Developing Predictive Approaches to Characterize Adaptive Responses of the Reproductive Endocrine Axis to Aromatase Inhibition: I. Data Generation in a Small Fish Model

Daniel L. Villeneuve,*,† Miyuki Breen,‡ David C. Bencic,§ Jenna E. Cavallin,*,¶ Kathleen M. Jensen,*, Elizabeth A. Makynen,*, Linnea M. Thomas,*,2 Leah C. Wehmas,*,3 Rory B. Conolly,¶ and Gerald T. Ankley*

*United States Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, Minnesota 55804; †Biomathematics Program, Department of Statistics, North Carolina State University, Raleigh, North Carolina 27695; ‡United States Environmental Protection Agency, Ecological Exposure Research Division, Cincinnati, Ohio 45268; §ORISE Research Participation Program, United States Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, Minnesota 55804; and ¶United States Environmental Protection Agency, Integrated Systems Toxicology Division, Research Triangle Park, North Carolina 27711.

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Adaptive or compensatory responses to chemical exposure can significantly influence in vivo concentration-duration-response relationships. This study provided data to support development of a computational dynamic model of the hypothalamic-pituitary-gonadal axis of a model vertebrate and its response to aromatase inhibitors as a class of endocrine active chemicals. Fathead minnows (Pimephales promelas) were either exposed to the aromatase inhibitor fadrozole (0.5 or 30 μg/l) continuously for 1, 8, 12, 16, 20, 24, or 28 days or exposed for 8 days and then held in control water (no fadrozole) for an additional 4, 8, 12, 16, or 20 days. The time course of effects on ovarian steroid production, circulating 17β-estradiol (E2) and vitellogenin (VTG) concentrations, and expression of steroidogenesis-related genes in the ovary was measured. Exposure to 30 μg fadrozole/l significantly reduced plasma E2 and VTG concentrations after just 1 day and those effects persisted throughout 28 days of exposure. In contrast, ex vivo E2 production was similar to that of controls on day 8–28 of exposure, whereas transcripts coding for aromatase and follicle-stimulating hormone receptor were elevated, suggesting a compensatory response. Following cessation of fadrozole exposure, ex vivo E2 and plasma E2 concentrations exceeded and then recovered to control levels, but plasma VTG concentrations did not, even after 20 days of depuration. Collectively these data provide several new insights into the nature and time course of adaptive responses to an aromatase inhibitor that support development of a computational model (see companion article).

Key Words: steroidogenesis; endocrine disruption; reproduction; alternative species; compensation; time course.

Aromatase (cytochrome P450 [CYP] 19) catalyzes a key rate-limiting step in estrogen biosynthesis—conversion of C19 androgens such as androstenedione or testosterone (T) to C18 estrogens such as estrone or 17β-estradiol (E2) (Miller, 1988). From a therapeutic perspective, aromatase inhibitors have been sought and developed primarily to treat estrogen-dependent cancers. However, aromatase has also been recognized as a target for chemical contaminants in the environment. For example, numerous fungicides, certain polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, organotins, etc. have been reported to inhibit aromatase activity, at least in vitro (Drenth et al., 1998; Sanderson, 2006; Vinggaard et al., 2000). This has led to recognition of aromatase inhibitors as an important class of endocrine disruptors and inclusion of screening for aromatase inhibitors specifically, and steroid biosynthesis inhibitors more broadly, in the Endocrine Disruptor Screening Program of United States Environmental Protection Agency (U.S. EPA) (http://www.epa.gov/endo/). In vitro aromatase inhibition assays have also been incorporated into nascent high-throughput screening programs such as ToxCast (Reif et al., 2010). As chemicals are screened in these programs, identification of additional environmental contaminants that inhibit aromatase activity can be expected. Based on adverse outcome pathway knowledge developed by our research program, such chemicals are likely to function as reproductive toxicants in fish (Ankley et al., 2010).

