EFFECTS OF 4-NONYLPHENOL ON FECUNDITY AND BIOMARKERS OF ESTROGENICITY IN FATHEAD MINNOWS (PIMEPHALES PROMELAS)

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Abstract—Adult fathead minnows (Pimephales promelas) were exposed to waterborne concentrations of 4-nonylphenol (NP) ranging from 0.05 to 3.4 μg NP/L for 42 d. Results were similar, but slightly different, for two experiments conducted during July and August, near the beginning of the breeding season, and a second experiment conducted during September and October, at the end of the breeding season, during which the adults were maintained continuously in breeding condition. Inverted U-type dose-response relationships were observed for egg production and for concentrations of vitellogenin (Vtg) and 17β-estradiol (E2) in blood plasma. Concentrations of plasma Vtg were significantly different between males and females, with plasma concentrations in females ranging from 20 to 110 μg Vtg/ml. Both experiments had no statistically significant, dose-dependent effect of NP on plasma Vtg in males but significant effects of NP on Vtg concentrations in females. In the first experiment, Vtg concentration generally increased with NP concentration, whereas the second experiment showed a negative correlation. Plasma E2 concentrations in both males and females were significantly affected by NP. The concentration of total estrogen equivalents in the plasma increased 900% because of exposure to NP. Most of this increase resulted from increased plasma E2 concentrations, with only a 4% increase resulting from the estrogen agonist activity of NP. The effects of NP on adult fathead minnows seem not to result from a direct-acting estrogen agonist mechanism but rather from changes in the endogenous concentrations of E2 through an indirect activation mechanism of action.

Keywords—Vitellogenin Hormones Endocrine Reproduction

INTRODUCTION

Recently, the effects of endocrine disrupters on wildlife [1] and humans [2] have received much interest. One type of endocrine disruption occurs when xenobiotics mimic steroid hormones. One class of hormone mimics is the xenoestrogens [3,4]. Fish living in the vicinity of or caged below wastewater treatment plants have altered hormone status or gonadal histology [5,6], and monitoring of plasma vitellogenin (Vtg) concentrations, which have been proposed as a reliable measure for exposure of fish to environmental estrogens, [7], has indicated that some wild and feral fish are currently being exposed to environmental estrogens [8–11]. One such class of compounds are the alkylphenol ethoxylate nonionic surfactants and their intermediate degradation products, the alkylphenols [4]. Some of the estrogenic activity of wastewaters has been attributed to degradation products of alkylphenol ethoxylate, primarily nonylphenol (NP) and octylphenol (OP), which are intermediate transformation products of para-substituted phenols and mono- and di-ethoxylates [12]. These compounds are weak estrogen agonists, and they have been proposed to act as estrogen mimics by direct action at the estrogen receptor (ER) [4,13].

The current study was conducted to determine the relative potency of NP for affecting biomarkers of exposure to estrogenic compounds, to calibrate these responses to reproductive performance, to investigate the probability that NP causes effects by acting as an estrogen agonist, and to determine the relative sensitivities of the estrogen-specific biomarkers compared with ecologically relevant parameters such as survival and egg production.

MATERIALS AND METHODS

Nonylphenol

4-Nonylphenol was obtained from Schenectady International (Freeport, TX, USA). The mixture, which was composed of several isomers, had a purity of greater than 98% but contained trace concentrations of cumyl- and dodecalphenol. However, these could not be observed during mass spectral analyses performed on the mixture. The NP was dissolved in ethanol as a carrier solvent, which was diluted to give the desired concentrations of NP in water.

The NP concentrations in water were measured at the beginning, middle, and end of the experiments. The analytic procedure for NP determinations has been described elsewhere [14]. Briefly, 1-L water samples from the experimental system were spiked with OP as an internal standard and then filtered through glass-fiber filters (nominal pore size, 0.7 mm) and extracted with a 47-mm, styrene-divinylbenzene Empore® disk (3M, St. Paul, MN, USA). Analyses were performed using reverse-phase, high-pressure liquid chromatography with fluorescence detection. The method limit of quantification for NP was approximately 5 ng in a 1-L sample of water. Individual quantifications of NP were corrected for recovery of the tert-butylphenol internal standard, the average of which was 88 ± 4% (mean ± SD, n = 6). The coefficient of variation for triplicate measurements of a water extract was less than 2%. The measured concentrations averaged approximately one-third of the nominal concentrations (Tables 1 and 2).

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Methods used for determining the bioaccumulation of NP to fathead minnows have been described elsewhere [15]. Whole fish were homogenized with sodium sulfate and then spiked with OP as an internal standard. The homogenate was extracted using Soxhlet’s apparatus for 12 h with dichloromethane. Gel-permeation chromatography was used to remove bulk lipids, and acidic silica gel was used for further cleanup of the samples. Concentrations of NP were quantified by NP–high-performance liquid chromatography with fluorescence detection.

