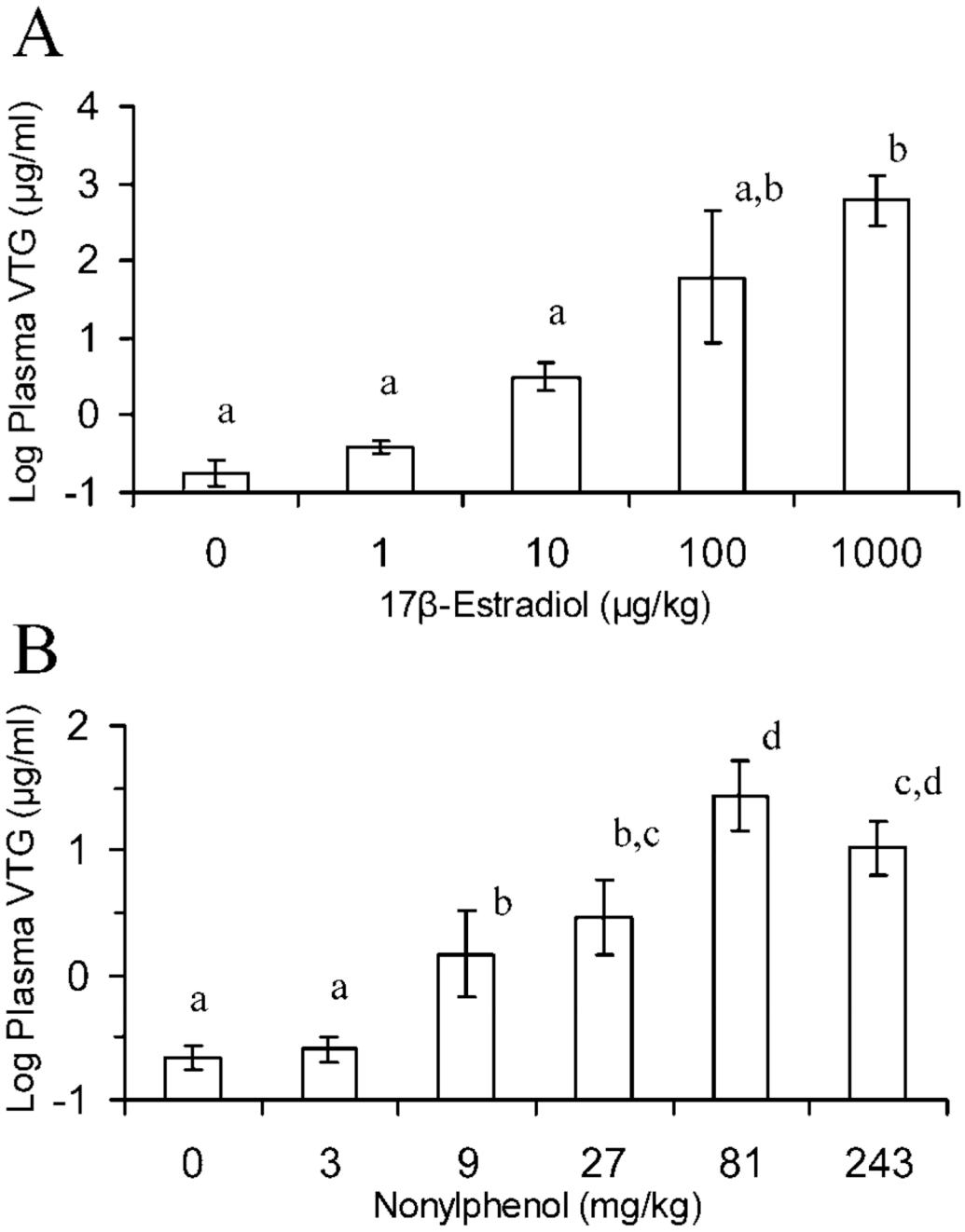

Fig. 1.

Vitellogenin (VTG) response of white sturgeon (*Acipenser transmontamus*) to 17β-estradiol (**A**) and *p*-nonylphenol (**B**). Fish were injected once intraperitoneally, and blood was sampled 7 d later. Each bar represents the mean ± standard deviation ($n = 3$), and lowercase letters indicate the Tukey grouping ($p < 0.05$) from analysis of variance of log plasma vitellogenin.