Indeed, previous study has demonstrated that in vivo exposure to aromatase inhibitors can cause reproductive impairment in fish (Ankley et al., 2002, 2005; Sun et al., 2007). Based on endpoints measured in fish at multiple levels of biological organization, an adverse outcome pathway has been defined whereby aromatase inhibition leads to decreased estrogen synthesis in...
the gonad, decreased concentrations of circulating E2, reduced hepatic vitellogenesis, reduced uptake of vitellogenin (VTG) into oocytes, impaired oocyte growth, and ultimately decreased fecundity (Ankley et al., 2010). Early life exposures to aromatase inhibitors have also been reported to cause adverse effects on growth, eye development, sexual differentiation, and sperm quality in zebrafish (Hamad et al., 2007; McAllister and Kime, 2003), as well as complete sex reversal in Xenopus laevis (Olmstead et al., 2009). In birds, exposure to aromatase inhibitors has been linked to altered behavior and impaired osteogenesis (Deng et al., 2010; Wacker et al., 2008). Additionally, gestational exposures of rodents to aromatase inhibitors have been associated with in utero developmental abnormalities and altered sexual behaviors in adulthood (Gerardin et al., 2008; Tiboni et al., 2008). Collectively, given the important role of estrogens in many aspects of developmental, reproductive, and behavioral biology, it is clear that chemical inhibition of aromatase poses a potential risk in vivo to a diversity of vertebrates. However, the challenge of understanding at what concentrations and under what exposure conditions adverse in vivo effects are likely to occur remains.

To more effectively utilize in vitro aromatase inhibition data for quantitative risk assessment and predictive toxicology, there is a need to develop computational models that can support prediction of complex concentration-duration-response relationships that may occur in vivo (Villeneuve and Garcia-Reyero, 2011). To address this, our research team has conducted a series of time-course experiments to characterize direct effects of exposure to endocrine active chemicals (EACs) such as aromatase inhibitors, adaptive/compensatory responses that occur during exposure, and the rapidity and extent to which organisms recover following exposure (Ankley et al., 2009a,b; Ekman et al., 2011; Villeneuve et al., 2009). In earlier studies in which fathead minnows were exposed to 3 or 30 μg fadrozole/l for 1, 2, 4, or 8 days, followed by varying durations of depuration (experiment 1; Villeneuve et al., 2009) or exposed to 2, 5, 15 or 50 μg fadrozole/l for 7 days (Villeneuve et al., 2006), we documented a series of ovarian gene expression changes consistent with a compensatory feedback response to declining E2 concentrations. Although we observed some recovery in both rates of E2 production and plasma E2 concentrations, circulating concentrations of VTG did not return to control levels, even after 8 days of depuration, and after 21 days of continuous exposure, reproductive impacts were still observed (Ankley et al., 2002; Villeneuve et al., 2009).

This study had four primary objectives. First, we tested the hypothesis that plasma VTG concentrations in female fathead minnows would return to control levels over a more extended depuration/recovery period. Second, we sought to evaluate whether compensatory responses observed in an 8-day exposure would be sustained over a longer period of continuous exposure. Third, we wanted to examine the dynamics of the compensatory responses at a lower fadrozole concentration (i.e., 0.5 μg fadrozole/l) that had been used in the previous studies (e.g., experiment 1; Villeneuve et al., 2006, 2009). Finally, we wanted to measure the concentrations of fadrozole in plasma. Collectively, these data contribute to increased understanding of the nature and time course of compensatory responses and recovery processes that can affect concentration-duration-response relationships and toxicological outcomes for aromatase inhibitors. The results also inform the development of computational models of the hypothalamic-pituitary-gonadal axis in fish that could support the use of data from in vitro screening assays to predict probable reproductive outcomes in fish. The initial steps toward development of such a model are described in the companion article by Breen et al. (2013).

