Propiconazole Inhibits Steroidogenesis and Reproduction in the Fathead Minnow (Pimephales promelas)

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Conazoles are designed to inhibit cytochrome P450 (CYP) 14α-demethylase, an enzyme key to fungal cell wall formation. In vertebrates, conazoles may inhibit other CYPs, potentially disrupting processes like sex steroid synthesis. Propiconazole is a current-use pesticide that is among the first chemicals being tested in the U.S. Environmental Protection Agency endocrine disruptor screening program. Fathead minnows (Pimephales promelas) were exposed to 0, 5, 50, 500, or 1000 µg propiconazole/l in a 21-day study that evaluated apical reproductive endpoints (fecundity, fertility, hatch); measures of endocrine function and steroid synthesis, such as cholesterol, vitellogenin (VTG), and sex steroid (testosterone [T], 17β-estradiol [E2]) concentrations in the plasma; and changes in gonadal expression of steroidogenic genes. Plasma E2 and VTG concentrations in females were reduced by exposure to propiconazole, and egg production was decreased in the 500 and 1000 µg/l treatment groups. These in vivo effects coincided with inhibition of E2 synthesis by ovary explants exposed to propiconazole in vitro. We also observed a compensatory response in females exposed to propiconazole, manifested as increased gonad weight and upregulation of genes coding for key steroidogenic proteins, including CYP19 (aromatase), CYP17 (hydroxylase/lyase), CYP11A (cholesterol side-chain-cleavage), and steroidogenic acute regulatory protein. Other than an increase in relative testis weight, effects on endocrine function in males were less pronounced than in females. This study provides important data relative to the potential endocrine activity of propiconazole in fish and, more generally, to the further delineation of pathways for the reproductive effects of steroid synthesis inhibitors in fish.

Key Words: propiconazole; fish; reproduction; steroidogenesis; endocrine disruption.

One manner through which chemicals can impact endocrine function is via alterations in sex steroid concentrations. A number of chemicals have been shown to interfere with steroid synthesis pathways in fish, thereby affecting sexual development and reproductive success (Ankley et al., 2002, 2007; Noaksson et al., 2003a, b; Tillitt et al., 2010; Villeneuve et al., 2008; Zhang et al., 2008). In steroid biosynthesis, the conversion of cholesterol to physiologically active hormones involves a series of reactions catalyzed by enzymes in the cytochrome P450 (CYP) superfamily and hydroxysteroid dehydrogenases (HSDs) (Miller, 1988; Parker and Schimmer, 1995). Briefly, steroidogenic acute regulatory protein (STAR) transports cholesterol from the cytosol into the inner mitochondrial membrane where CYP side-chain cleavage (CYP11A) catalyzes its conversion to pregnenolone. Pregnenolone is metabolized to progesterone by 3β-HSD, and progesterone is converted to androstenedione by the bifunctional enzyme CYP hydroxylase/lyase (CYP17). Androstenedione then is metabolized to testosterone by 17β-HSD. The key enzyme that converts androgens to estrogens is aromatase (CYP19), which is expressed at a low level in testes and more prominently in ovaries. The complexity of the steroidogenesis pathway allows for multiple targets for chemical disruption. For example, studies with different chemicals in fish have documented adverse reproductive endocrine effects associated with inhibition of CYP11A (Ankley et al., 2007), CYP17 (Ankley et al., 2005, 2007), 3β-HSD (Villeneuve et al., 2008), and CYP19 (Ankley et al., 2002, 2005; Noaksson et al., 2003b).

Conazoles are a class of fungicides, used both as pesticides and drugs, that function through inhibition of CYP51 (14α-demethylase), which is important for the synthesis of ergosterol used in cell wall formation (Henry and Sisler, 1984; van den Bosche et al., 1978). The conazole moiety binds to the heme group of the CYP51 protein, inhibiting functionality of the enzyme (Henry and Sisler, 1984; Hitchcock et al., 1990). However, many conazoles are not particularly specific for inhibition of prokaryotic CYP51s as they also inhibit a variety of CYPs in other phyla, including vertebrates (Mason et al., 2008).
Propiconazole is a prototypical inhibitor of CYP51 commonly used in agriculture to control fungal growth on fruit, vegetable, and cereal crops (Battaglin et al., 2011; Schwinn, 1984). Propiconazole is one of 60-plus chemicals being evaluated in the first round of Tier 1 testing of the U.S. Environmental Protection Agency (USEPA) Endocrine Disruptor Screening Program (EDSP) (Federal Register, 2009). The Tier 1 EDSP battery of assays for screening potential endocrine disruption is comprised of a number of in vivo mammalian and nonmammalian assays, including a short-term (21-day) fathead minnow reproduction test (USEPA, 2009). Based on past studies with other fungicides in fish, we hypothesized that propiconazole would disrupt endocrine function through depressing steroid synthesis via inhibition of one or more steroidogenic CYPs and subsequently would decrease plasma VTG concentrations and egg production. To assess the veracity of this predicted pathway, we employed an expanded version of the EDSP short-term reproduction assay. Besides the endpoints utilized for the basic Tier 1 test, we made a number of additional measurements to more completely characterize biological activity of propiconazole, including determination of sex steroid synthesis/plasma concentrations, expression of key gonadal and hepatic genes involved in compensatory responses (including xenobiotic metabolism), and biochemical alterations allowing comparison of our fish data with effects of propiconazole on cholesterol metabolism in mammals (Murphy et al., 2012; Nesnow et al., 2011; USEPA, 2011).

