TOXICOLOGICAL SCIENCES **86(2)**, 300–308 (2005)
doi:10.1093/toxsci/kfi202
Advance Access publication May 18, 2005

Effects of Two Fungicides with Multiple Modes of Action on Reproductive Endocrine Function in the Fathead Minnow (*Pimephales promelas*)

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Received March 30, 2005; accepted May 6, 2005

Many chemicals that adversely affect reproduction and/or development do so through multiple pathways within the reproductive tract and hypothalamic–pituitary–gonadal axis. Notable in this regard are fungicides, such as prochloraz or fenarimol, which in mammals have the potential to impact endocrine function through inhibition of CYP enzymes involved in steroid metabolism, as well as through antagonism of the androgen receptor(s). The objective of our studies was to assess the effects of prochloraz and fenarimol on reproductive endocrine function in a model small fish species, the fathead minnow (*Pimephales promelas*), using both *in vitro* and *in vivo* assays. The two fungicides inhibited *in vitro* CYP19 aromatase activity in brain and ovarian homogenates from the fish, with prochloraz exhibiting a greater potency than fenarimol. Prochloraz and fenarimol also bound competitively to the cloned fathead minnow androgen receptor expressed in COS-1 cells. The two fungicides significantly reduced fecundity of the fish in a 21-day reproduction assay at water concentrations of 0.1 (prochloraz) and 1.0 (fenarimol) mg/l. The *in vivo* effects of prochloraz on plasma steroid (17β-estradiol, testosterone, 11-ketotestosterone) and vitellogenin (an estrogen-responsive protein) concentrations, as well as on gonadal histopathology, were consistent with inhibition of steroidogenesis. Fenarimol also affected several aspects of endocrine function *in vivo*; however, the suite of observed effects did not reflect either aromatase inhibition or androgen receptor antagonism. These studies contribute to a better mechanistic understanding of the extrapolation of effects of endocrine-disrupting chemicals across vertebrate classes.

Key Words: **fathead minnow; prochloraz; fenarimol; reproductive toxicity.**

INTRODUCTION

Environmental contaminants that act as estrogen receptor agonists have received much attention in terms of possible effects on reproduction and development in fish and wildlife (WHO, 2002). However, chemicals can adversely affect endocrine function through many other biologically relevant pathways within the reproductive tract and the hypothalamic–pituitary–gonadal (HPG) axis. For example, there is increasing evidence that environmental contaminants that interact with androgen receptors could cause significant adverse effects on individuals and populations (for a review, see Gray *et al.*, 2005). Several studies indicate that androgen receptor agonists are affecting fish exposed to some types of discharges, including pulp and paper mill effluents and feedlot runoff (Howell *et al.*, 1980; Larsson *et al.*, 2000; Orlando *et al.*, 2004; Parks *et al.*, 2001). Although data from the field are less definitive in terms of existing impacts, evidence from lab studies indicates that there are a number of relatively common environmental contaminants that act as antagonists of androgen receptors in vertebrates, including some organochlorine insecticides and variety of fungicides (Gray *et al.*, 2005). In addition to chemicals that interact directly with receptors, function of the HPG axis can be affected by xenobiotics that affect the metabolism of sex steroids. One enzyme in vertebrates that appears particularly critical in this regard is CYP19 aromatase, which converts testosterone to 17β-estradiol. A variety of pesticides, many of which are in use throughout the world, have been shown to affect (generally inhibit) *in vitro* aromatase activity in different vertebrate systems (Heneweer *et al.*, 2004; Mason *et al.*, 1987; Sanderson *et al.*, 2002; Vinggaard *et al.*, 2000; Zarn *et al.*, 2003). Controlled experimentation has demonstrated that aromatase inhibitors can produce profound effects on the reproductive endocrine system and spawning success of fish (Ankley *et al.*, 2002), and recent

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studies suggest that altered steroidogenesis is associated with adverse effects observed in fish from the field (Lavado *et al.*, 2004; Noaksson *et al.*, 2003).

One complication in assessing the potential human health or ecological risk of environmental contaminants that affect HPG function is that a single compound may elicit effects at different points along the axis; *i.e.*, a chemical can have multiple (or mixed) toxic modes of action (MOA). Several fungicides broadly characterized as aromatic heterocyclic amines have the potential to exert toxicity via multiple MOA within the HPG axis. Because these types of fungicides are designed to depress fungal growth through inhibition of a CYP enzyme key to ergosterol biosynthesis (14α-demethylase; Van den Bossche *et al.*, 1978, 1982), it is not surprising that several fungicides also inhibit CYP enzymes such as aromatase in vertebrates (Mason *et al.*, 1987; Sanderson *et al.*, 2002; Vinggaard *et al.*, 2000). Recent *in vitro* data suggest that, in addition to inhibiting aromatase, some fungicides may act as androgen receptor antagonists (Andersen *et al.*, 2002; Birkhøj *et al.*, 2004; Kojima *et al.*, 2004). Based on *in vitro* studies as well as more limited *in vivo* research, two fungicides that could act as both aromatase inhibitors and anti-androgens are prochloraz and fenarimol (Noriega *et al.*, 2005; Vinggaard *et al.*, 2000, 2002, 2005).