MATERIALS AND METHODS

Fadrozole exposure. Sexually mature adult fathead minnows (Pimephales promelas; 5–6 months old), obtained from an onsite culture facility at the U.S. EPA Mid-Continent Ecology Division (Duluth, MN), were exposed to 0, 0.5, or 30 μg fadrozole/l. Solvent-free solutions of fadrozole were delivered to 20-l tanks containing 10 l of test solution via a continuous flow (approximately 45 ml/min) of UV-treated, filtered Lake Superior water. Four male and four female fathead minnows were exposed in each tank. The experiment was initiated by transferring random groups of fish directly to tanks that had been receiving a continuous flow of test solution for approximately 48 h. Addition of fish was staggered by replicate within each treatment to permit all samples from a given exposure tank to be collected within 45 min of the desired exposure duration. Fish were either exposed continuously and sampled after 1, 8, 12, 16, 20, 24, or 28 days of exposure or exposed for 8 days, after which chemical delivery ceased and the tanks received a continuous flow of control Lake Superior water (no fadrozole) for an additional 8, 12, 16, or 20 days (Supplementary fig. 1), with fadrozole concentrations steadily diminishing as the volume in the tank was exchanged (6.5 water exchanges per day at 45 ml/min flow rate). There were two replicate tanks for each unique exposure condition (i.e., combination of treatment and time point). Fish survival, water temperature, and dissolved oxygen concentrations were monitored daily in all test tanks. Flow rates and pH were monitored one to two times each week.

Fadrozole concentrations in exposure tanks were measured on 19 occasions over the course of the experiment. Water from control tanks was also analyzed for fadrozole on 14 occasions over the study duration. Fadrozole concentrations in the tank water were quantified by high-pressure liquid chromatography (LC) with mass spectral (MS) detection. The LC/MS (1946 LC/MSD, Agilent, Wilmington, DE) method consisted of injecting 50 μl of tank water onto a Kinetex C18 2.0 x 50mm column (Phenomenex, Torrance, CA) held at 25°C with an isocratic elution using a mobile phase of 50% 0.025M ammonium acetate buffer/methanol at a flow rate of 200 μl/min. Fadrozole was measured in the selective ion monitoring mode using the response of positive ions 224 and 225 amu, with an atmospheric pressure photoionization source with a toluene dopant. Recoveries (mean ± SD) in water samples spiked with 0.43 and 21.8 μg fadrozole/l, respectively, were 98±3% (n = 30) and 101±4% (n = 28). Average percent agreement among duplicates was 97±3% (n = 59). The method detection limit was 0.05 μg/l.

On appropriate sampling days, fish were euthanized in a buffered solution of tricaine methanesulfonate (MS-222; Finquel, Argent, Redmond, WA). Whole-body wet weight was measured, and urine was collected from males using nonheparinized microcapillary tubes. The presence or absence of sperm in each urine sample was noted and urine was stored at −80°C for future metabolomic analyses (e.g., Collette et al., 2010). Blood was collected from both males and females using heparinized microhematocrit tubes then centrifuged to separate the plasma. Plasma samples were stored at −80°C until analyzed. For males, the remaining carcass (sans urine and blood) was wrapped in solvent-rinsed aluminum foil and stored at −20°C for tissue residue analysis. For females, liver tissue was removed, snap frozen in liquid nitrogen, and stored at −80°C. Ovary tissue was removed, weighed, and then split into three subsamples. A 16±8mg (mean ± SD) portion of the center of the right ovary was transferred...
to a glass test tube containing 500 μl medium 199 (M2520; Sigma, St Louis, MO) supplemented with 0.1mM isobutylmethylxanthine (Sigma I7018) and 1 μg 25-hydroxycolesterol (Sigma)/ml for use in an ex vivo steroid production assay. The entire left ovary and the anterior portion of the right ovary was snap frozen in liquid nitrogen and stored at ~80°C for subsequent metabolomic analysis, whereas the posterior portion of the right ovary was similarly frozen and stored for RNA extraction. Female brain tissue was removed, snap frozen, and stored at ~80°C. Pituitary glands (< 0.5 mg) were removed with a fine forceps, transferred to 100 μl RNAAlater (Ambion, Austin, TX) and stored at ~20°C. All dissection tools were washed with RNaseZap (Ambion) between samples to prevent cross-contamination and/or RNA degradation. All procedures involving animals were reviewed and approved by the U.S. EPA Animal Care and Use Committee in accordance with Animal Welfare Act and Interagency Research Animal Committee guidelines.