Fish

Fathead minnows (Pimophales promelas) were cultured in the Michigan State University Aquatic Toxicology Laboratory (East Lansing, MI, USA) culture facility under a light-dark cycle of 16 h: 8 h continuously flowing well water at 17 to 19°C. Fish were fed a 1:1 (v:v) mixture of Tetramin® (Tetra Werke, Melle, Germany) and ground Sterling Silver Cup Trout Chow (pellet size, 2–4 mm; Murray, UT, USA) once daily at a rate of approximately 0.5% of fresh body weight. The dry food was supplemented with Artemia nauplii (brine shrimp). Freeze-dried Spirulina sp. algae were fed to fry as long as 2.5 cm in length. Reproductively mature fish (age, 12–18 months) were transferred to randomly assigned, 19-L glass aquaria in a circulating water-jacketed bath (Frigid Units Living Stream, Toledo, OH, USA). Each tank received treated well water (3 L/h) through a solenoid-controlled, proportional-flow diluter apparatus (Ace Glass, Vineland, NJ, USA). Each tank received vigorous aeration, and escape of aerosols from the tanks was prevented by glass lids. The temperature of the water jacket was maintained at 26°C.

Exposures

Fish were randomly assigned to 20 groups, with each group containing two males and two females and placed into 19-L aquaria. Two studies were conducted. The first study (EX I) was conducted during July and August, whereas the second study (EX II) was conducted during September and October. To simulate ambient environmental conditions, the fish were maintained in breeding condition for the entire experimental period. Treatments were in triplicate, with the exception of the solvent control group, which due to the restriction of space in the water bath contained only two aquaria. Fish were allowed to acclimate in control water for 7 d and then exposed to NP for 7 d before the commencement of breeding. Breeding was initiated by the introduction of two terra-cotta pot halves (spawning tiles) into each aquarium. These pot halves acted as spawning substrata, without which the fish would not spawn. Fish were exposed to treatments for a total of 42 d. Spawning substrata were removed for 7 d (days 22–29) to allow the fish to rest and then returned for the final 2 weeks of the exposure. The aquaria were randomly assigned to receive treatment concentrations of NP or control solutions delivered by a proportional-flow diluter. Treatments included control, which received only water, and a solvent control, which received the same concentration of solvent (0.0001% ethanol) as the NP treatments. Fathead minnows were exposed to target (i.e., nominal) concentrations of 0.1, 0.3, 1.0, 3.0, and 10 µg NP/L. The actual, measured concentrations were similar but differed between the two experiments. Because the same nominal concentrations resulted in slightly different actual concentrations in the two experiments, they were designated as A, B, C, D, and E, respectively, so they could be combined. In EX I, the measured concentrations were 0.05 (A), 0.16 (B), 0.4 (C), 1.1 (D), and 3.4 (E) µg NP/L (Table 1). In EX II, the mean measured concentrations were 0.09 (A), 0.10 (B), 0.33 (C), 0.93 (D), or 2.4 (E) µg NP/L (Table 2).

Fish maintenance and monitoring

As mentioned, fish were fed once daily throughout the exposure with freshly hatched brine shrimp and a 1:1 (v:v) mixture of Tetramin and ground Sterling Silver Cup Trout Chow (Purina, St. Louis, MO, USA). Water temperatures ranged from 24.8 to 26°C. Spawning tiles were checked daily for eggs, which were carefully removed from the spawning tiles, counted under a dissecting microscope, and placed into hatching chambers according to treatment. Eggs were then hatched and reared for future study. Four general water-quality parameters were measured throughout the exposure period: dissolved oxygen and water temperature on every other day, and pH and alkalinity every week. Alkalinity was determined by titration of bromocresol green indicator to a pH of 4.3 according to American Public Health Association Method 403 [16]. Water samples were taken three times during the exposure to be analyzed for NP. Effluent from the aquariums was treated to remove NP by trickle filtration through activated charcoal.

Blood collection

At the end of the experiments, fish were euthanized with tricaine methanesulfonate (MS-222). Their weights and standard lengths were recorded, and blood was collected from the caudal vein with a heparinized hematocrit tube. Blood was allowed to clot for 1 h on ice and then centrifuged at 3000 g for 10 min at 4°C. Hematocrit was determined, and then plasma was drawn off and stored at −80°C for later analysis of Vtg and 17β-estradiol (E2).