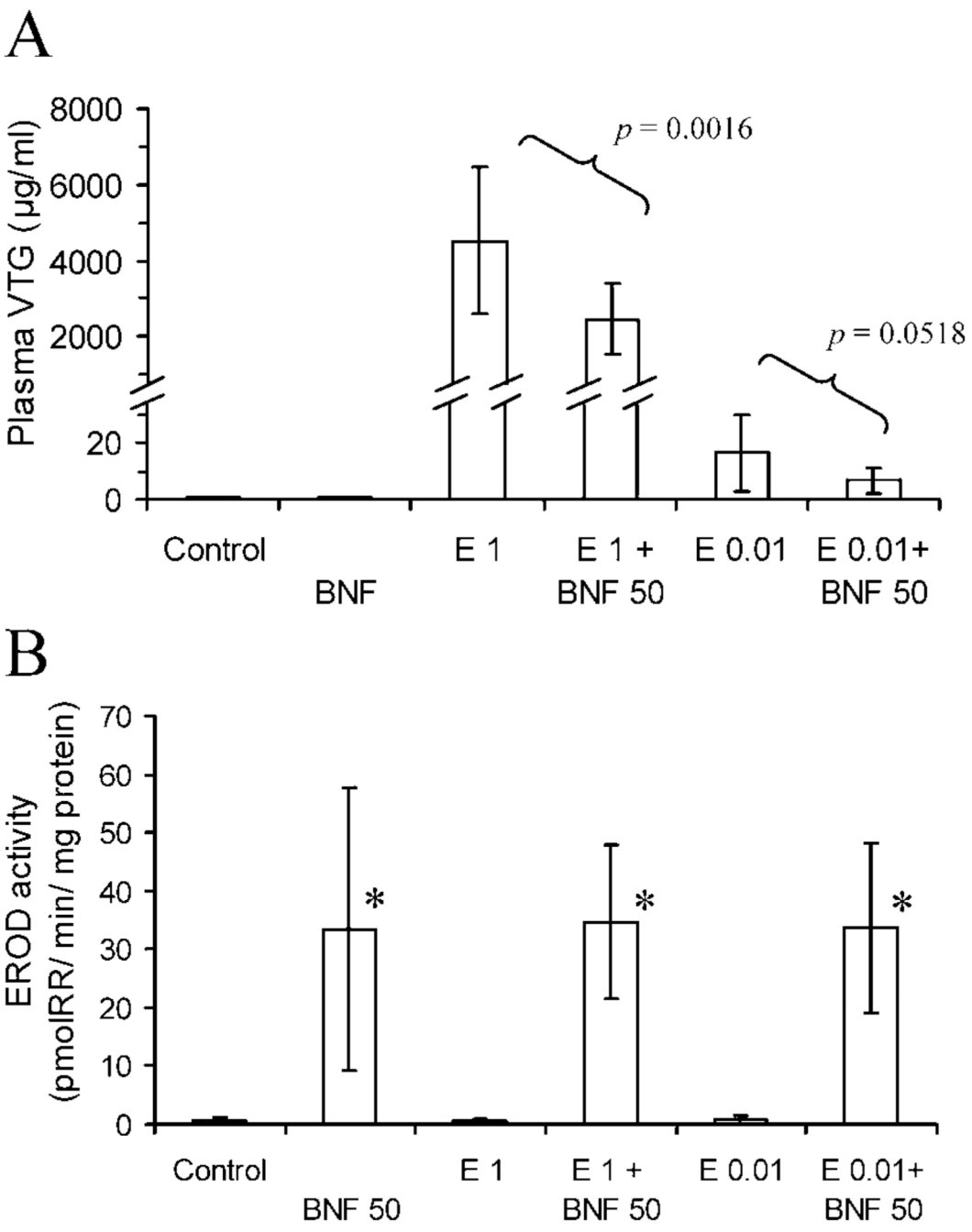

Fig. 2.

β-naphthoflavone (BNF) reduced 17β-estradiol (E)–induced vitellogenin (VTG; **A**) and induced ethoxyresorufin-*O*-deethylase (EROD) activity expressed as the rate of resorufin (RR) formation (**B**). Fish were simultaneously given two separate intraperitoneal injections of E and BNF (or vehicle control) once, and blood and liver were sampled 7 d later. Dose (mg/kg) is indicated after the compound abbreviation on the *x* axis. Each bar represents the mean ± standard deviation ($n = 9$). (**A**) A factorial analysis of variance (ANOVA) of log plasma VTG from all four treatments of E and BNF+E showed a significant effect of BNF ($p = 0.0038$). Additionally, ANOVA was used to assess the effects in treatment pairs as noted in the figure. (**B**) Asterisks indicate a significant difference from control ($p < 0.05$).