An additional set of tanks loaded with four males and four females per tank, as above, were included in the overall experimental design to allow for measurement of fadrozole concentrations in plasma. Fish in these tanks were exposed to 0.5 or 30 μg fadrozole/l for 1 day, 8 days, or 8 days followed by a single day of depuration or to control Lake Superior water for 8 days. There were two replicate tanks per time × treatment combination. However, unlike in the main experiment, only blood samples were collected from these fish. After blood collection, the rest of the carcass was preserved intact and stored at ~20°C for possible tissue residue analysis. Plasma was prepared by centrifugation and samples stored at ~80°C for subsequent determination of plasma fadrozole concentrations. For fadrozole quantification, 15 μl of plasma was combined with 50 μl acetonitrile, vortexed, and then sonicated for 15 min. Following sonication, samples were centrifuged for 10 min at 12,000 × g at 0°C, and the supernatant was transferred to a clean sample vial. The remaining extract was subjected to a second round of extraction with 100 μl of 50% acetonitrile/water and the supernatant of the second round of extraction combined with the first. The volume was brought to 500 μl, and 50 μl of the resulting final extract was injected into the LC/MS. The subsequent fadrozole quantification was the same as that described for the tank water. The effective method detection limit for plasma fadrozole measurements was 0.3 pg/μl.

Biochemical analyses. Plasma VTG concentrations were quantified by enzyme-linked immunosorbent assay using a polyclonal antibody to fathead minnow VTG and purified fathead minnow VTG as a standard (Korte et al., 2000). Ex vivo steroid production assays were conducted using methods adapted from McMaster et al. (1995) as described previously (Amlkey et al., 2007; Martinovic et al., 2008). Steroids were extracted from medium (ex vivo) or plasma samples by liquid-liquid extraction with diethyl ether and quantified by radioimmunoassay (Jensen et al., 2001). For culture medium samples from ex vivo steroid production assays, both E2 and T were quantified; however, due to limited volumes, only E2 could be quantified in female plasma.

Gene expression analyses. Relative abundance of mRNA transcripts coding for aromatase (cyp19a1a), follicle-stimulating hormone receptor (fshr), cytochrome P450 cholesterol side-chain cleavage (cyp11a), and steroidogenic acute regulatory protein (star) in oocytes was measured by real-time quantitative PCR (QPCR). Ovary samples were extracted and DNase treated (DNA free; Applied Biosystems/Ambion), and then 250 ng total RNA was reverse transcribed to cDNA and transcripts were quantified by Dynamo Sybrgreen (Bio-Rad, Hercules, CA) using methods and reaction conditions detailed previously (Biales et al., 2007). Primer specificity was determined through melting curve analysis. Both minus reverse transcription and no-template controls were used to confirm a lack of genomic DNA contamination and/or primer dimer formation. Relative quantity values, unadjusted for amplification efficiency, were calculated using the 2ΔΔCT method (Livak and Schmittgen, 2001) using 18S rRNA as a normalizer. QuantumRNA Universal 18S primers (Ambion) were used for 18S rRNA, and all other primer sequences used for the analyses are provided as Supplementary table 1.

Statistical analysis. Analyses were conducted such that samples from all relevant treatment groups (i.e., control, continuously exposed to 0.5 or 30 μg fadrozole/l, and 8 days exposed to 0.5 or 30 μg fadrozole/l then depurated/allowed to recover [0.5R, 30R]) for each time point were analyzed in the same assay (e.g., radioimmunoassay and QPCR), whereas samples from different time points were often analyzed in separate assays. As a result, interassay variability was generally not a factor in comparisons among treatments but could contribute increased variability to comparisons among time points. Therefore, unless otherwise stated, statistical analyses were focused on comparisons within rather than between time points. Data normality was evaluated using a Kolmogorov-Smirnov test. Homogeneity of variance was evaluated using Levene’s test. Data conforming to parametric assumptions were analyzed using one-way ANOVA followed by Duncan’s multiple range test. Data that did not conform to parametric assumptions were either transformed to meet parametric criteria or analyzed using a nonparametric Kruskal-Wallis test followed by Dunn’s nonparametric post hoc test. There were no significant differences among replicate tanks within a treatment group and time point for the endpoints examined. Therefore, individual fish were considered the unit of replication for statistical purposes. Differences were considered significant at p < 0.05 unless otherwise noted.