E2 quantification

Concentrations of E2 in blood plasma were determined by enzyme immunoassay with Estradiol EIA Kits (Cayman...
Chemical, Ann Arbor, MI, USA). The methods were those specified by the manufacturer [17], with the exception that samples and standards were extracted in diethyl ether and reconstituted in buffer for analyses. E₂ (7.8 to 1000 pg/ml) standard curves were assayed in duplicate on each plate. The specificity of the E₂ antibody was as follows: 17β-estradiol, 100%; estrone, 7.5%; estriol, 0.3%; testosterone, 0.1%; and 5α-dihydrotestosterone, 0.1%. An external standard of pooled male and female goldfish plasma was assayed in at least triplicate on each plate for determination of the variability between assays. Log-logit transformation of E₂ concentrations (pg/ml) as a function of the percentage of maximum binding (%B/B₀) was calculated, and a standard curve was established by plotting absorbance units as a function of E₂ concentration in a log-logit regression. Samples were analyzed in at least duplicate, and samples or standards exceeding a 20% coefficient of variation were reassayed. Samples that resulted in less than 20% binding on the standard curve were diluted by 50% and reanalyzed.

**Vtg quantification**

Concentrations of Vtg in blood plasma were determined by a competitive enzyme-linked immunosorbent assay using a polyclonal rabbit antigoilfish Vtg antiserum [18]. This antiserum was found to recognize fathead minnow Vtg and optimized for maximum sensitivity and minimum interference in fathead minnows. Standards ranging from 0.135 to 75.3 ng Vtg/well and dilute samples were assayed in at least duplicate, and most of the time in triplicate, on each 96-well plate. Samples or standards that exceeded a 15% variability among absorbance values were discarded or reanalyzed depending on availability of the sample. Samples resulting in less than 20% of maximum binding on the standard curve were reassayed at greater dilutions. Interassay coefficients of variation were determined from an average of measured, standard Vtg concentrations on each plate (standard, 4.34 ng Vtg/well). All samples for Vtg measurement were assayed on the same day, with intra- and interassay coefficients of variation of 7.6% and 10.9% (n = 3), respectively. The method detection limit was 0.27 μg Vtg/ml plasma.

**Statistical analyses**

Fecundity and concentrations of E₂ or Vtg were tested for assumptions of normality and homogeneity of variance. Rejection of these assumptions resulted in the use of nonparametric statistical comparisons for all parameters. For egg production, data from the two experiments were pooled by treatment and the combined data analyzed, as were the results from the two experiments separately. All parameters were analyzed by Kruskal-Wallis one-way analysis of variance, which was conducted on the ranks of the values (PROC GLM, SAS Statistical Software Institute, Cary, NC, USA). All statistical tests of significance used a type I error (α) of 0.05 unless otherwise indicated. Means ± standard errors of the means are reported here and plotted in figures.

Nonlinear (inverted U-type) dose-response relationships were observed for fecundity and plasma E₂ concentrations, which complicated determination of the threshold for effects. Methods exist to extract EC50 values from hormetic response relationships [19], but determination of a lowest-observed-adverse-effect concentration was complicated by the difficulty in knowing whether an increase in a parameter such as egg production during a particular period was an adverse effect.

In this study, any change, either positive or negative, was used as the point at which to determine a lowest-observed-effect concentration. The lowest-observed-effect-concentration was defined as being the lowest NP concentration that caused a statistically significant change in a parameter from those values observed in the solvent control. The no-observable-effect concentration was defined as being the greatest NP concentration that did not cause a significant change in a response relative to the controls.

### RESULTS AND DISCUSSION

**Survival and health of adult fish**

The NP was not acutely toxic to adult fathead minnows at the concentrations studied, and fatalities of adult fathead minnows during exposures to NP exhibited no dose-dependent relationship to NP (Table 3). Fourteen fish (seven males and seven females) died in EX I, whereas seven fish (three males and four females) died in EX II. Hematocrit was measured to assess the general health of the adult fish [20]. Hematocrit values were not statistically different among treatments for males (EX I, p < 0.9219; EX II, p < 0.9900) or females (EX I, p < 0.5653; EX II, p < 0.3262) in EX I. Thus, adult fish were not suffering from general stress in the range of NP concentrations to which they were exposed.

**Fecundity**

When the number of eggs produced was examined by Friedman’s test (i.e., nonparametric two-way analysis of variance on ranks of values), a marginally statistically significant difference was found in responses between EX I and EX II (p
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Fig. 1. Mean number of eggs produced per female fathead minnow as a function of nonylphenol (NP) concentration in experiment I. Confidence intervals represent the standard error of the mean. Treatments with the same uppercase letter are not significantly different from one another (Kruskal-Wallis and Tukey test, \( \alpha = 0.05 \)). C = control, SC = solvent control.