Our main study objectives were to (1) support the further development of toxicity/adverse outcome pathways for reproductive effects of steroid synthesis inhibitors, such as conazoles, in fish and (2) specifically assess whether propiconazole would disrupt endocrine function in an assay used for the EDSP. However, this work also provided an opportunity to generate, at least some, toxicity information relevant to assessing potential environmental risks of the fungicide. Propiconazole has been detected in surface water at concentrations of 0.291 (mean) and 1.15 (maximum) μg/l in agricultural settings (Battaglin et al., 2011) and has been shown to have relatively low acute toxicity in fish, typically causing lethality only at low mg/l concentrations in water (USEPA, 2000). However, there are no published studies concerning the possible sublethal (e.g., reproductive) effects of propiconazole in fish.

**MATERIALS AND METHODS**

Propiconazole dosing and exposure characterization. Propiconazole (97% purity) was provided by Syngenta Crop Protection Inc. (Greensboro, NC). Solvent-free stock solutions were generated by initially dissolving 100 mg of propiconazole in 1 l of Lake Superior water using sonication for 2 h at 45°C. Four 1 l solutions subsequently were combined and diluted to 1.9 l with Lake Superior water to serve as a primary stock, which was further diluted to target test concentrations when delivered to the exposure tanks. Test concentrations of 0 (control), 5, 50, 500, and 1000 μg propiconazole/l were chosen based on initial data from a 96-h range-finding test with fathead minnows, which demonstrated no lethality at these concentrations (data not shown).

Water samples were collected from each exposure tank and analyzed for propiconazole twice a week during the 21-day exposure. Water was directly analyzed using an Agilent (Wilmington, DE) model 1100 liquid chromatography–mass selective detector equipped with an atmospheric pressure photoionization interface. An aliquot of sample was injected onto a Zorbax SB-C18 (Agilent) column (2.1×150 mm) and eluted isocratically at a flow rate of 0.2 ml/min with a mobile phase composed of 60% acetonitrile/water. A toluene dopant also was used at a flow rate of 0.05 ml/min. Propiconazole concentrations were determined using selective-ion monitoring (positive-ion mode), with ions at 342 and 344 amu. Propiconazole exists as two diastereomers, each with two enantiomers (Aebi et al., 1999). Our concentrations were the summation of these two major diastereomers as they were not individually baseline resolved. Concentrations were calculated with an external standard method of quantitation. Quality control samples such as procedural blanks, spiked recoveries, and duplicate analyses comprised 10% of the sample load. The mean percent agreement (± SD) between duplicate analyses was 99% (±1.1, n = 27), and the average propiconazole recovery was 98% (±2.2%, n = 15).

The concentration of propiconazole in tissues of the fish also was determined at the conclusion of the test using an extraction method described elsewhere (Skolness et al., 2012). Three females and three males from each treatment were analyzed, except for controls where a total of nine fish (five males, four females) were analyzed. Analysis of fish extracts used the same analytical method described above for the water samples. The quantitation limit for tissues ranged from 45 to 250 μg propiconazole/kg, depending on weight of fish. The mean recovery (± SD) of propiconazole from spiked tissue samples was 103.5% (± 9.5, n = 7).

Reproductive toxicity test. A 21-day fathead minnow reproduction study was conducted using the basic protocol described by Ankley et al. (2001). Fathead minnows (5–6 months old) from an on-site culture facility at the USEPA lab in Duluth, MN were used for the experiment. Twenty-liter aquaria were divided by a nylon mesh screen into two equal chambers. One male and one female were placed together with a spawning substrate on each side of the divider. During a chemical free 14-day acclimation period, egg production was assessed and recorded daily. Eggs were removed from the spawning substrate, counted, and examined microscopically to determine fertility. Pairs that did not reproduce during the acclimation period were excluded from the chemical exposure. After the 14-day acclimation, pairs that had reproduced successfully during acclimation were randomly assigned to experimental treatment groups. Propiconazole exposures were then initiated with six replicate tanks (12 pairs of fish) per treatment. During both the acclimation and exposure phases, fish were maintained in continuously flowing Lake Superior water at a flow rate of 45±2 ml/min at 25.0°C ± 1.0°C, with a photoperiod of 16:8 h light:dark. Temperature and dissolved oxygen were monitored daily, and hardness and alkalinity were measured two or three times weekly. All values remained within acceptable limits (Ankley et al., 2001). Fish were fed frozen (thawed prior to feeding) brine shrimp _Artemia_ (Artemia; San Francisco Bay Brand, Newark, CA) twice daily. During the 21-day propiconazole exposure, spawning was monitored in a similar manner as...
during the acclimation period. In addition to spawning activity, hatching success of eggs held in clean water was evaluated as previously described in Skolness et al. (2012). Animal research protocols were approved by the on-site Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