RESULTS

Measured concentrations of fadrozole in the 0.5 and 30 μg/l treatment tanks over the course of the study (mean ± SD) were 0.35 ± 0.03 (n = 158) and 25.3 ± 1.0 (n = 158) μg/l, respectively. No fadrozole was detected (≤ 0.05 μg/l) in water from control tanks. In tanks where fish were exposed to 0.5 μg fadrozole/l for 8 days, followed by depuration (0.5R treatments), fadrozole concentrations were nondetectable by day 12 (i.e., after 4 days of depuration) and remained so throughout the rest of depuration. In contrast, in tanks where fish were exposed to 30 μg fadrozole/l for 8 days, followed by depuration (30R treatments), 0.1 μg fadrozole/l could be detected after 4 days of depuration (exposure day 12), and small fadrozole peaks, estimated as less than 0.02 μg fadrozole/l, were detected after 8 days of depuration (exposure day 16). However, after 12 days of depuration, all fadrozole concentrations were less than the method detection limit of 0.05 μg/l and no discernible fadrozole peaks were evident on the chromatograms.

After 1 day of exposure to 30 μg fadrozole/l, measured plasma concentrations (mean ± SD) were 30.3 ± 8.8 pg fadrozole/μl in males (n = 4) and 26.7 ± 7.4 pg fadrozole/μl in females (n = 3). After 8 days of exposure to 30 μg fadrozole/l, plasma concentrations were 27.7 ± 6.0 pg fadrozole/μl in males (n = 6) and 21.9 ± 4.5 pg fadrozole/μl (n = 6) in females. After a single day of depuration, those concentrations had dropped to 1.43 ± 0.95 pg fadrozole/μl in males (n = 4) and 2.07 ± 0.32 pg fadrozole/μl in females (n = 3). No fadrozole was detected in plasma from males or females exposed to control Lake Superior water for 8 days (detection limit, 0.3 pg/μl).

Fish survival was high over the duration of the study. No males and just two of 304 females in the study died prior to sampling. Mean (± SD) temperature of the water in the test tanks was 25.5 ± 0.6°C. Mean (± SD) dissolved oxygen concentrations was 6.2 ± 0.6 mg/l, and pH was 7.8 ± 0.16. There were no significant treatment-related differences in survival or water quality conditions.

Exposure to fadrozole had significant effects on plasma E2 concentrations in females but only in those exposed to 30 μg...
fadrozole/l (Fig. 1A). Plasma E2 concentrations were significantly reduced within 1 day of exposure to 30 µg fadrozole/l. After 8 days of exposure, plasma E2 concentrations in females exposed to 30 µg fadrozole/l had rebounded slightly and were not significantly different from those in control females. However, for those females exposed to 30 µg fadrozole/l continuously for 12 or more days, plasma E2 concentrations remained significantly reduced. In females exposed to 30 µg fadrozole/l for 8 days, then exposed to control Lake Superior water over subsequent days, plasma E2 concentrations rebounded dramatically and were actually significantly greater than those detected in control females 4 and 8 days postexposure, before returning to control levels following 12 or more days of depuration/recovery (Fig. 1A). Plasma E2 concentrations in females exposed to 0.5 µg fadrozole/l (nominal) were not significantly different from those in control females (Fig. 1B).

Concentrations of the estrogen-responsive protein, VTG, in female plasma were significantly affected at both 0.5 and 30 µg/l fadrozole concentrations (Figs. 1C and 1D). Exposure to 30 µg fadrozole/l reduced plasma VTG concentrations more than 2.4-fold within 24 h and over 40-fold within 8 days. Plasma VTG concentrations remained significantly depressed (> 65- to 400-fold) in the females continuously exposed beyond 8 days. For females exposed to 30 µg fadrozole/l for 8 days and control water on subsequent days, plasma VTG concentrations generally remained significantly depressed compared with controls (except 16 days postexposure; day 24). However, the VTG concentrations in those fish were only about 1.5- to 2.5-fold less than those in control fish. That was similar to the magnitude of VTG depression observed in fish exposed continuously to 0.5 µg fadrozole/l for 12 or more days (Fig. 1D). As in fish exposed to the greater concentration of fadrozole, depuration in Lake Superior water after 8 days of exposure to 0.5 µg fadrozole/l resulted in some recovery of VTG concentrations compared with fish that were continuously exposed; however, they still tended to be lower than those in females never exposed to fadrozole.