< 0.0609) (Figs. 1 and 2). No statistically significant effect of NP treatments was found \((p < 0.2541)\), but a statistically significant interaction was found between the treatment and experiment terms \((p < 0.0309)\). Variation in fecundity among replicate tanks along with the small sample size made it difficult to demonstrate statistical differences, especially with use of the less powerful, nonparametric statistical tests. EX I showed no difference in the number of eggs produced in the control and solvent controls; the mean number of eggs produced in the two control groups was 150 eggs per female. Exposure to the least concentration of NP studied resulted in greater egg production than that observed in the controls. Whereas the mean was greater than 340 eggs per female for the entire exposure, this effect was not statistically different from the fecundity of the control groups. In EX I, fewer eggs were produced at NP concentrations of 0.16, 0.4, and 1.6 \( \mu g \) NP/L than in the controls, but again, using nonparametric statistics, the differences from the controls were not significantly different. Exposure to 3.4 \( \mu g \) NP/L caused an almost-complete elimination of egg production (Fig. 1). The number of eggs produced per female in the greatest NP concentration studied was significantly less than that produced in the 0.05 \( \mu g \) NP/L exposure, but it was not significantly less than those in the three other exposures or the two controls.

In EX II, the effects of NP on fecundity were similar (Fig. 2) to those observed in EX I. No significant difference was found among the two controls and exposure to 0.09, 0.10, 0.33, or 0.93 \( \mu g \) NP/L. Fecundity was significantly greater in the 0.1 \( \mu g \) NP/L exposure group, but this was not statistically significantly different from that in the group with the greatest exposure (2.4 \( \mu g \) NP/L). Thus, a no-observed-effect or lowest-observed-effect concentration could not be determined based on fecundity from either of the experiments individually.

To assess the potential effects of exposure to NP over an entire reproductive season, production of eggs for the two experiments was summed by treatment to give a cumulative number of viable eggs per treatment (Fig. 3). This metric represents the total fecundity of a population over the entire summer and is a realistic estimation of what might happen in nature. When the data were combined, little difference was found among the control, the solvent control, and the three treatments (C, D, and E), but a statistically significant effect of NP treatment was found for the combined data. Both the control and treatment B (Fig. 3) resulted in fecundities significantly greater \((p < 0.0395)\) than that in the solvent control but not significantly different from one another. This result suggests an inverted U-type dose-response relationship, which indicates a possible hormetic response of fecundity to NP exposure. Because exposure to the greatest doses (i.e., treatment E) eliminated reproduction in the EX I but not in EX II, it is impossible from these data to accurately determine a threshold for the effects of NP on fecundity. However, concentrations
Concentrations ranged from less than 20 of greater than 0.3 to 0.4 μg NP/L seem to have depressed egg production.

There is no explanation for the differences in response between the two replicate studies. The first exposure was conducted during July and August, and the experiment was repeated during September and October. Despite the exposures being conducted back to back from the middle of summer, the reproductive capacity of the fish was less in EX I. All control and exposure groups commenced spawning during the first week of exposure in EX I, whereas in EX II, it was more than a week to the first spawning, with the exception of the fish exposed to the least concentration (0.09 μg/L NP). Exposed fish in EX I produced threefold as many eggs as those exposed in EX II, and NP-exposed fish from the treatments of 0.09 μg/L in EX I and 0.1 μg/L in EX II produced more eggs than the control fish.

**Plasma Vtg**

Concentrations of plasma Vtg were different in males and females (p < 0.0001), between EX I and EX II (p < 0.0129), and in response to NP exposure (Figs. 4 and 5). In general, plasma concentrations of Vtg were greater in females than in males (Figs. 4 and 5), but no overall, statistically significant effect of NP treatment on the plasma Vtg was found among males in either experiment (p < 0.627). However, in both experiments, a significant increase in plasma Vtg was observed among some males exposed to the least concentration of NP, which resulted in an increased variance of Vtg concentrations. This increase in plasma Vtg concentration was approximately fivefold in both EX I and EX II. A statistically significant treatment effect of NP on the plasma Vtg of females was also found in both EX I (p < 0.0016) and EX II (p < 0.0085). Concentrations ranged from less than 20 μg/ml to a maximum of 110 μg/ml. In EX I, no statistically significant differences were found in plasma Vtg of females among the control group, solvent control, and three least concentrations of NP. Females exposed to the two greatest concentrations of NP contained significantly less plasma Vtg, but the plasma Vtg concentrations resulting from exposure to these two NP concentrations were not significantly different from one another. Only in females exposed to the greatest concentration of NP were plasma Vtg concentrations significantly less than those in all other treatments. In EX II, the concentration of plasma Vtg in females was statistically greater among fish exposed to all NP treatments relative to controls. The greatest plasma concentration of Vtg in females was observed among fish exposed to the least concentration of NP (0.09 μg NP/L). In EX I, a statistically significant, negative correlation was found between the plasma Vtg concentration of females and exposure to NP (r² = 0.59, p < 0.001), whereas in EX II, no statistically significant correlation was found (r² = 0.013, p > 0.5) between these two parameters. Whereas NP caused greater plasma concentrations of Vtg relative to that in control fish, the U-shaped curve resulted in a poor correlation even though a significant treatment main effect resulted from exposure to NP.