The purpose of these studies was to assess the effects of prochloraz and fenarimol on reproductive endocrinology in the fathead minnow (*Pimephales promelas*). The fathead minnow has been widely used as a model species for identifying and assessing the effects of a variety of xenobiotics that affect different pathways within the HPG axis, including (anti-) estrogens and androgens, and chemicals that affect steroidogenesis (for review see Ankley and Johnson, 2004). To assess effects of the two fungicides on the HPG axis we used a combination of mechanism-oriented *in vitro* assays for androgen receptor binding (Wilson *et al.*, 2004a) and aromatase inhibition (Ankley *et al.*, 2002) in the fathead minnow, in conjunction with a short-term (21-day) multi-endpoint reproduction test (Ankley *et al.*, 2001). As such, these studies lend insights into the MOA of the fungicides in the fish, as well as their effects on reproduction, an endpoint of direct relevance to the health of individual animals and populations. This type of approach is critical to understanding the basis for extrapolation of the effects of endocrine-disrupting chemicals across species, as well as helping to establish linkages across biological levels of organization to support the use of mechanistic data for predicting ecological risk (Miracle and Ankley, 2005).

MATERIALS AND METHODS

Biological techniques. Prochloraz (99.5% purity) and fenarimol (99.8% purity) were obtained from Sigma (St. Louis, MO). Solvent-free stock solutions of the two chemicals were prepared in Lake Superior water using a shell-coating technique (USEPA, 2002). The stocks were diluted with Lake Superior water to achieve target test concentrations of 0.03, 0.1, and 0.3 mg/l for prochloraz, and 0.1 and 1.0 mg/l for fenarimol. Flow rates of clean water (for controls) or chemical solutions to the test tanks were 46 ml/min. General chemical characteristics of the Lake Superior water measured in the test system over the course of the studies were hardness, 46 mg/l as $CaCO_3$; alkalinity, 40 as $CaCO_3$; pH, 7.3; and dissolved oxygen 6.2 mg/l.

The basic experimental design for these studies was the same as that described by Ankley *et al.* (2001), except that a paired rather than group spawning approach was employed. Two pairs of fish were held in glass aquaria containing 10 l of test solution, separated from one another by nylon mesh screen. Each half of the tank had a single spawning substrate constructed of PVC pipe (USEPA, 2002). Adult fish from an on-site culture unit were held for an acclimation period in clean water in the test system, during which fecundity was assessed daily. The animals were kept at 25° ± 1°C under a 16:8 h light:dark cycle, and fed adult brine shrimp twice per day. After 15 days, chemical exposures were initiated with animals that had successfully spawned during acclimation. Six pairs of fish were exposed to each concentration of prochloraz, and four pairs of animals were exposed to each fenarimol treatment. Fewer replicates were used for the fenarimol exposure because of the expense of the chemical, particularly in a flow-through test. Eight pairs of fish were used as common controls for the prochloraz and fenarimol tests.

During the 21-day chemical exposure, fecundity and fertility of the spawned eggs were assessed daily. At the conclusion of the assay, the fish were anesthetized with buffered tricaine methylsulfonate, and blood was collected with a heparinized micro-hematocrit tubule (USEPA, 2002). Plasma was obtained by centrifugation and stored with a protease inhibitor at −80°C until determination of vitellogenin concentrations was accomplished with an enzyme-linked immunosorbent assay with a fathead minnow polyclonal antibody (Korte *et al.*, 2000; Parks *et al.*, 1999), and sex steroid (17β-estradiol, testosterone, 11-ketotestosterone) concentrations were measured via radioimmunoassay (Jensen *et al.*, 2001). Brains of the fish were removed and snap-frozen in liquid nitrogen until they were analyzed for aromatase activity, as described below.

Gonads from both sexes were removed and fixed in 1% gluteraldehyde/4% formaldehyde in 0.1 M phosphate buffer (Leino *et al.*, 2005). The remainder of the fish carcass was frozen at −20°C until it was analyzed for tissue concentrations of the prochloraz or fenarimol (see below). Gonad tissues were embedded in paraffin, sectioned at 4 to 5 μm in a step-wise fashion, and stained with hematoxylin and eosin. A total of three sections were assessed from each gonad, beginning at the midline and then at 50 μm intervals following the first section. The gonad sections were evaluated by Experimental Pathology Laboratories (Herndon, VA).

Brain aromatase activity in fish from the *in vivo* exposures was determined by measuring release of tritium-labeled water from the C1 carbon of tritiated androstenedione (Lephart and Simpson, 1991; Thompson and Siiteri, 1974), with an assay optimized for fathead minnow post-mitochondrial supernatant preparations (Ankley *et al.*, 2005). The direct effects of prochloraz and fenarimol on aromatase activity *in vitro* were assessed using the same protocol with brain and ovary homogenates (post-mitochondrial supernatants or S9 fractions) prepared from sexually mature, untreated females from the on-site culture unit. Eight concentrations of prochloraz and fenarimol were prepared in methanol by threefold serial dilution from a concentrated stock. Methanol stocks were diluted in phosphate buffer to yield prochloraz concentrations ranging from 0.05 to 57 μM, and fenarimol concentrations ranging from 0.06 to 77 μM. The final concentration of methanol in each reaction vial was 0.66%. Solvent control responses were not significantly different from non-solvent controls. All chemical concentrations, controls, and solvent controls were assayed in triplicate.