Upon conclusion of the exposure, the fathead minnows were euthanized in buffered tricaine methanesulfonate (MS-222; 100/ mg buffered with 200 mg NaHCO3/l; Argent, Redmond, WA) and weighed. Nuptial tubercles, a secondary sex characteristic on the heads of male fathead minnows, were scored as described elsewhere (Jensen et al., 2001). Blood was collected from the caudal vasculature with a heparinized microhemocrit tube and centrifuged to obtain plasma, which was stored at -80°C until analyzed for T and E2, cholesterol, triglycerides, and VTG. Liver was collected and divided into two subsamples, which were flash frozen in liquid nitrogen and stored at -80°C until used for gene expression or metabolomic analyses (latter are not reported herein). Gonads were removed and weighed for the calculation of the gonadal-somatic index (GSI = gonad wt/body wt × 100). Gonads were sectioned into two pieces. Two portions of the gonad were snap-frozen in liquid nitrogen and stored until used for gene expression analyses. A third portion of the gonad was preserved in Davidson’s fixative for histological analysis. The final piece of the gonad was used immediately for an ex vivo steroid production assay. Brain and pituitary were collected together, flash frozen in liquid nitrogen, and stored at -80°C. The remaining carcass was wrapped in solvent-rinsed aluminum foil and stored frozen at -20°C until analyzed for propiconazole tissue residues.

**Biochemical measurements.** Plasma VTG concentrations were quantified using an enzyme-linked immunosorbent assay with a polyclonal antibody to fathead minnow VTG and purified fathead minnow VTG as a standard (Korte et al., 2000). Plasma concentrations and ex vivo production of T and E2 were determined using radioimmunoassay (RIA) as described elsewhere (Jensen et al., 2001; McMaster et al., 1995; Villeneuve et al., 2009).

In addition to measuring ex vivo steroid production, we determined the direct effects of propiconazole on T and E2 synthesis using an in vitro assay in which ovary explants from untreated fathead minnows were incubated with different concentrations of the fungicide. The approach for the in vitro assay is described in detail by Villeneuve et al. (2008) and utilized the same basic materials and procedures as the ex vivo steroid production assay. Briefly, ovary tissue was collected from 10 reproductively mature, untreated females from the culture facility and divided into approximately 10 mg subsamples. Ovary subsamples from each female were incubated with each treatment (0, 0.5, 5, 50, 500, 1000, or 2500 µg propiconazole/l), yielding 10 orthogonal biological replicates per treatment. Each propiconazole exposure solution contained 0.1% methanol, and there was both a nonsolvent (no methanol, no propiconazole) and solvent control (0.1% methanol). After a 12-h incubation, media were collected and stored at -80°C until steroids were extracted and measured using RIA. Total cholesterol and triglyceride concentrations in plasma samples were quantified using assay kits (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer’s instructions. Briefly, for the cholesterol measurement, 1 µl of the plasma was diluted 1:400 in Assay Buffer, and 50 µl of dilute plasma or an appropriate cholesterol standard was added per well. Assay cocktail, 50 µl (Assay Buffer, cholesterol detector, horseradish peroxidase, cholesterol oxidase, and cholesterol esterase) was then added to all wells. The plate was covered, incubated for 30 min at 37°C, and protected from light. For the triglyceride kit, samples were diluted 1:10, and 10 µl of sample or standard was added to wells accordingly. Diluted Enzyme Buffer, 150 µl, was added to all wells. Triglyceride assay plates were incubated at room temperature for 15 min, protected from light. Plates for both kits were read with an automated BioTek plate reader (Biotek, Synergy 4; Gen5 Software, Winooski, VT). The cholesterol determination used a fluorescence excitation wavelength at 530 nm and emission wavelength at 585 nm, whereas the triglyceride measurement was based on a colorimetric absorbance reading at 530 nm. Concentrations were calculated by regression against the appropriate standard curve.

**Gene expression analyses.** Total RNA was extracted from liver and gonads as previously described by Skolness et al. (2012) with TRI Reagent (Sigma-Aldrich, St Louis, MO), following manufacturer’s protocols. Gene expression was measured using QPCR with a one-step procedure with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following manufacturer’s instructions, as described in detail elsewhere (Ankley et al., 2009b; Skolness et al., 2012).