**Plasma E₂**

Exposure to NP caused effects on plasma E₂ concentrations that were similar for male and female fathead minnows (Figs. 6 and 7). No statistically significant differences were found in responses between EX I and EX II (p < 0.129). Statistically significant effects of NP on plasma E₂ concentrations were found (p < 0.0001), but a statistically significant difference between the responses of males and females was not (p < 0.1157). In EX I, the four concentrations of NP ranging from 0.05 to 1.1 μg NP/L resulted in statistically significant greater concentrations of plasma E₂ than those in the controls. Exposure to 2.4 μg NP/L produced plasma E₂ concentrations in both males and females that were not significantly different from those in the controls. In females, exposure to 0.1 μg NP/L resulted in the greatest mean plasma concentration of E₂ (24 ng/ml), which was approximately 10-fold greater than in the controls. Thus, in both males and females, an inverted U-type response was found. In EX II, an increase in plasma E₂ was found, but the response was more variable. In contrast to EX I, the greatest concentration of NP in EX II did not result in plasma E₂ concentrations similar to levels in the control fish. Instead, the plasma E₂ concentrations of fish exposed to the...
different from one another (Kruskal-Wallis and Tukey test, mean. Treatments with the same uppercase letter are not significantly different from one another (Kruskal-Wallis and Tukey test, α = 0.05). C = control, SC = solvent control.

The greatest NP concentration were similar to those of fish exposed to the three next lesser NP concentrations.

Concentrations of plasma E2 in fish from the control and solvent control groups were similar in both EX I and EX II. Concentrations of plasma E2 in fish exposed to NP in both EX I and EX II were approximately two- to threefold greater than those of the controls, with the exception of the 2.4 μg NP/L treatment in EX I. Fish exposed to 2.4 μg NP/L in EX I contained plasma E2 concentrations that were not significantly different from those of the controls. The trend for EX II was different from that for EX I. In EX II, as in EX I, the plasma E2 concentration was elevated in fish exposed to the lesser concentrations of NP, but the E2 concentration was not greater in fish exposed to the greatest concentration of NP. Also, the responses among fish were more variable in EX II than in EX I.

One of the major effects of NP was to increase the plasma concentrations of endogenous estrogen E2. In EX I, the increase in E2 was, on average, 10-fold, or from approximately 2 ng E2/ml to 20 ng E2/ml (Fig. 6). In EX II, the response was more variable, and overall, the concentrations of E2 were less. However, an increase of approximately threefold, or from 2 or 3 to 6 ng E2/ml, was observed (Fig. 7). The contribution of estrogen equivalents (E2-Eq) from NP acting as an estrogen agonist would be approximately 9.1 × 10^-2. On a relative basis, the increase in plasma E2-Eq contributed by E2 and that contributed by NP (E2-Eq-NP) in EX I would have been 900% and 4.5%, respectively, and in EX II, 200% and 3% respectively.

**Relationship between plasma E2 and Vtg**

The trends in plasma Vtg concentrations as a function of dose were opposite in EX I and EX II (Figs. 4 and 5). In EX I, plasma Vtg decreased as a function of NP dose, whereas in EX II, plasma Vtg tended to increase as a function of NP dose. The reason for this difference is unexplained, because plasma E2 increased as a function of NP dose in both experiments. When the relationship between Vtg and E2 was examined by a correlation analysis stratified by experiment and sex, no significant correlation was found between plasma E2 concentration and Vtg for females (Table 4). In males, Vtg in the plasma was significantly correlated with E2 in EX II, when the average concentration of E2 was less, but Vtg was not significantly correlated with E2 in EX I, when the concentration of E2 in the plasma of males was greater.

Induction of Vtg synthesis by the liver for release into the plasma is controlled by the ER [21–23]; however, the relationship between concentrations of E2 and Vtg in the plasma may not be as strong [24–25]. The results observed here were similar to those for rainbow trout (Onchorhynchus mykiss), in which during the entire season, no correlation between plasma E2 concentration and plasma Vtg was found [23]. In cyprinid fishes such as the roach (Rutilus rutilus L.), which spawns once in a reproductive season, and in bleak (Alburnus alburnus L.) and white bream (Blicca bjoerkna L.), which have multiple spawnings per season, plasma Vtg concentrations were weakly yet positively correlated with plasma E2 concentrations [26]. Induction of Vtg has been suggested to be a sensitive, E2-specific biomarker [8,27–29] for the exposure of oviparous animals, such as the fathead minnow, to estrogen agonists [30], and it has been used to monitor for exposure of fish to endogenous estrogens, exogenous estrogens, and xenoestrogens [3,12,31]. However, in those experiments, a relatively great increase in plasma E2 concentration resulted in a rather modest increase in plasma Vtg concentration. This indicates that induction of plasma Vtg in adult, multiple-spawning, cyprinid fishes such as the fathead minnow might not be a very sensitive measure of exposure to estrogenic