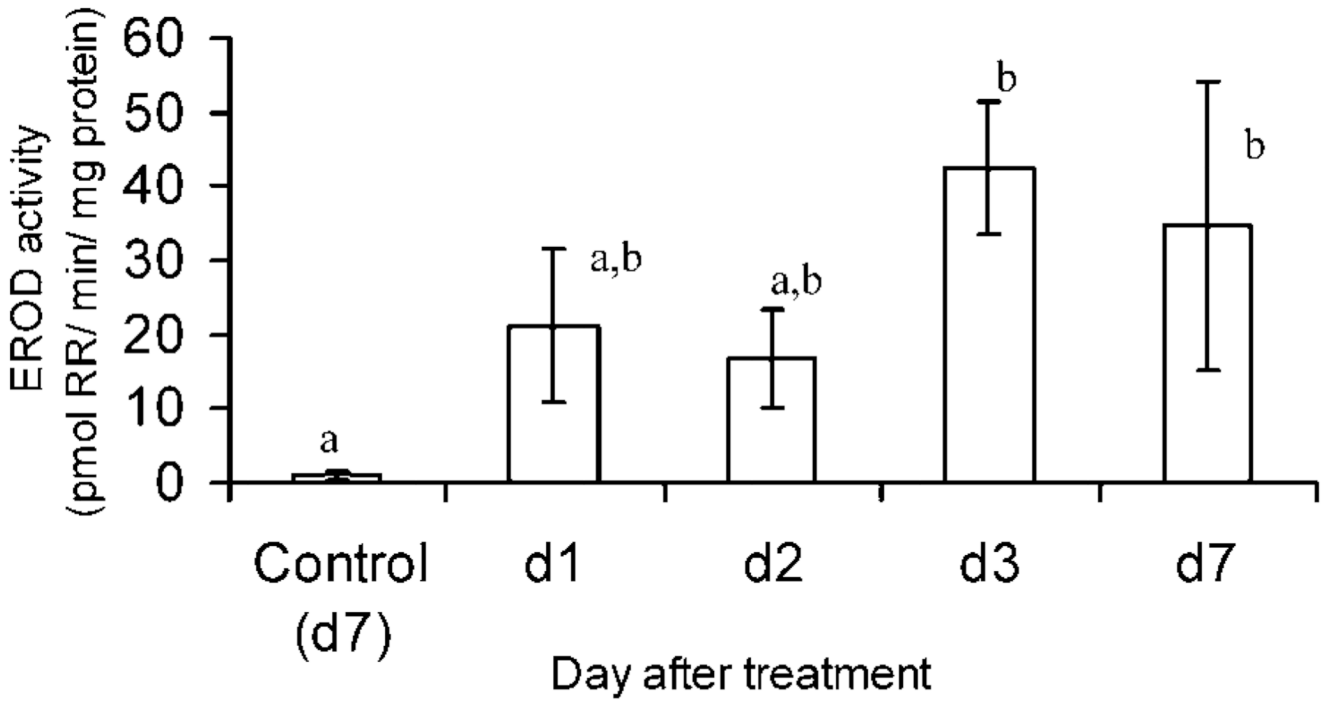

Fig. 3.

Time course of ethoxyresorufin-*O*-deethylase (EROD) activity expressed as the rate of resorufin (RR) formation after β-naphthoflavone (BNF) treatment. Fish were once injected intraperitoneally with 50 mg/kg of BNF, and liver was sampled after the indicated duration. Each bar represents the mean ± standard deviation ($n = 3$), and letters indicate the Tukey grouping ($p < 0.05$).