Expression of several genes involved in endocrine function in gonadal tissue was determined. These included genes coding for proteins involved in steroid synthesis: star, cyp11a1, cyp17, and cyp19a1a. Additional genes whose expression was measured in the gonad included follicle-stimulating hormone receptor (fsr), low-density lipoprotein receptor (ldlr), 3-hydroxy-3-methyl-glutaryl-CoA reductase (hmgr), and cyp51. Expression of genes related to lipid metabolism was measured in the liver and included hmgr, cyp51, farnesyl diphosphate synthase (fpds), and fatty acid synthase (fasm). Two additional transcripts measured in the liver code for enzymes involved in the phase I oxidative metabolism of xenobiotics, cyp1a1, and cyp3a.

**Statistical analysis.** Basic statistical analyses were conducted using Statistica 8 (StatSoft Inc., Tulsa, OK) and GraphPad Instat v. 3.01 (GraphPad Software, San Diego, CA). When parametric assumptions were met, a one-way ANOVA was performed with Duncan’s multiple comparisons test to determine differences across treatment groups. When data did not meet parametric assumptions, a Kruskall-Wallis test was performed with Dunn’s test for multiple comparisons. Plasma cholesterol and triglyceride concentrations and gene expression data are depicted as fold-change (log 2) relative to control values within each sex. Data are presented as means with SEM. Differences were considered significant at p < 0.05.

**RESULTS**

**Exposure Characterization.**

Water propiconazole concentrations were close to nominal and did not vary greatly over the course of the exposure (Table 1). No propiconazole was detected in any control tank sample over the course of the exposure (detection limit = 1 µg/l). Similarly, for the tissue residue analysis, no propiconazole was detected.
in the controls or procedural blanks above the quantitation limit. Tissue concentrations of propiconazole in fish exposed to the chemical for 21 days exhibited a concentration-dependent increase, reflective of the water concentrations to which the fish were exposed (Table 1). Female fish had consistently higher tissue concentrations of propiconazole than males. Measured bioconcentration factors (BCFs) for females ranged from about 13 to 38, whereas those for males ranged from 7.5 to 18 (Table 1).

**Biological Responses: Apical and Biochemical Endpoints**

Over the course of the exposure, there were no mortalities. There were also no changes noted relative to general feeding or swimming behavior. There was no effect on the weight of the fish (Table 2). The GSI of both females and males was increased in a concentration-dependent manner in the 500 and 1000 µg propiconazole/l treatment groups (Table 2).

The cumulative number of eggs per female was significantly reduced by exposure to propiconazole in three of the four treatments, with the largest effects occurring in the 500 and 1000 µg propiconazole/l treatment groups (Fig. 1). Neither fertility nor hatching success of the deposited eggs was affected by propiconazole (data not shown).

Propiconazole caused a concentration-dependent decrease in plasma E2 concentrations in the female fish from the 21-day exposure (Fig. 2A). Corresponding with the depression in E2, plasma VTG concentrations also were decreased in a concentration-dependent manner in the females (Fig. 2B). Although plasma E2 levels were depressed, *ex vivo* E2 production in ovarian tissue was not decreased at 21 days; in fact in the 500 µg propiconazole/l treatment, E2 production was significantly increased (Fig. 2C). *Ex vivo* ovarian production of T did not vary significantly between the control and treatment groups (Fig. 2D).

The effects of propiconazole on steroid production in an *in vitro* assay with explants of ovaries from untreated females from the culture unit are shown in Figure 3. There was no significant difference between the nonsolvent and solvent controls;

<table>
<thead>
<tr>
<th>Nominal concentrations (µg/l)</th>
<th>Measured water concentrations (µg/l)</th>
<th>Propiconazole in tissue (µg/kg)</th>
<th>Female</th>
<th>BCF</th>
<th>Male</th>
<th>BCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N.D.</td>
<td>N.D</td>
<td>—</td>
<td>—</td>
<td>N.D</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>5.8 (0.04)</td>
<td>110 (11)</td>
<td>19 (1.8)</td>
<td>75 (23)</td>
<td>13 (4.0)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>53 (0.2)</td>
<td>693 (140)</td>
<td>13 (2.6)</td>
<td>400 (39)</td>
<td>7.5 (0.7)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>563 (6.5)</td>
<td>9792 (1651)</td>
<td>17 (2.9)</td>
<td>5264 (1264)</td>
<td>9.4 (2.3)</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1056 (6.0)</td>
<td>39,604 (7010)</td>
<td>38 (6.6)</td>
<td>19,203 (556)</td>
<td>18 (5.2)</td>
<td></td>
</tr>
</tbody>
</table>

*Data represent mean (± SE; n = 49). No propiconazole was detected in any control tanks over the course of the experiment (detection limit = 1 µg/l).

*Data represent mean (± SE; n = 3). No propiconazole was detected in any control fish (n = 9; quantitation limit was from 45–250 µg/kg depending on the weight of the fish).

N.D., not detectable.