**Table 4. Correlations between 17β-estradiol and vitellogenin**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sex</th>
<th>Correlation coefficient (r)</th>
<th>Probability (p)</th>
<th>Sample size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>M</td>
<td>0.12010</td>
<td>0.5349</td>
<td>29</td>
</tr>
<tr>
<td>I</td>
<td>F</td>
<td>−0.21871</td>
<td>0.2456</td>
<td>30</td>
</tr>
<tr>
<td>II</td>
<td>M</td>
<td>0.34677</td>
<td>0.0445</td>
<td>34</td>
</tr>
<tr>
<td>II</td>
<td>F</td>
<td>0.08001</td>
<td>0.6478</td>
<td>35</td>
</tr>
</tbody>
</table>

*F = female; M = male.
plasma E2 concentrations reaching their greatest levels early in the cycle and then subside, which is followed by an increase in Vtg secretion by the liver [32]. In salmonid fishes that spawn seasonally in fish [33,34]. In rainbow trout (O. mykiss), increases in plasma E2 concentration are associated with a decrease in the GhI concentration. Supplementation of E2 by injection caused a decrease in the plasma GhI concentration but did not affect the GhII concentrations in previtellogenic females [35]. In other studies of mature salmonids, supplementation with plasma E2 caused an increase in GhII concentration [36]. Thus, changes in the plasma E2 concentration with concomitant changes in the plasma concentrations of GhI and GhII could explain the results regarding egg production observed in this study.

Several reports have described the seasonal changes in plasma steroid hormone concentrations and their relationships to plasma Vtg concentrations, but most of these have involved fish, and particularly salmonids, that spawn only once annually [33]. It is inappropriate to compare results from studies of those species to those of a multiple-spawner species such as the fathead minnow [26]. In the bittersweet (Rhodeus ocellatus), a multiple-spawning cyprinid, plasma E2 concentrations remain fairly great throughout the 5-d spawning cycle, with plasma E2 concentrations reaching their greatest levels early and then decreasing gradually throughout the vitellogenic phase [37]. In another multiple-spawning cyprinid, the common goldfish (Carassius auratus), plasma concentrations of E2 increase slowly before ovulation, remain elevated during ovulation, and then decrease [37,38]. Aida [37] suggests that a gradual decrease in E2 levels followed by an increase in testosterone at the end of vitellogenesis might stimulate the GtH surge that precedes ovulation. This suggests that if all the other conditions are appropriate, an increase in plasma E2 concentration might result in decreased ovulation. This type of cycle is similar to that of several other multiple-spawning cyprinids [26]. In EX I, in which endogenous plasma E2 concentrations, fecundity, and induction of E2 by NP was greater, the greater E2 concentration seemed to have a negative effect on egg production. This is similar to the results of a study that exposed fathead minnows to waterborne E2 [39], in which increased E2 concentrations caused a dose-dependent decrease in egg production. In EX II, which was conducted later in the spawning season, when egg production was declining, exposure to NP and a subsequent increase in plasma E2 concentration resulted in a positive correlation. These results underscore the importance of timing as well as of duration and intensity of exposure to endocrine-modulating chemicals.

The differential effects observed in this study may have resulted from the differential ability of the fish to secrete E2 during the two periods studied. Because the adult fish were maintained in breeding condition at a temperature of 26°C from May until September, during EX II the fish may have been nearing the end of the season during which they typically spawn. Also, during EX II, the plasma E2 concentrations were less, so that at that time, an increase in E2 levels may have resulted in greater reproductive output.

Induction of Vtg is considered to be one of the most sensitive endpoints when monitoring for exposure to estrogenic substances [7,29], yet in this study, effects on reproduction were observed at NP concentrations that did not induce Vtg. This brings into question whether the effects on egg production resulted from the direct estrogenic properties of NP. When rainbow trout were exposed to NP, an induction of Vtg occurred that was negatively correlated with the gonadosomatic index in males [40]. These results are consistent with those caused by exposure of fish to E2; thus, the authors concluded that the effects were caused by the estrogenic potential of NP. However, in that study, no measurements of plasma E2 concentrations were made. Thus, it is unknown whether an elevation of plasma E2 concentrations occurred that could explain the results. The mechanism by which exposure to NP causes an increase in plasma E2 concentration is yet to be elucidated, but the effects observed in the present study are unlikely to have resulted from a direct estrogenic mechanism.