Competitive binding of prochloraz and fenarimol to the fathead minnow androgen receptor was determined as described elsewhere (Wilson *et al.*, 2004a). Briefly, cloned fathead minnow androgen receptor (Wilson *et al.*, 2004a) was transiently transfected into COS-1 cells (African green monkey kidney cells; ATCC, Rockville, MD), and binding of the two fungicides was

assessed with 0.5 nM ^{3}H-R1881 (New England Nuclear Life Sciences Products, Boston, MA) as a competitor. Prochloraz and fenarimol were each tested at 10 concentrations ranging from 30 nM to 1 mM. The binding assays were conducted in duplicate, with three replicate assays per chemical.

Fungicide concentrations that inhibited 50% binding (IC_{50}) of the synthetic androgen R1881 to the fathead minnow androgen receptor were calculated with logistic regression (Wilson *et al.*, 2004a). IC_{50} values for inhibition of aromatase activity also were determined by nonlinear regression. Other *in vitro* and *in vivo* biological data initially were analyzed by analysis of variance (ANOVA) and Dunnett's procedure to determine differences between control and treatment groups. When necessary, data were transformed for normalization and/or to reduce heterogeneity of variance. Analyses were conducted using SAS 9.0 (SAS Institute, Cary, NC). Differences were considered significant at $p \leq 0.05$.

Chemical analyses. Water samples (1 ml) were collected from the control and treatment tanks on six different occasions over the course of the 21-day reproduction study, and immediately analyzed for prochloraz or fenarimol using reversed-phase high-performance liquid chromatography (HPLC). Instrumentation for the analysis was an Agilent (Wilmington, DE) model 1100 HPLC comprised of a capillary pump, chilled auto sampler (4°C), heated column compartment (25°C), and diode-array detector. Sample aliquots (20 µl for prochloraz; 5 µl for fenarimol) were injected onto a Zorbax (Agilent) SB-C18 column (2.1 × 75 mm) and eluted isocratically with 75% methanol/water (prochloraz), or 70% methanol/water (fenarimol) at a flow rate of 0.4 ml/min. Concentrations of the two fungicides were determined using the response at a wavelength of 220 nm, and an external standard method of quantification. Analytical quantification limits for prochloraz and fenarimol were 0.015 and 0.05 mg/l, respectively. No prochloraz or fenarimol was detected in the control tanks ($n = 24$) or procedural blanks ($n = 7$). The mean (SD, n) percent recovery of prochloraz in spiked Lake Superior water was 97 (2, 6), and the mean (SD, n) percentage agreement among duplicate samples was 98 (1, 11). The mean (SD, n) percent recovery of fenarimol in spiked Lake Superior water was 85 (4, 6), and the mean (SD, n) percentage agreement among duplicate samples was 99 (1, 7).

We also determined prochloraz and fenarimol concentrations in the carcasses of animals from the reproduction assay. Fish (from which blood, brain, liver, and gonads had been removed) were placed in acetonitrile (5 ml/g of tissue) and homogenized with a high-speed homogenizer. After settling, a 5 ml aliquot of the sample was transferred to a clean tube, placed in a −20°C freezer for at least 2 h, and then centrifuged at 3000 × g for 20 min at 4°C. The supernatant was concentrated under nitrogen gas, chilled, and centrifuged again. The resultant supernatant was diluted to 10 ml with 10% methanol in water, and analyzed by HPLC as described above. The analytical quantification limits for prochloraz and fenarimol in the fish tissue were 0.015 and 0.05 mg/kg (wet wt), respectively. Neither analyte was detected in control animals. The mean (SD, n) percent recovery of prochloraz from spiked tissue samples was 79 (12, 6), and the percentage agreement between duplicate samples was 89 (7, 3). The mean (SD, n) percent recovery of fenarimol from spiked tissue samples was 97.4 (2, 2), and agreement percentage between duplicate samples was 95.4 (4, 1).

RESULTS

Water concentrations of prochloraz remained stable and relatively close to target concentrations over the course of the 21-day test. Mean (SD, $n = 6$) measured concentrations over the 21-day test were 0.032 (0.012), 0.116 (0.042), and 0.311 (0.107) mg/l, respectively, in the 0.03, 0.1, and 0.3 mg/l nominal treatments. The mean (SD, $n = 6$) measured concentration of fenarimol in water from the 0.1 mg/l treatment was 0.096 (0.036) mg/l. Mean (SD, $n = 6$) measured concentration of fenarimol in the high treatment group (1.0 mg/l) was somewhat less than nominal at 0.569 (0.17) mg/l. Mean (SD, n) concentrations of prochloraz in tissues of the fish (sans liver, gonads, and blood) at the conclusion of the 21-day test were 0.92 (0.12, 4), 4.6 (0.78, 11), and 25.5 (4,12) mg/kg in the 0.03, 0.1, and 0.3 mg/l treatments, respectively. Mean (SD, $n = 4$) concentrations of fenarimol in fish from the 0.1 and 1.0 mg/l treatment groups were 1.06 (0.28) and 19.2 (8.7) mg/kg, respectively. There were no evident sex-related differences in accumulation of the two fungicides.