<table>
<thead>
<tr>
<th>Propiconazole (µg/l)</th>
<th>Weight (g)</th>
<th>GSI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.32 (0.10)</td>
<td>9.60 (0.62)</td>
</tr>
<tr>
<td>5</td>
<td>1.41 (0.07)</td>
<td>11.30 (0.87)</td>
</tr>
<tr>
<td>50</td>
<td>1.26 (0.10)</td>
<td>11.14 (0.88)</td>
</tr>
<tr>
<td>500</td>
<td>1.37 (0.13)</td>
<td>13.55 (1.46)*</td>
</tr>
<tr>
<td>1000</td>
<td>1.60 (0.09)</td>
<td>18.42 (0.88)**</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.04 (0.16)</td>
<td>1.15 (0.10)</td>
</tr>
<tr>
<td>5</td>
<td>3.31 (0.21)</td>
<td>1.20 (0.13)</td>
</tr>
<tr>
<td>50</td>
<td>3.20 (0.19)</td>
<td>1.35 (0.11)</td>
</tr>
<tr>
<td>500</td>
<td>2.80 (0.12)</td>
<td>1.80 (0.14)**</td>
</tr>
<tr>
<td>1000</td>
<td>2.78 (0.20)</td>
<td>2.48 (0.15)**</td>
</tr>
</tbody>
</table>

*Notes. Data indicate the mean (± SE; n = 12).

*Asterisks indicate a significant (*p ≤ 0.05; **p ≤ 0.01) difference from control values.*

**FIG. 1.** Effects of 21-day propiconazole exposure on cumulative fecundity in fathead minnows. Asterisks indicate a significant (*p ≤ 0.05; **p ≤ 0.01) difference from control values.
therefore, data from treated tissue were compared with that from nonsolvent controls. In vitro E2 production was decreased in a concentration-dependent manner, with significant effects observed in the 1000 and 2500 µg/l treatment groups (Fig. 3A). No significant modulation of T production was observed for the propiconazole-treated ovary explants (Fig. 3B).

In the male fathead minnows, propiconazole did not cause changes in plasma T concentrations (Fig. 4A) although ex vivo T production was significantly decreased at propiconazole concentrations of 50 and 1000 µg propiconazole/l (Fig. 4B). Expression of nuptial tubercles in the males was not affected by propiconazole, with mean (± SE) tubercle scores of 20.9 (1.30), 21.0 (0.99), 20.4 (1.2), and 19.8 (1.1) in the 0, 5, 50, 500, and 1000 µg propiconazole/l treatments, respectively.

Histological analysis of ovaries indicated that four of the six females in the 1000 µg propiconazole/l treatment had mild to moderate increases in oocyte atresia (Table 3, Fig. 5C). There was no histological evidence of treatment-related reductions in yolk formation in the developing oocytes or changes in the
gonad stage score (Table 3, Figs. 5A–C). In the males, there was an increased prevalence of spermatozoa and distended seminiferous tubules in fish from the highest treatment group (Fig. 5F). No other pathologies were observed (Table 3, Figs. 5D–F).

Relative plasma cholesterol concentrations were significantly reduced in females from three of the four treatments, and there also was a trend, albeit not significant, toward decreased plasma cholesterol in the males (Fig. 6A). Propiconazole did not have significant or consistent effects on plasma triglyceride concentrations in either sex (Fig. 6B).

**Biological Responses: Gene Expression Changes**

In the females, exposure to propiconazole increased ovarian expression of several genes related to steroidogenesis (fsrh, star, cyp11a, cyp17, and cyp19a1) in one or more treatment groups (Figs. 7A–E). Expression of all five genes was increased in females from the 1000 µg/l treatment group, and four of the five were upregulated in the 500 µg/l treatment group. Far fewer effects on steroidogenic-related genes were observed in the males, where only cyp17 expression was significantly increased in the 500 µg propiconazole/l treatment (Fig. 7D).

Three genes related to cholesterol metabolism also were measured in gonadal tissue (ldlr, hmgr, and cyp51). No significant effects were observed in males, but in females, hmgr and cyp51 had decreased expression in the 500 and 1000 µg propiconazole/l treatment groups (Figs. 7G and H).

In the females, hepatic hmgr expression was significantly reduced in the 500 and 1000 µg propiconazole/l treatments, whereas expression of hmgr, fdps, cyp51, and fasn in males was significantly reduced in one or more treatment groups (Figs. 8A–D). An important gene related to hepatic xenobiotic metabolism in fish, cyp1a1, was significantly upregulated by propiconazole in a concentration-dependent manner in the females and also upregulated in males from the 500 and 1000 µg propiconazole/l treatment groups (Fig. 8E). Another phase I xenobiotic-metabolizing gene, cyp3a, was also upregulated at one or more test concentrations in the exposed fish (Fig. 8F).