NP versus estradiol potency

Nonylphenol has been suggested to be an environmental estrogen or xenobiotic that can mimic the effects of E2 [41]. The fact that NP has been reported to be estrogenic in mammals [42] has lead to speculation that it also might be estrogenic to fish [3,12,13,34]. Nonylphenol has been reported to bind to the ER of rainbow trout liver cells and to induce Vtg mRNA transcription [34,44]. The induction of Vtg mRNA has been blocked by the antiestrogen 4-hydroxytamoxifen and ICI 164–384 [44], which indicates that the effect of NP on Vtg production was via an ER-mediated process, and that NP was acting as an ER agonist in rainbow trout liver tissue. Based on in vitro bioassays, including receptor binding and expression assays with an ER-binding affinity, the relative potency of NP is approximately $4.0 \times 10^{-5}$ as great as that of E2 [45]. In rainbow trout hepatocytes, NP has been reported to bind to the ER with an affinity of approximately $1.2 \times 10^{-3}$ less than that of E2 [44]. Similarly, NP induces responses that are under the control of the ER among in vitro expression assays [3,13,45,46]. Previous studies with NP have reported increased in vitro production of Vtg in rainbow trout primary hepatocytes.
4-Nonylphenol effects on fathead minnows

Assuming that the plasma had a density of 1.0 g/ml and contained approximately the same concentration of NP as found in the whole body, then as observed in the greatest exposure to NP, a plasma concentration of 912 ng NP/ml can be estimated. Applying the relatively conservative (likely maximum) relative potency of 10^{-4} to the maximum concentration of 912 ng NP/ml, a value of 9.12 × 10^{-6} M, which is between 5.0 × 10^{-2} and 5.0 × 10^{-1} less potent than E_2. However, the response to NP did not reach the same maximum as that caused by E_2, which indicates that NP is a partial estrogen agonist.

For this reason, it is difficult to accurately estimate the relative potency of NP, which has an estrogenic potency to fish of between 1 × 10^{-4} to 1 × 10^{-6} relative to that of E_2 [12]. The relative potency of NP to induce Vtg expression in rainbow trout primary hepatocytes is approximately 9 × 10^{-6} [12]. The relative potency to induce expression of the lac-Z reporter gene under the control of an exogenous ER in yeast for induction of between 10^{-3} and 10^{-4} [12]. The relative potency of NP to induce Vtg expression in rainbow trout hepatocytes is approximately 9 × 10^{-6} [12]. The relative potency to induce expression of the lac-Z reporter gene under the control of an exogenous ER in yeast is 1.4 × 10^{-4} [13] and, to induce expression of E_2-specific responses in the MCF-7 immortal human breast cancer cell line, between 10^{-3} and 10^{-4} [3] and 4 × 10^{-5} [45]. Based on a range of relative potencies from 10^{-4} to 10^{-3} and a threshold concentration of E_2 to induce Vtg in fathead minnows of between 10 and 100 ng E_2/L, it can be concluded that the estrogenic potency of NP relative to E_2 is between 10^{-3} and 10^{-4}. Using the maximum relative potency of NP and the minimum threshold concentration of E_2 results in a threshold concentration of 10 µg NP/L, whereas the minimum relative potency of NP considered with the maximum plasma concentration of E_2 required for induction of Vtg results in a concentration of 1,000 µg NP/L required to induce Vtg in male fathead minnows. The other two possible combinations result in estimates of 100 µg NP/L. All these estimates indicate that the concentrations of NP studied here would be unlikely to induce measurable concentrations of Vtg in male fathead minnows through an E_2-agonist mechanism, which is consistent with the fact that little induction of Vtg was observed in male fathead minnows exposed to the greatest NP concentration.

The potential for NP to increase plasma Vtg concentrations by acting as a direct E_2 agonist was further investigated by calculating the number of E_2-Eq that would be contributed by NP relative to the number contributed by endogenous E_2 in the plasma. This calculation requires estimates for the plasma concentrations of NP and E_2 in fish and for the potency of NP relative to E_2. This calculation also requires knowledge of the relative availability or biologically active fraction of both potential ER ligands. The small volume of plasma available in fathead minnows and the need for other assays precluded measuring the concentrations of NP in the plasma; however, an estimate of the possible concentration could be made from the whole-body measurements of NP.

Assuming that the plasma had a density of 1.0 g/ml and contained approximately the same concentration of NP as found in the whole body, then as observed in the greatest exposure to NP, a plasma concentration of 912 ng NP/ml can be estimated. Applying the relatively conservative (likely maximum) relative potency of 1 × 10^{-4} to the maximum concentration of 912 ng NP/ml, a value of 9.12 × 10^{-2} ng E_2-Eq-NP/ml as contributed by NP is estimated. Concentrations of E_2 measured in fish exposed to the greatest concentrations of NP (EX I, 3.4 µg NP/L; EX II, 2.4 µg NP/L) were 1.45 and 5.8 ng E_2/ml, respectively. The average of these two values is 3.6 ng E_2/ml. The E_2-Eq-NP would contribute approximately 2.5% to the total E_2-Eq. Thus, this line of evidence suggests that NP is unlikely to contribute significantly to the total E_2-Eq in the plasma. Because the male plasma Vtg normally is used as an indicator of exposure to environmental estrogens, exposure to this level of NP would not be expected to cause an increase in the plasma Vtg concentration. In fact, in male fathead minnows, no induction of plasma Vtg was observed with any concentration of NP tested.