One fish, a female from the 0.1 mg prochloraz/l treatment, died on day 5 of the test; this did not appear to be treatment-related as there was no evidence of abnormal behavior in any of the other control or pesticide-exposed animals. The two fungicides caused significant concentration-dependent decreases in fecundity of the fathead minnows (Fig. 1 a and b). There were no treatment-related effects of either prochloraz or fenarimol on fertility or hatching success (data not shown).

Histological examination of gonads of males exposed to prochloraz indicated increased spermatogonia in 50% (three of six) of the fish from both the 0.03 and 0.3 mg/l treatments. None of the eight control fish exhibited this condition. In the fenarimol-exposed males, 75% (three of four) of the fish from

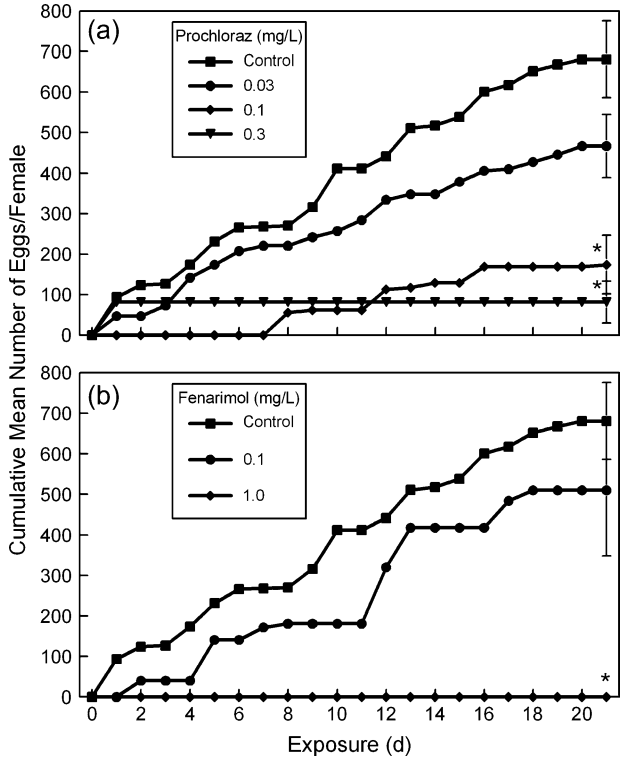

FIG. 1. Effects of exposure to (a) prochloraz or (b) fenarimol on cumulative fecundity of the fathead minnow during a 21-day test. Data are mean (21-day standard error) values for pairs of control fish ($n = 8$), and animals exposed to prochloraz ($n = 6$ for the 0.03 and 0.3 mg/l treatments, and $n = 5$ for the 0.1 mg/l group) or fenarimol ($n = 4$). Asterisks indicate a significant difference from control values.

the 1.0 mg/l treatment also exhibited increased spermatogonia. There also appeared to be an increase in testicular stage in males from the 0.1 and 0.3 mg/l prochloraz treatments. The majority (five of eight) of control males were in stage 2 (mostly primary and secondary spermatogonia; Leino *et al.*, 2005), whereas in the prochloraz-exposed fish, a relatively greater number of males were at stage 3, where the majority of the germ cells are primary and secondary spermatocytes. Both prochloraz and fenarimol increased oocyte atresia in female fathead minnows. Two of eight (25%) of the control animals had minimal to mild atresia, whereas all six females from the 0.3 mg/l prochloraz treatment exhibited mild to moderate degrees of oocyte atresia, and 75% (three of four) fish from the 1.0 mg/l fenarimol treatment had this pathology. There was no marked effect of either fungicide on ovarian staging in the females.

Plasma vitellogenin concentrations in males were low to non-detectable and were not affected by exposure to either fungicide (Fig. 2a). Plasma estradiol concentrations in males also were not altered by either prochloraz or fenarimol (Fig. 2b). Concentrations of testosterone and 11-ketotestosterone were significantly decreased in the highest prochloraz treatment, and they were unaffected by fenarimol (Fig. 2 c and d). Prochloraz caused a significant decrease in brain aromatase activity in males from the 0.3 mg/l treatment group (Fig. 2e). Fenarimol did not affect brain aromatase activity in the males (Fig. 2e).

There were concentration-dependent decreases in plasma vitellogenin concentrations in females exposed both to prochloraz and fenarimol (Fig. 3a). Exposure to prochloraz also decreased plasma estradiol concentrations in a concentration-dependent fashion (Fig. 3b). Both test concentrations of fenarimol increased estradiol in the females (Fig. 3b). Plasma testosterone concentrations in females exposed to the two fungicides were not consistently affected; although the average testosterone concentration in fish exposed 0.3 mg prochloraz/l was about 50% of that in controls, the difference was not statistically significant (Fig. 3c). Neither fungicide affected brain aromatase activity in the females (Fig. 3d).