**DISCUSSION**

Propiconazole, a putative inhibitor of a number of CYPs, depressed plasma sex steroid concentrations and VTG synthesis and overall egg production in female fathead minnows exposed to the chemical for 21 days. The fungicide also inhibited production of E2 in an in vitro assay with ovarian tissue from untreated fish. This suite of responses was consistent with expectations based on adverse outcome pathways derived from other chemical inhibitors of steroidogenesis in fish. For example, 21-day reproduction studies with a number of suspected/known inhibitors of steroidogenesis including fadrozole, prochloraz, trilostane, and ketoconazole resulted in decreased egg production in fathead minnows (Ankley et al., 2002, 2005, 2007; Villeneuve et al., 2008). The fungicide prochloraz, an inhibitor of multiple CYPs involved in steroid synthesis, and the drug fadrozole, a more specific inhibitor of CYP19, reduced E2 and VTG concentrations in female fathead minnows within 1 day of exposure, and both parameters remained depressed through the duration...
of a 21-day reproduction test (Ankley et al., 2002, 2005, 2009b; Skolness et al., 2011; Villeneuve et al., 2009). The fungicide ketoconazole, also an inhibitor of multiple steroidogenic CYPs, decreased plasma E2 and VTG concentrations in female fathead minnows exposed in a short-term (≤ 8 days) exposure, but in a 21-day test, E2 and VTG had returned to baseline levels in the fish due, it appears, to different compensatory mechanisms (Ankley et al., 2007, 2012). Finally, in the 21-day fathead minnow reproduction assay, the drug trilostane, an inhibitor of the steroidogenic enzyme 3β-HSD, reduced female VTG

FIG. 5. Effects of propiconazole on fathead minnow gonads. Fish were exposed to 0 (control), 500, or 1000 µg propiconazole/l for 21 days. (A) Control ovary, (B) ovary from fish exposed to 500 µg/l, (C) ovary from fish exposed to 1000 µg/l, (D) control testis, (E) testis from fish exposed to 500 µg/l, and (F) testis from fish exposed to 1000 µg/l. Arrows in panel C indicate atretic oocytes. This figure appears in color online.
production and fecundity (Villeneuve et al., 2008). This was consistent with the direct inhibitory effects of trilostane on in vitro production of E2 in ovarian explants from untreated fathead minnows (Villeneuve et al., 2008). Although there are some variations in the effects of these different inhibitors of sex steroid synthesis, the overall pattern of responses is remarkably consistent: reduced steroid production, depressed VTG, and decreased fecundity. Hence, the present work with propiconazole reinforces and builds upon previously derived adverse outcomes for the effects of inhibitors on steroid synthesis in female fish.

The effects of propiconazole on endocrine function in the male fathead minnows were much less pronounced than in the females. Although propiconazole appeared to decrease ex vivo synthesis of T, the fungicide did not decrease plasma T concentrations or reduce spermatogenesis (as evidenced by histological changes). Conversely, prochloraz decreased plasma T concentrations in males in both 8- and 21-day exposures (Ankley et al., 2005, 2009b). Ketoconazole also decreased plasma T concentrations in males in an 8-day exposure; however, due to seeming compensatory alterations, this effect did not persist for 21 days (Ankley et al., 2007, 2012). The aromatase inhibitor fadrozole actually slightly increased plasma T concentrations in male fathead minnows following 8- or 21-day exposures (Ankley et al., 2002; Villeneuve et al., 2009). The differential effects of these chemical inhibitors on steroid synthesis in female versus male fish likely are due to the specific CYPs with which the chemicals preferentially interact. For example, there is evidence that both prochloraz and ketoconazole interact with CYPs other than CYP19 (e.g., CYP11A and CYP17), which are “upstream” of T production. Accordingly, these chemicals tend to decrease T in males and both T and E2 in females. Conversely, chemicals (like fadrozole) that interact more specifically with CYP19 than other steroidogenic CYPs should tend to decrease E2 in females and would not be expected to reduce plasma T in either females or males. Based on this, we speculate that propiconazole may have a higher affinity for CYP19, than for example, CYP11A or CYP17. This hypothesis is, in fact, consistent with the in vitro inhibition data from the current study, in which propiconazole was found to decrease E2 synthesis and not alter T production (at a concentration up to 2500 µg/l). Our relative sensitivity data also are consistent with studies conducted with the human adrenocortical carcinoma cell line (H295R), in which propiconazole reduced E2 and T production by 50% at concentrations of 0.9 and 5.3 µM, respectively (Kjaerstad et al., 2010).

Prior work has shown that chemicals that inhibit sex steroid production in fish consistently cause responses indicative of adaptation and/or compensation. For example, in this study, propiconazole upregulated several genes involved in gonadal steroidogenesis, fshr, star, cyp11a, cyp17, and cyp19a1a, in females. Upregulation of one—and more commonly several—of these same genes has been noted in studies with multiple fish species (fathead minnow, zebrafish, medaka) exposed to chemical inhibitors of steroidogenesis (Ankley et al., 2009b, 2012; Baudiffier et al., 2012; Villeneuve et al., 2007, 2009; Zhang et al., 2008). Although most of the work concerning the effects of steroid synthesis inhibitors on gonadal gene expression has emphasized females, changes in testicular expression of steroidogenic enzymes also have been observed in male fish. For example, Ankley et al. (2007) suggested that ketoconazole’s apparent lack of effect on circulating concentrations of T in males may, in part, have been due to upregulation of cyp11a and cyp17. Similarly, Baudiffier et al. (2012) noted upregulation of several genes in testicular tissue involved in steroidogenesis in male zebrafish exposed to clotrimazole and suggested that this may have offset potential decreases in concentrations of the androgen 11-ketotestosterone in males. Propiconazole, however, did not consistently affect testicular expression of genes involved in steroidogenesis in this study.