In addition to effects on circulating concentrations of E2 and VTG, steroid production by ovary tissue was affected by the in vivo exposure to fadrozole (Figs. 2A and 2B). Ex vivo

**FIG. 1.** Concentrations of 17β-estradiol (E2; panels A and B) and vitellogenin (VTG; panels C and D) measured in the plasma of female fathead minnows exposed to 0, 0.5 (panels B and D), or 30 µg (panels A and C) fadrozole/l either continuously for 1, 8, 12, 16, 20, 24, or 28 days, or exposed to 0.5 or 30 µg fadrozole/l for 8 days followed by an additional 4, 8, 12, 16, or 20 days of depuration in control Lake Superior water (no fadrozole; 0.5 R, 30 R). Error bars represent SE and n = 8 for most conditions (treatment × time point combinations). Vertical dashed line indicates cessation of chemical delivery to 0.5 R and 30 R treatments. * indicates statistically significant difference from control (0) within time point.
E2 production by ovary tissue was significantly reduced after 24 h of exposure to 30 µg fadrozole/l. However, in contrast with plasma E2 concentrations, that significant effect did not persist when the exposure period was extended beyond 8 days. What was similar to the plasma E2 effect was the significant increase in ex vivo E2 production by ovary tissue collected from fish that were exposed for 8 days, and then held in control water only for the subsequent 4 days. Over the 12-h incubation period, ovary tissue from these fish produced, on average, twice as much E2 as ovary tissue from control fish and 6.5 times more than ovary tissue from fish exposed continuously for 12 days. Ex vivo T production was also significantly affected (Figs. 2C and 2D). Specifically, ovary tissue from females exposed to 30 µg fadrozole/l for 8 or more days produced, on average, about five times more T than ovary tissue from control fish. In the fish exposed to 30 µg fadrozole/l for 8 days before being held in control water, ex vivo T production returned to the control range within 8 days postexposure.

At the molecular level, there was significant modulation of the abundance of two transcripts coding for proteins thought to be key for production of E2 by the fish ovary. In the females that experienced significant reductions in circulating E2 concentrations (i.e., those exposed continuously to 30 µg fadrozole/l), both cyp19a1a and fshr expressions were significantly elevated throughout the entire exposure duration (except day 1 in the case of cyp19a1a; Fig. 3). The abundance of transcripts coding for StAR and CYP11A was not significantly affected by fadrozole exposure (Supplementary fig. 2).

**DISCUSSION**

In an earlier study (experiment 1), Villeneuve et al. (2009) reported that exposure to 3 or 30 µg fadrozole/l caused significant reductions in ex vivo E2 production and plasma E2 concentrations within 24 h and that over subsequent days of exposure (through day 8), effects on ex vivo E2 production (both doses) and plasma E2 concentrations (3 µg fadrozole/l only) gradually decreased. The gradual reduction in impact was accompanied by rapid and persistent increases in the expression of cyp19a1a and fshr (particularly at the 30 µg/l concentration) and more transient upregulation of star and cyp11a (Villeneuve et al., 2009). After fadrozole delivery ceased, there was a period of increased ex vivo E2 production compared with the controls, suggesting that E2 production rates by the gonad had increased as part of a compensatory response to aromatase inhibition by fadrozole. This study (experiment 2) largely confirmed those initial results. Ex vivo E2 production and plasma E2 concentrations in fish exposed to 30 µg fadrozole/l recovered slightly from exposure day 1 to exposure day 8, such that on day 8 the effect of fadrozole was not statistically significant (Figs. 1A and 2A). Fish exposed to 30 µg fadrozole/l for
8 days followed by control water on subsequent days exhibited a period during which their \textit{ex vivo} and plasma E2 concentrations exceeded those of the controls, again, suggesting that the fish had mounted a compensatory response to the stressor. Just as in the previous study (experiment 1), both \textit{cyp19a1a} and \textit{fshr} expression, thought to be localized primarily within the granulosa cells, increased in response to fadrozole exposure. Additionally, this study (experiment 2) showed that this change in gene expression was sustained over the entire period of time that the fadrozole exposure was continued. In contrast, the more transient increases in \textit{cyp11a} and \textit{star} transcripts (Villeneuve et al., 2009), both of which are thought to be localized primarily in the theca cells, were not observed in this study. Given that elevated \textit{ex vivo} production of T was observed in both this study (experiment 2) and the previous one (experiment 1; Villeneuve et al., 2009), it is not clear whether increased expression of \textit{cyp11a} and \textit{star} is not a consistent response to aromatase inhibition and/or declining E2 concentrations or whether the timing of sample collection in this study (experiment 2) simply missed this more transient upregulation. Either way, there were several distinct consistencies between this study (experiment 2) and that of Villeneuve et al. (2009) (experiment 1), which suggest a mechanism of compensation that should be incorporated into dynamic concentration-response models intended to predict effects of aromatase inhibitors.