Assays with post-mitochondrial supernatant from the brains and ovaries of untreated female fathead minnows indicated that both prochloraz and fenarimol significantly inhibited *in vitro* aromatase activity (Fig. 4 a and b). The IC_{50} values for inhibition of brain and ovarian aromatase activity by prochloraz were 11.2 and 7.2 μM, respectively (Fig. 4 a and b). Although fenarimol also reduced both brain and aromatase activity *in vitro*, it was less potent than prochloraz (Fig. 4). The degree of inhibition of aromatase by fenarimol was too low to permit reliable calculation of IC_{50} values (Fig. 4 a and b).

Both prochloraz and fenarimol exhibited specific, saturable binding to the fathead minnow androgen receptor in the COS-1 whole-cell assay (Fig. 5). The IC_{50} values were 10 and 25 μM, respectively, for prochloraz and fenarimol.

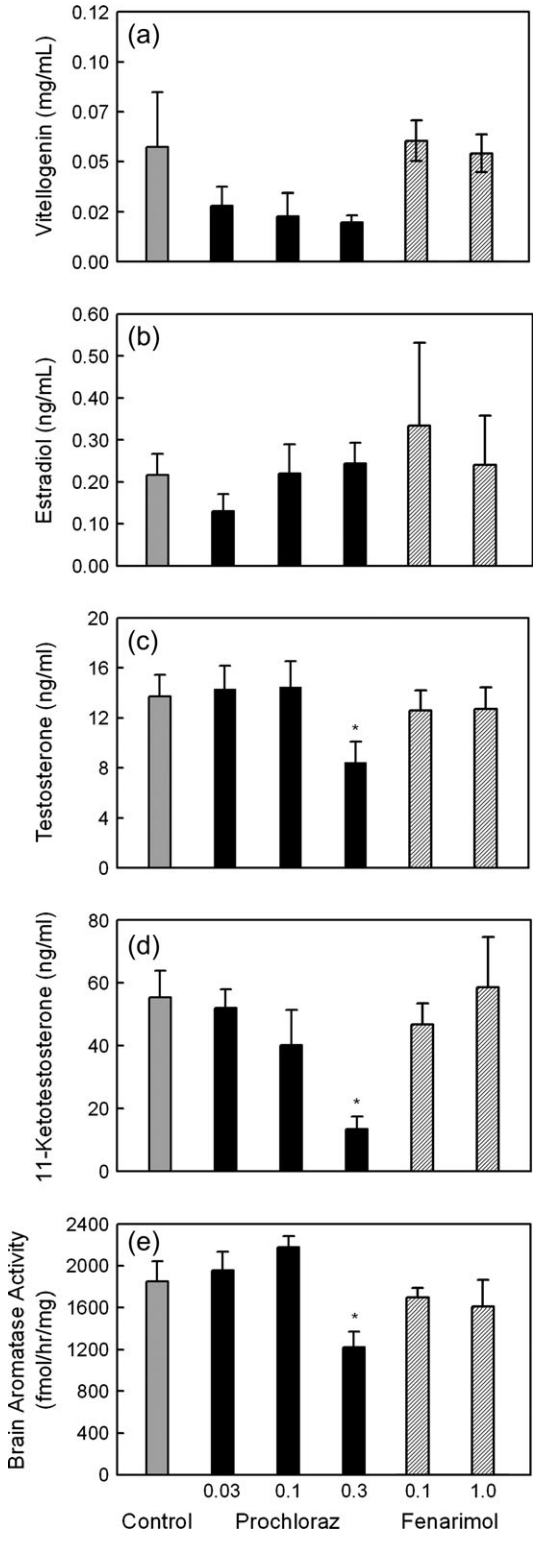

FIG. 2. Effects of different water concentrations (mg/l) of prochloraz or fenarimol on plasma concentrations of (a) vitellogenin, (b) 17β-estradiol, (c) testosterone, and (d) 11-ketotestosterone, and (e) brain aromatase activity in male fathead minnows exposed to the fungicides for 21 days. Data are depicted as the mean (standard error of the mean) for $n = 8$ (controls), $n = 6$ (prochloraz), or $n = 4$ (fenarimol) individuals. Asterisks indicate a significant difference from the controls.