Compensatory/adaptive responses in the HPG axis in response to chemical stressors can be manifested in ways other than changes in gene expression. For example, ketoconazole significantly increased the size of the gonads (GSI) in both male and female fathead minnows, thereby, ostensibly, increasing the overall ability of the fish to produce sex steroids.
FIG. 7. Effects of a 21-day exposure to propiconazole on gonadal gene expression from female (open bars) and male (closed bars) fathead minnows. Results of gene expression are expressed as fold-change relative to control; log 2. Data are for (A) fshr, (B) star, (C) cyp11a, (D) cyp17, (E) cyp19a1a, (F) ldlr, (G) hmgr, and (H) cyp51. Bars indicate mean ± SEM (n = 8–12). Asterisks indicate a significant (*p ≤ 0.05; **p ≤ 0.01) difference from control values.
In this study, propiconazole also significantly increased GSI of both sexes, almost doubling the relative weight of the gonad in the highest treatment group (1000 µg/l). Assuming a relatively consistent rate of steroid production per unit gonad weight, increases in overall tissue mass could significantly offset decreases in rates of T and/or E2 production on a per unit mass basis. In addition to changes in gonad size, some inhibitors of steroidogenesis can cause apparent compensatory responses through alterations in the cellular composition of the gonads; for example, in fathead minnow males exposed to ketoconazole, a proliferation of interstitial cells, which are responsible for steroid synthesis, was observed (Ankley et al., 2007). However, we did not see a similar histological alteration in testis of propiconazole-exposed animals. Consequently, in this study, we cannot rule out the possibility that increased mass was associated with impaired spawning, and associated
Propiconazole has relatively low acute toxicity to fish, but little degree of "pseudo persistence" in situations where there are concentrations in aquatic environments and may exhibit some with this, propiconazole has been detected at 0.009–1.15 µg/l (USEPA, 2006). Based on data from this study and depending on a weight-of-evidence analysis of the results of all the Tier 1 EDSP assays, the fungicide may proceed to Tier 2 testing to provide a more comprehensive understanding of endocrine-related effects at a broad range of doses. Among the Tier 2 tests potentially applicable to more fully exploring the endocrine effects of propiconazole is a longer term study (e.g., multigeneration) with the Japanese medaka (USEPA, 2012).

Ciba-Geigy first registered propiconazole in 1981 for use on grass seed, and since then, additional uses on agricultural commodities such as grains, rice, celery, corn, fruits, and peanuts have been approved (USEPA, 2006). Over a 5-year period, it was estimated that almost 157,000 kg of propiconazole-active ingredient was used for U.S. agriculture (USEPA, 2006). Given this degree of agricultural use, there is the potential for propiconazole to enter aquatic environments due to runoff or erosion. An estimate of potential environmental concentrations of propiconazole, based on use patterns as of 2006, indicated that the 90-day average concentrations of the fungicide in surface water could range from 1 to 11.9 µg/l (USEPA, 2006). Consistent with this, propiconazole has been detected at 0.009–1.15 µg/l concentrations in aquatic environments and may exhibit some degree of “pseudo persistence” in situations where there are continuous agricultural applications and, hence, constant input to aquatic systems (Battaglin et al., 2011; Kahle et al., 2008).

Propiconazole has relatively low acute toxicity to fish, but little is known concerning potential risks of the fungicide in longer term, sublethal fish exposures. In our 21-day fathead minnow exposure, propiconazole significantly impacted egg production at 500 and 1000 µg/l concentrations of the fungicide in water. Effects on egg production occurred at a concentration as low as 5 µg propiconazole/l, but because fecundity in the 50 µg/l treatment did not differ from the controls, the reliability of 5 µg/l as a lowest observable effect concentration for reproductive effects is uncertain (particularly given the lack of effect on other endocrine endpoints in the treatment group). In an unpublished study, propiconazole also was reported to reduce fecundity in a life-cycle study with an estuarine fish, the sheepshead minnow, at water concentrations above 150 µg/l (USEPA, 1988). Unfortunately, the sheepshead minnow study was deemed invalid for pesticide registration purposes due to diluter malfunction and loss of chemical delivery for an undetermined amount of time. Given uncertainties as to possible reproductive effects and the fact that some significant biological responses were observed in the 5 and 50 µg/l treatment groups (e.g., decreased plasma cholesterol and VTG; expression of hepatic cyp1a1), additional studies focused on potential chronic effects of propiconazole in fish would be desirable.