In addition to confirming reproducibility of our previous study with fadrozole, the current, more temporally intensive study (experiment 2) enabled a more thorough mechanistic evaluation of compensatory responses to aromatase inhibition. For example, in experiment 1, Villeneuve et al. (2009) noted that although plasma VTG concentrations recovered considerably over the 8 days depuration/recovery period included in that study, concentrations of the lipoprotein never returned to control levels (Villeneuve et al., 2009). Therefore, we were curious whether full recovery of circulating VTG concentrations would occur over a more extended recovery period as this has significant implications for predicting/modeling the potential reproductive impacts of aromatase inhibition in fish (Miller et al., 2007; Li et al., 2011a,b). We found that even when the depuration period was extended 2.5 times longer than that in experiment 1 (Villeneuve et al., 2009), plasma VTG concentrations did not return to levels comparable to those in females that had never been exposed to fadrozole (Fig. 1C) despite the fact that plasma E2 concentrations had fully recovered or even exceeded those of control fish (Fig. 1A). Remarkably, even at the lower concentration of fadrozole (0.5 µg/l), which caused no discernible effect on plasma E2 concentrations (Fig. 1B), some reduction in VTG concentrations was evident over roughly half of the depuration/recovery phase of the experiment (Fig. 1D).

These results suggest that, of the endpoints examined in this
study (experiment 2), plasma VTG concentrations were the most sensitive to the effects of fadrozole. Furthermore, even modest effects on VTG concentrations were quite persistent, further affirming the utility of VTG concentrations, in female fish, as a useful biomarker (Miller et al., 2007). This extended duration of VTG depression following a transient exposure to an aromatase inhibitor can, in theory, be incorporated into modeling efforts. However, remaining uncertainties as to just how long the VTG depression may persist and the subtle mechanisms through which VTG production is reduced even at concentrations that cause no detectable change in E2 production or circulation make accurate modeling of VTG responses challenging.

A second major question that arose from our previous time course study with fadrozole (experiment 1) was whether the apparent compensatory response that led to a recovery in ex vivo E2 production and plasma concentrations (at least for the 3 µg fadrozole/l treatment; Villeneuve et al., 2009) could be sustained over a longer exposure duration. At the molecular level, the response in this study (experiment 2) was maintained, with increased cyp19a1a and fshr being sustained throughout the duration of fadrozole exposure. Ex vivo E2 production was maintained at a level that was not significantly different from the control. However, consistent with experiment 1 (Villeneuve et al., 2009), neither plasma E2 nor VTG concentrations recovered despite what might be viewed as successful compensation, at least in terms of ex vivo steroid production. This is consistent with results of a 21-day reproduction experiment with fadrozole in which exposure to concentrations greater than 10 µg fadrozole/l caused significant reductions in plasma E2 and VTG concentrations and associated reductions in fish fecundity (Ankley et al., 2002). Unfortunately, the 0.5 µg fadrozole/l treatment concentration included in this study (experiment 2) did not cause significant reductions in ex vivo E2 production or plasma E2 concentrations, so it was not feasible to directly discern whether compensation could be successfully maintained at lower fadrozole concentrations. However, the VTG effect at the 0.5 µg/l concentration does suggest that some impact was occurring, even if it could not be resolved statistically for endpoints other than VTG. Thus, at present, although there is evidence that some compensatory response to fadrozole exposure occurs, it is not clear that it ultimately does much to mitigate the potential negative effects on plasma VTG production and, ultimately, reproduction, in long-term exposures.