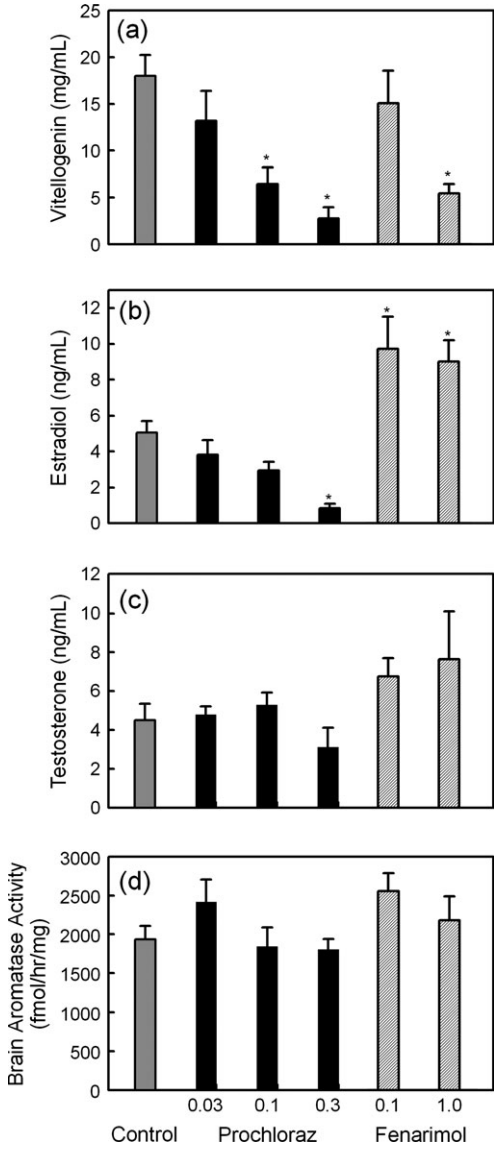

FIG. 3. Effects of different water concentrations (mg/l) of prochloraz or fenarimol on plasma concentrations of (a) vitellogenin, (b) 17β-estradiol, and (c) testosterone and (d) brain aromatase activity in female fathead minnows exposed to the fungicides for 21 days. Because of one mortality in the 0.1 mg prochloraz/l treatment, and limited plasma volumes from the females, samples sizes for vitellogenin and steroids varied. Vitellogenin and aromatase data are depicted as the mean (standard error) for $n = 8$ (controls), $n = 5$–6 (prochloraz), or $n = 4$ (fenarimol) individuals. Sample sizes (treatment group) for estradiol/testosterone concentrations were 7/5 (control), 5/4 (0.03 mg prochloraz/l), 3/3 (0.1 mg prochloraz/l), 4/5 (0.3 mg prochloraz/l), 4/3 (0.1 mg fenarimol/l), and 3/3 (1.0 mg fenarimol/l). Asterisks indicate a significant difference from the controls.

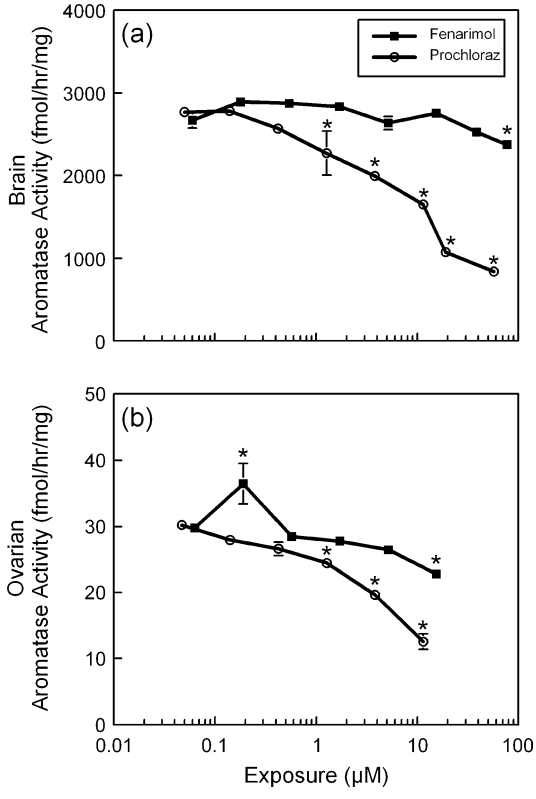

FIG. 4. Effects of prochloraz and fenarimol on *in vitro* aromatase activity in (a) brain and (b) ovary homogenates from female fathead minnows. Data indicate the mean (standard deviation) for triplicate determinations at each test concentration. Asterisks indicate a significant difference from the controls.

DISCUSSION

Recent *in vivo* and/or *in vitro* studies in mammalian systems indicate the potential for a number of aromatic heterocyclic amine fungicides, including prochloraz and fenarimol, to affect endocrine function both through inhibition of CYP enzymes involved in steroidogenesis and antagonism of the androgen receptor (Birkhøj *et al.*, 2004; Noriega *et al.*, 2005; Sanderson *et al.*, 2002; Vinggaard *et al.*, 2000; 2002; 2005; Wilson *et al.*, 2004b). Given the degree of conservation in structural and functional aspects of the HPG axis across vertebrates, it is reasonable to hypothesize that these types of chemicals would act in a similar fashion in fish (Kime, 1998). However, there has been little systematic comparative research to ascertain the extent to which effects of different endocrine-disrupting chemicals can accurately be extrapolated across vertebrate classes. In the present study, we demonstrate that both prochloraz and fenarimol inhibit fathead minnow aromatase activity *in vitro* and bind to the cloned androgen receptor of this species. Not surprisingly, suites of *in vivo* responses elicited by the two fungicides in a short-term reproduction assay were more complex with regard to interpretation of MOA; however, both caused significant alterations in endocrine function of the fish which ultimately affected reproductive success (fecundity).