Feeding studies with propiconazole in rainbow trout indicated little potential for significant tissue accumulation of the fungicide from dietary sources due to a relatively short half-life of the ingested chemicals (Konwick et al., 2006). However, there are no published data concerning the bioconcentration of propiconazole relative to water-borne exposures. In this study, the fungicide accumulated in the tissues of fathead minnows in a concentration-dependent manner, peaking at 39,600 ± 3,000 µg/kg in females from the 1000 µg/l treatment group (Table 1). The females accumulated roughly twice as much propiconazole as males due possibly to differential lipid content between the sexes (reproductively active females have about twice the lipid as males; Ankley et al., 2001). However, propiconazole did not accumulate in the fish to the degree expected from a predicted BCF for the chemical derived from its log octanol/water partition coefficient (kow) of 3.65 (Syngenta, 2012). Based on previous work done in fathead minnows with chemicals with a range of kow values, a predicted steady-state BCF for propiconazole (in the absence of metabolism/elimination) is about 250 (Veith et al., 1979). Our measured BCF values based on water and tissue concentrations in this study ranged from 9.4 to 37.5 (Table 1), suggesting efficient metabolism of propiconazole by the fish. A route of metabolism of propiconazole could be via phase I oxidative processes in the liver, catalyzed by enzymes such as CYP1A1 and CYP3A, both of which are involved in xenobiotic metabolism in fish (Xu et al., 2005). Consistent with possible enhanced metabolism of the fungicide via this route, there was a concentration-dependent upregulation of cyp1a1 and cyp3a in both females and males exposed to propiconazole. The possibility of induced hepatic metabolism of propiconazole is supported by the work of others both with fish and mammals. For example, in a 14-day exposure with brown trout,
propiconazole was shown to increase hepatic CYP1A activity (Egaas et al., 1999). Similarly, propiconazole has been shown to induce various CYP genes and associated activities in the livers of rodents (Chen et al., 2009; Hester et al., 2012; Sun et al., 2005). Although further work is required to fully understand potential ramifications, it is noteworthy that environmentally relevant concentrations of propiconazole apparently are capable of inducing hepatic xenobiotic-metabolizing enzymes in fish.

A recent review by an external science advisory group to the USEPA assessed possible mechanisms via which propiconazole (and related conazoles) could act as carcinogen(s) in mammalian systems (USEPA, 2011). Long-term (2 years) feeding studies with mice had indicated that dietary exposure to propiconazole increased hepatocellular adenomas/carcinomas, so there was an interest in understanding pathway(s) responsible for these effects. A multistep cascade of key events, identified as either causal or associated with tumor formation in mice, was described by USEPA (2011). Initial DNA damage by propiconazole was proposed to occur via induction of hepatic CYPs involved with xenobiotic metabolism (through activation of nuclear receptors), causing increased oxidative stress and subsequent mutations (Hester et al., 2012). Decreased plasma cholesterol in propiconazole-exposed mice is thought to occur via inhibition of CYP51-catalyzed cholesterol biosynthesis (Nesnow et al., 2011). The subsequent increased flux of cholesterol biosynthesis caused by negative feedback was hypothesized to increase levels of hepatic mevalonic acid, a molecule that acts as a growth stimulant, enhancing cell proliferation and tumor formation (Murphy et al., 2012). It is informative to consider this proposed carcinogenesis pathway in the context of endpoints measured in this study with the fathead minnow. For example, we observed upregulation of genes coding for hepatic CYPs involved in xenobiotic metabolism in propiconazole-exposed fish. We also noted decreased plasma concentrations of cholesterol and alterations in the expression of several genes related to cholesterol biosynthesis (hmgr, fdps, and cyp51). Although we did not consider endpoints directly related to carcinogenesis in this study, several of the responses we observed in propiconazole-exposed fathead minnows are consistent with the proposed pathway resulting in cancer in rodents (USEPA, 2011).

**SUMMARY AND CONCLUSIONS**

In summary, the fungicide propiconazole impacted reproductive endocrine function in 21-day exposures with the fathead minnow. The molecular event that initiates this adverse outcome pathway is likely direct inhibition of CYPs involved in synthesis (such as CYP19 aromatase), as supported by the impact of propiconazole on steroid production by ovary tissue exposed in vitro. Depression of steroidogenesis in females leads to decreased plasma concentrations of E2 in the female fish and subsequent decreases in VTG production, which is associated with inhibition of oocyte development and release. Our study also provides evidence of a compensatory response in the fish to the effects of propiconazole, likely mediated through negative feedback (in response to depressed E2), as indicated by upregulation of several genes coding for key proteins involved in ovarian steroidogenesis and, possibly, increased GSI. Although our studies were not specifically designed to formally assess the possible ecological risks of propiconazole, the resultant effects data are substantive enough to suggest that additional chronic fish studies with the fungicide would seem warranted.

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