This study (experiment 2) also provided critical knowledge concerning the kinetics of fadrozole uptake and excretion in fish as it relates to the responses observed in vivo. Based on fadrozole’s estimated log Kow of 3.20 (estimated using KOWWIN v. 1.67; http://www.epa.gov/opptintr/exposure/pubs/episuite.htm) and assuming strictly branchial uptake and elimination, one would predict an equilibrium blood:water partition coefficient ($P_{bw}$) of 35 (Fitzsimmons et al., 2001). Total measured concentrations in plasma (not differentiating free fadrozole from protein bound) were approximately equal to water concentrations, suggesting rapid metabolism of fadrozole by fathead minnows. This was further supported by the plasma data that showed that fadrozole was rapidly cleared from the body during depuration. Based on these results, it appears that for modeling purposes, plasma concentrations of fadrozole can be assumed to be similar to water concentrations under steady-state conditions.

Finally, although it did not relate directly to our initial study objectives or hypotheses, the time-dependent profile of cyp19a1a expression in fish exposed continuously to 30 µg fadrozole/l was notable. Increased cyp19a1a expression can be viewed as one of the primary compensatory responses to declining E2 concentrations. Over the course of the 28-day exposure, average cyp19a1a expression, although elevated, seemed to oscillate. Although interesting in its own right, what was even more remarkable was that plasma E2 concentrations followed a parallel time-dependent pattern (Fig. 4). Ex vivo E2 production, which, in a cause-effect sense, would be considered more temporally proximal to cyp19a1a expression, did not closely parallel the cyp19a1a expression profile (Supplementary fig. 3). Although by no means definitive, these behaviors raise the question of whether circulating E2 concentrations have a direct influence on cyp19a1a expression in the fathead minnow ovary. As opposed to cyp19a1b, which is an isoform of the aromatase gene predominantly expressed in brain and known to be regulated by estrogen responsive elements, the cyp19a1a isoform is not thought to contain a functional estrogen responsive element as part of its gene regulatory region (Callard et al., 2001; Kishida and Callard, 2001). Therefore, there is currently no evidence to suggest that direct interaction of E2 with the estrogen receptor in ovary tissue could account for the parallel profiles. However, it has been suggested that T can stimulate follicle-stimulating hormone–independent expression of cyp19

![FIG. 4.](https://academic.oup.com/toxsci/article-abstract/133/2/225/1615381) Comparison of the time-dependent profile of cyp19a1a transcript abundance (normalized to 18S rRNA) with the time-dependent profile of plasma 17β-estradiol (E2) concentrations in female fathead minnows continuously exposed to 30 µg fadrozole/l for 1, 8, 12, 16, 20, 24, or 28 days. Error bars represent SE and n = 8 for most conditions (treatment x time point combinations). Y-axis is split to allow measurements made in different units to be displayed on the same graph.
in mammalian granulosa cells via interaction with liver receptor homolog-1 LRH-1 (Wu et al., 2011). It is not known whether a similar mechanism operates in fish, nor was it feasible to measure circulating T concentrations in the females from this study due to limited available plasma volumes. Nonetheless, the ex vivo T production results suggest that excess accumulation of T in ovary tissue and/or plasma was possible. Assuming that impaired ability to aromatize T to E2 was the major cause of the nearly fivefold increase in ex vivo T release from the ovaries of fish exposed to 30 µg fadrozole/l, one could hypothesize that direct, local, T-dependent regulation of cyp19a1a expression could yield an oscillatory expression pattern. Such regulation, if it exists, would be an important regulatory pathway to include in a mechanistic model of steroidogenesis. Thus, follow-up investigations are warranted.

As a whole, these data contribute to on-going efforts to understand and model biological responses to EACs, like aromatase inhibitors. Development of computational system models that incorporate knowledge concerning the compensatory feedback mechanisms that modulate the impacts of EACs can lead to improved prediction of complex concentration-duration-response behaviors. If appropriately coupled to complementary toxicokinetic models, such computational system models have potential to aid the extrapolation of in vitro toxicity pathway assay data into probabilistic predictions of potential adverse outcome(s). Additionally, iterations of model development and empirical testing can lead to an improved understanding of the biology underlying toxicological responses to EACs and other environmental contaminants (see companion paper; Breen et al. 2013).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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