In the fathead minnow, we found that prochloraz was a much more potent inhibitor of *in vitro* brain and ovarian aromatase activity than fenarimol. This is similar to what has been reported from mammalian studies. For example, Vinggaard *et al.* (2000) found that prochloraz was about 250 times more effective than fenarimol in inhibiting aromatase activity in

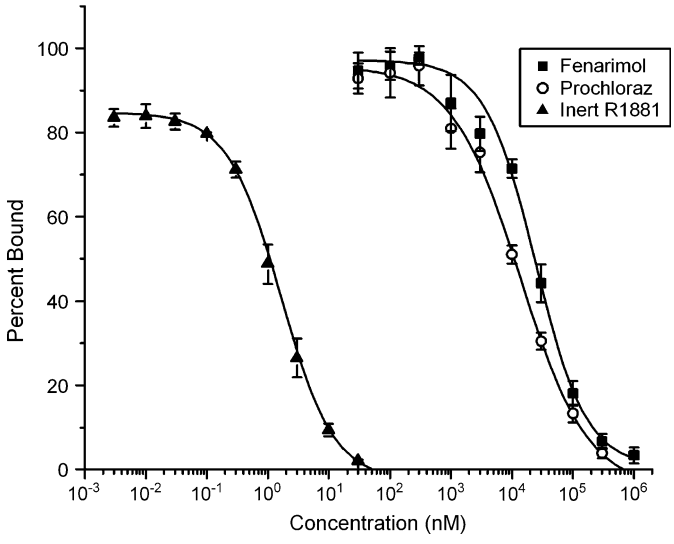

FIG. 5. Competitive binding (with R1881) of prochloraz and fenarimol to the fathead minnow androgen receptor expressed in COS-1 cells. Data indicate the mean (standard deviation) for triplicate determinations.

human placental microsomes. Sanderson *et al.* (2002) also reported that fenarimol was a less potent inhibitor than prochloraz of aromatase in human adrenocortical carcinoma (H295R) cells. Ability of the two pesticides to bind to the fathead minnow androgen receptor is reasonably similar to what has been found in mammalian systems. In the present study, the IC_{50} values for inhibition of binding of R1881 to the fish receptor were 10 μM and 25 μM, respectively, for prochloraz and fenarimol. Andersen *et al.* (2002) reported an inhibitory concentration for the two pesticides of 20 μM for R1881-induced transactivational activity in Chinese hamster ovary cells transiently transfected with a human androgen receptor–reporter gene construct. In a similar assay system, Kojima *et al.* (2004) reported that fenarimol was slightly less potent than prochloraz in inhibiting transactivational activity induced by dihydroxytestosterone. Overall, despite the fact that there are substantial variations not only between the species under consideration (*i.e.,* humans vs. fathead minnows) but also between the actual test endpoints (*e.g.*, inhibition of receptor binding vs. transactivational activity), it appears that both pesticides would be classified as aromatase inhibitors and androgen receptor antagonists, irrespective of whether the *in vitro* assays were based on mammalian or fish enzymes/receptors. With regard to comparability across species in chemical binding to the androgen receptor, conclusions from this study are similar to those of Wilson *et al.* (2004a), who measured the competitive binding of several known androgen receptor agonists and antagonists to the fathead minnow receptor, and compared the data to results obtained with the human androgen receptor expressed in COS-1 cells.

In vivo studies with rats have documented the anti-androgenic nature of prochloraz and fenarimol, both of which induce a suite of morphological alterations similar to those caused by the known androgen receptor antagonist flutamide (Noriega *et al.*, 2005; Vinggaard *et al.*, 2002, 2005). *In vivo* responses of rats to the two fungicides have been less apparent in terms of inhibition of steroidogenic CYP enzymes, such as aromatase, although this may be due in part to experimental design issues (*e.g.*, exposure duration; Noriega *et al.*, 2005). Compared to findings in rats, prochloraz in the fathead minnow caused a suite of *in vivo* responses clearly consistent with inhibition of steroidogenesis. In studies with fadrozole, a comparatively specific and potent inhibitor of aromatase, Ankley *et al.* (2002) reported that a 21-day exposure caused decreased (brain) aromatase activity and depressed concentrations of plasma estradiol and vitellogenin (an estrogen-responsive protein) in female fathead minnows. These responses were accompanied by altered gonadal histopathology (in both sexes) and a complete cessation of spawning at a fadrozole water concentration of 10 μg/l (Ankley *et al.*, 2002). Prochloraz caused a relatively similar suite of effects in the fish, including depressed brain aromatase activity (in males) and decreased plasma estradiol and vitellogenin concentrations in females. Prochloraz caused oocyte atresia in the female gonads, as well as an increased production of spermatogonia in male testes, both pathologies observed in fathead minnows exposed to fadrozole (Ankley *et al.*, 2002). Like fadrozole, prochloraz significantly decreased fecundity of the fish, albeit at higher test concentrations. Unlike fadrozole, prochloraz reduced plasma androgen concentrations in male (and, perhaps, female) fathead minnows, suggesting inhibition of CYP enzymes, in addition to aromatase, involved in steroidogenesis. One potential candidate in this regard is CYP17α lyase which, in rats appears to be inhibited by prochloraz (Wilson *et al.*, 2004b). Reactions mediated by lyase are critical to the production of androgens in vertebrate steroidogenesis, so inhibition of this enzyme could explain decreased plasma concentrations of testosterone and 11-ketotestosterone in the fish.