There is an uncertainty of approximately 100-fold in the estimate of the relative contributions of NP and E_2 to the total E_2-Eq. However, based on the relative potency of NP to induce Vtg in rainbow trout of 1 × 10^{-6}, the calculations would seem to be conservative, with even lesser contributions of NP to the total E_2-Eq being likely. If the assumption of a density of 1.0 g/ml for blood plasma is violated, the amount of NP in the plasma would be overestimated, which would result in an overestimate of the E_2-Eq-NP. Thus, this calculation is a conservative estimate for the maximum contribution of NP to the total E_2-Eqs.

The analysis of the relative contributions to the E_2-Eq does not consider the relative proportions of NP and E_2 that might be bound to plasma-carrier proteins. E_2 is selectively bound with high affinity to the steroid hormone-binding globulin (SHBG), whereas weak xenoestrogens such as OP bind less tightly to SHBG [47,48]. Three of the most important characteristics for determining the bioavailability of ER ligands are lipid solubility, biologic half-life, and amount of protein binding. It has been suggested that weak xenoestrogens may be more active in vivo than E_2, because they are free rather than bound like E_2 [48]. However, in humans, except during the third trimester of pregnancy, less than 40% of E_2 is bound to SHBGs. Most E_2 is bound to albumin and other serum proteins, with only approximately 2% being available as free E_2. Exact values for NP distribution in the blood are not available, but a high capacity for the binding of compounds of this type to serum proteins does exist [31, 49]. The binding of OP, a compound similar to NP, to human albumin is approximately 1.7-fold less than that for E_2 [48]. The binding of OP to human SHBG is approximately fourfold less than that for E_2. Therefore, assuming the greatest degree of binding by E_2 and the least degree of binding by NP could result in a greater probability, by factor of as much as 100-fold, of NP reaching ER in an available form than that for E_2. However, the contribution of E_2-Eq-NP could be approximately 20%, or only one-fifth that of E_2. Because much of the E_2 and NP probably would be bound to albumin-type proteins and both E_2 and NP have similar values for K_{app} and, thus, similar polarities, they would be expected to bind similarly to these less-specific proteins. Therefore, the effect of differential availability is probably less than a factor of 10, and there may be no difference at all. To reduce the uncertainty in calculations of this type, more information on the relative concentrations of bound and free E_2 and of synthetic E_2 agonists is needed.

The particular mechanism of action by NP to be considered here is its potential as an endocrine disruptor and, in particular, its potency as an environmental estrogen mimic. Previously, results of both in vivo and in vitro studies have suggested that NP is a weak estrogen agonist, and that the toxic effects of NP result from its mimicking estrogen [3,5,6,10,12,13,43,46,48]. However, because of the relatively great concentrations of ER in E_2-responsive tissues and the relatively great pool of unoccupied receptor, NP is unlikely to act as an E_2 agonist. To further assess the likelihood that the effects of NP result from an ER-mediated mechanism of action,
the relative potencies of NP to cause various effects were compared to those for E₂ (Table 5). The potencies were estimated and compared by making a ratio of the concentration of NP and that of E₂ to elicit the same level of response, such as the EC₅₀. The greater the ratio, the more potent E₂ is relative to NP. Based on what is known of the potency of NP as determined from in vitro receptor binding and expression assays, it would be expected that for specifically ER-mediated responses, the ratio would be in the range of 10² to 10³, which is also in the range of responses reported for E₂-specific in vitro expression assays (Table 5). Receptor binding also resulted in a relatively great ratio, but not as great as would be expected if receptor occupancy because of binding affinity was the major determinant of ER-mediated effects. Reasons exist to believe that receptor binding affinity and potency for ER-mediated responses may not always be highly correlated [47,50]. This might be an artifact of the relatively great concentration of NP needed to achieve an IC₅₀. It was not possible to calculate a ratio for other endpoints, such as survival and hematocrit, because no effects were seen for NP at the concentrations tested. In the case of egg production, the ratio was 7.5, which indicates that NP is more effective at causing this result than would be expected based on its potency as an E₂ agonist. Similarly, histologic effects of NP in male fish were observed when the fish were exposed to concentrations much less than would have been predicted from the relative potency of NP as an estrogen agonist [51]. In fact, the results presented here suggest that the effects of both NP and E₂ on egg production may not be ER-specific responses. Taken together, these results indicate that the effects on egg production are unlikely to be the results of NP acting as an ER agonist.

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