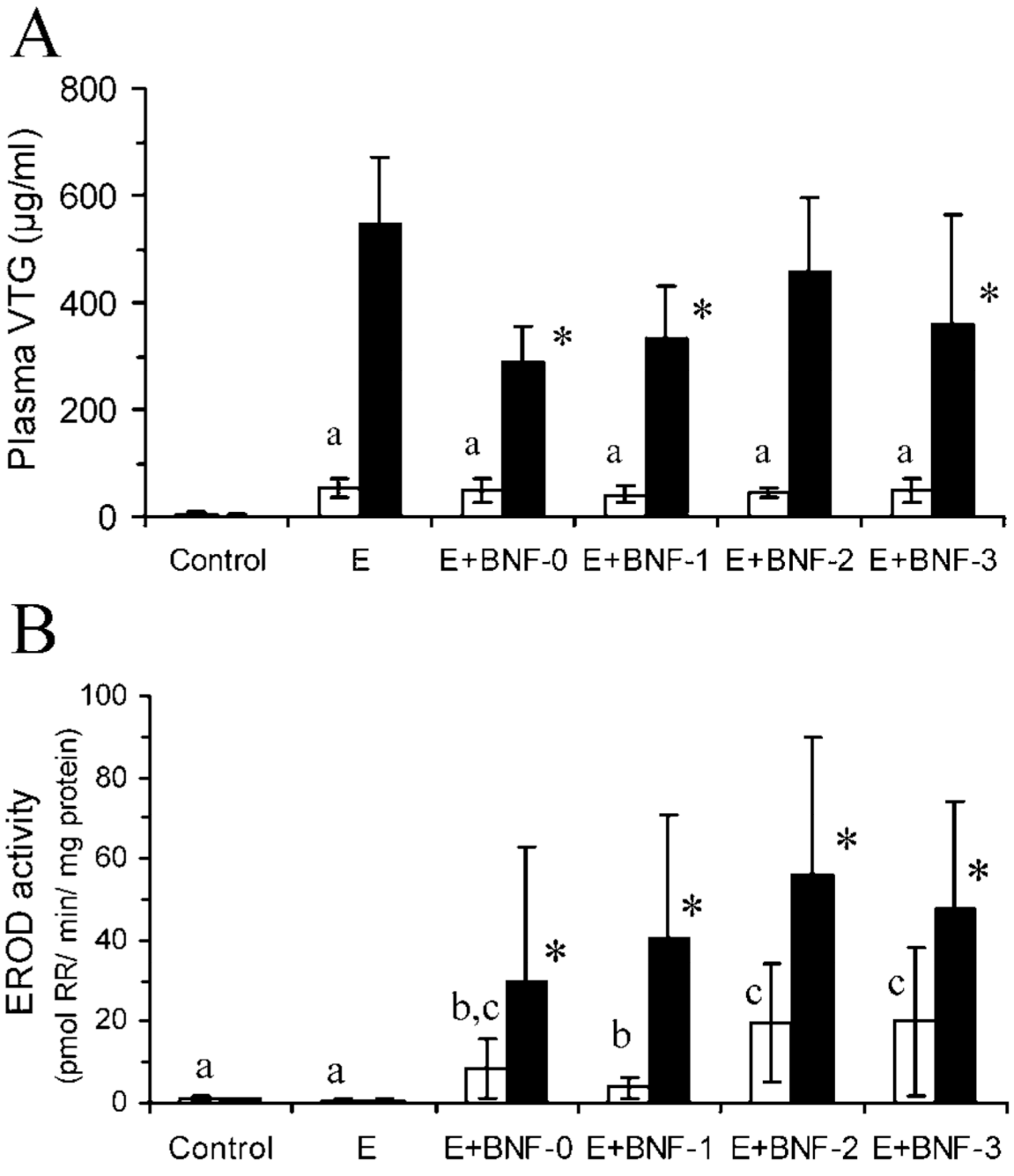

Fig. 4.

Influence of treatment timing on induced vitellogenin (VTG; **A**) and ethoxyresorufin-*O*-deethylase (EROD) activity expressed as the rate of resorufin (RR) formation (**B**). Fish were injected once intraperitoneally with 17β-estradiol (E; 1 mg/kg) and β-naphthoflavone (BNF; 50 mg/kg) or vehicle control. The number of days that BNF was administered before estrogen is indicated on the *x* axis. Fish were sampled at 1 d (□) or 2 d (■) after estrogen treatment. Each bar represents the mean ± standard deviation (where $n = 9, 8, 9, 6, 6,$ and 6, respectively). (**A**) Statistically significant difference between the estrogen-only treatment and any of the last four treatments is indicated by an asterisk ($p < 0.05$), and the lowercase letter a indicates a statistical difference from control ($p < 0.05$). (**B**) Letters indicate the

Tukey grouping ($p < 0.05$), and asterisks indicate a significant difference from control ($p < 0.05$).