Fenarimol also significantly reduced fecundity of the fish during the 21-day assay. However as opposed to prochloraz, which caused a pattern of responses consistent with inhibition of steroidogenic enzymes (especially aromatase), fenarimol exposure resulted in a more ambiguous suite of responses in the fathead minnow. For example, like prochloraz, fenarimol decreased plasma vitellogenin concentrations in female fathead minnows; however, this was accompanied by an increase rather than a decrease in plasma estradiol concentrations. With respect to gonadal histopathology, the occurrence of spermatogonia was increased in testes of males exposed to fenarimol (a response consistent with exposure to an aromatase inhibitor; Ankley *et al.*, 2002), while oocyte atresia was observed in the females, a pathology seen in 21-day reproductive studies with fathead minnows exposed to chemicals with a variety of endocrine-related MOA, including aromatase inhibitors and anti-androgens (Jensen *et al.*, 2004; Leino *et al.*, 2005). Overall, the set of responses in fenarimol-exposed fish did not closely mimic those seen in comparable 21-day reproduction

assays with either aromatase inhibitors or anti-androgens (Ankley *et al.*, 2002; Jensen *et al.*, 2004), suggesting that fenarimol may be acting via an alternative (or additional) MOA in the fish. An intriguing observation, and one that may lend insights into how fenarimol might have affected endocrine function in the fish, was the decrease in vitellogenin coincident with an elevation in plasma estradiol. One possible explanation for this seemingly contradictory response would be direct antagonism of the estrogen receptor by fenarimol. Others have shown that estrogen receptor antagonists depress vitellogenin in fathead minnows (Panter *et al.*, 2002), and there is information from *in vitro* studies that fenarimol can bind to the human estrogen receptor (Andersen *et al.*, 2002; Kojima *et al.*, 2004). Directed studies as to whether/how fenarimol interacts with the fathead minnow estrogen receptor might provide insights as to the *in vivo* responses observed in the 21-day reproduction assay.

Data from this study illustrate one of the drawbacks to relying solely on *in vitro* assays to screen chemicals for their potential to affect endocrine function. Based on information from mammalian models, prochloraz and fenarimol were hypothesized to act as aromatase inhibitors and/or anti-androgens in fish. *In vitro* data for effects of the two chemicals on aromatase activity in preparations from fathead minnow brain and ovary, and binding to the cloned androgen receptor from the fish, were in concordance with this hypothesis. Although fenarimol was a weaker inhibitor of aromatase than prochloraz in the fish, both exhibited a qualitatively similar profile in the *in vitro* work. Prochloraz *in vivo* caused responses consistent with aromatase inhibition, although there was evidence that the chemical may also be operating via inhibition of an additional steroidogenic CYP enzyme (*i.e.,* lyase). In the case of fenarimol, *in vivo* responses were indicative of impacts on reproductive endocrine function, ranging from changes in plasma steroid and vitellogenin concentrations to effects on gonadal histology and, ultimately, fecundity. However, the endocrine pathway(s) through which the effects of fenarimol occurred was uncertain, and not predicted based on the results of the *in vitro* assays. The unexpected effects of fenarimol *in vivo* highlight the necessity of using whole-animal assays to augment *in vitro* tests when assessing endocrine-disrupting chemicals.

In summary, our studies demonstrate the effects of prochloraz and fenarimol, fungicides with multiple MOA, on reproductive endocrinology of the fathead minnow both *in vitro* and across multiple levels of biological organization *in vivo*. These results contribute to a better understanding of the fathead minnow as a model for assessing the occurrence and effects of endocrine-disrupting chemicals (Ankley *et al.*, 2001), and also provide information potentially useful for ecological risk assessments. Specifically, ecological risk assessments for chemicals historically have focused on endpoints directly indicative of survival, growth, and reproduction as a basis for predicting possible population-level effects (Suter *et al.*, 1987). Endpoints at biological levels of organization below the individual, such as *in vitro* data or *in vivo* changes in gene/protein/metabolite expression/production or histology (often collectively termed biomarkers), typically have not been used as a basis for quantitative risk assessment (SETAC, 1992). Unfortunately this obviates use of important mechanistic information that can aid in the extrapolation of effects across species, chemicals, and/or different exposure scenarios. To effectively apply biomarker data to risk assessments, it is necessary to understand the consequences of alterations at the molecular, biochemical, and cellular levels in terms of adverse responses at the individual and population levels (Miracle and Ankley, 2005). One impediment to this type of ''linkage'' research in ecotoxicology has been a general lack of emphasis on chemical MOA arising in large part from regulatory data needs (*i.e.,* endpoints related to survival, growth, and reproduction) typically used for assessing single chemicals. However, with the advent of regulatory programs focused on well-defined toxic MOA, such as certain classes of endocrine-disrupting chemicals, the opportunity to approach ecological risk assessments in a different manner is emerging. As a consequence, research integrated across biological levels of organization with well-defined toxicological models is needed to help serve as a basis for scientifically defensible ecological risk assessments.

ACKNOWLEDGMENTS

This work was supported in part by the Computational Toxicology Research Initiative sponsored by the Office of Research and Development of the U.S. Environmental Protection Agency (USEPA), and from the Office of Science Council and Policy within the Office of Pollution Prevention and Toxic Substances of the USEPA. We thank Sigmund Degitz and Ann Miracle for reviewing an earlier draft of this paper. Diane Spehar and Roger LePage assisted in manuscript preparation. This manuscript has been reviewed in accordance with official USEPA policy. Mention of products or trade names does not indicate endorsement by the federal government. Conflict of interest: none declared.

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