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Dynamic Nature of Alterations in the Endocrine System of Fathead Minnows Exposed to the Fungicide Prochloraz

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The vertebrate hypothalamic-pituitary-gonadal (HPG) axis is controlled through various feedback mechanisms that maintain a dynamic homeostasis in the face of changing environmental conditions, including exposure to chemicals. We assessed the effects of prochloraz on HPG axis function in adult fathead minnows (*Pimephales promelas*) at multiple sampling times during 8-day exposure and 8-day depuration/recovery phases. Consistent with one mechanism of action of prochloraz, inhibition of cytochrome P450 (CYP) 19 aromatase activity, the fungicide depressed *ex vivo* ovarian production and plasma concentrations of 17β-estradiol (E2) in female fish. At a prochloraz water concentration of 30 μg/l, inhibitory effects on E2 production were transitory and did not persist during the 8-day exposure phase. At 300 μg/l prochloraz, inhibition of E2 production was evident throughout the 8-day exposure but steroid titers recovered within 1 day of cessation of exposure. Compensation or recovery of steroid production in prochloraz-exposed females was accompanied by upregulation of several ovarian genes associated with steroidogenesis, including *cyp19a1a*, *cyp17* (hydroxylase/lyase), *cyp11a* (cholesterol side-chain cleavage), and follicle-stimulating hormone receptor. In male fathead minnows, the 8-day prochloraz exposure decreased testosterone (T) production, possibly through inhibition of CYP17. However, as for E2 in females, *ex vivo* testicular production and plasma concentrations of T recovered within 1 day of stopping exposure. Steroidogenic genes upregulated in testis included *cyp17* and *cyp11a*. These studies demonstrate the adaptability of the HPG axis to chemical stress and highlight the need to consider the dynamic nature of the system when developing approaches to assess potential risks of endocrine-active chemicals.

Key Words: **fish; prochloraz; endocrine function; compensation; recovery.**

Prochloraz is an imidazole fungicide registered for various agricultural uses throughout the world (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=73665). Prochloraz inhibits fungal growth by acting as an inhibitor of cytochrome P450 (CYP) 14α-demethylase (CYP51), an enzyme key to ergosterol synthesis (Van den Bossche *et al*., 1978, 1982). However, prochloraz can bind to and inhibit the activity of a wide variety of CYPs, including some involved in steroid synthesis in vertebrates. For example, studies have shown that the fungicide effectively inhibits aromatase (CYP19) activity in both fish and mammals, blocking the conversion of testosterone (T) to 17β-estradiol (E2) and acting *in vivo* as an antiestrogen (Ankley *et al*., 2005; Mason *et al*., 1987; Sanderson *et al*., 2002; Thorpe *et al*., 2007; Vinggaard *et al*., 2000). Prochloraz also can cause antiandrogenic effects in vertebrates either through direct antagonism of the androgen receptor or through inhibition of cytochrome P450 c17α-hydroxylase/17,20-lyase (CYP17), a steroidogenic enzyme involved in T production (Blystone *et al*., 2007; Noriega *et al*., 2005; Vinggaard *et al*., 2002, 2005). Due to its varied effects on the vertebrate hypothalamic-pituitary-gonadal (HPG) axis, prochloraz has been used as a model compound for studies focused on different mechanisms of endocrine disruption (Gray *et al*., 2006; USEPA, 2007).

Ankley *et al*. (2009) describe a research effort focused on a systems-based approach for studying reproductive effects of HPG-active chemicals with differing mechanisms of action in fish. The HPG axis is a highly dynamic system, which, through various feedback mechanisms, strives to maintain physiological conditions conducive to reproduction even in potentially stressful situations. Therefore, a component of predicting effects involves understanding how chemicals interact with the HPG axis temporally, in terms of both direct impacts and compensation during dosing, and recovery after cessation of chemical exposure. Some of our initial research on temporal changes in the HPG axis was described by Villeneuve *et al*. (2009). That work assessed effects of a model aromatase inhibitor, fadrozole, on reproductive endocrine function in the fathead minnow (*Pimephales promelas*). Consistent with its mechanism of action, fadrozole strongly inhibited synthesis of E2 by female fish and depressed production of the estrogen-responsive protein vitellogenin (VTG; egg yolk

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precursor) within 24 h of exposure. However, there was a subsequent time/dose-dependent compensatory response relative to E2 production by the fish, coincident with upregulation of several HPG axis genes that would contribute to increased steroid production (Villeneuve *et al*., 2009). Termination of the fadrozole exposure resulted in recovery of the fish in terms of gene expression and steroid (and to some degree VTG) production. Interestingly, shortly after removal of the chemical, a brief period of ''overcompensation'' by some components of the system was observed before the system returned to baseline (similar to control) conditions.

The objective of the present study was to employ an experimental design similar to that used by Villeneuve *et al*. (2009) to investigate responses of the fathead minnow HPG axis to prochloraz. Because prochloraz affects other targets in addition to aromatase, it provides a useful comparison to fadrozole in terms of revealing commonalities and differences in effects of the two chemicals on steroid synthesis and HPG function. The type of temporally intensive design employed for this work, while challenging to implement, is necessary for generation of the robust data sets needed for (1) identifying linkages between events occurring at differing biological levels of organization and (2) the development of dynamic modeling approaches for prediction of chemical effects on the reproductive system of fish (Ankley *et al*., 2009).

MATERIALS AND METHODS

Test animals and exposure conditions. Adult male and female fathead minnows (5–6 months old) from an on-site culture were used for this study. Although the fish were not held under conditions optimal for spawning, they were sexually mature, exhibiting typical male and female secondary sex characteristics. Solvent-free stock solutions of prochloraz (99.4% purity; Chem Services, West Chester, PA) were prepared as described elsewhere (Ankley *et al*., 2005) and diluted with filtered (control) Lake Superior water to achieve desired test concentrations of the fungicide. Exposures were conducted in glass aquaria containing 10 l of control Lake Superior water or prochloraz solutions (30 or 300 μg/l, nominal) delivered at a continuous flow of about 45 ml/min. The prochloraz treatments were chosen to bracket reproductive effect/no-effect concentrations based on results of a previous 21-day fathead minnow assay with this fungicide in our laboratory (Ankley *et al*., 2005). Experiments were conducted at 25°C ± 1°C under a 16:8 h light:dark photoperiod. Fish were fed adult brine shrimp to satiation twice daily.

Water samples (1 ml) were collected from all the test tanks containing fish on each sampling day of the exposure period (i.e., test days 1, 2, 4, and 8). The samples were immediately analyzed for prochloraz by reverse-phase high-performance liquid chromatography with diode array detection, as described by Ankley *et al*. (2005), with the slight modifications of larger sample injection volume (100 μl) and a lower flow rate (0.3 ml/min) to enhance detection limits. Tanks were also sampled on the first day of the recovery period (postexposure day 1) and thereafter until prochloraz concentrations had decreased to nondetectable levels (method detection limit = 5 μg/l). No prochloraz was detected in the control tanks ($n = 52$) or procedural blanks ($n = 4$). The mean (SD, n) recovery of prochloraz in spiked Lake Superior water samples was 92% (2.0, 4), and the agreement among duplicate samples was 99% (0.8, 8).

Experimental design and sample collection. The basic experimental design used for this study was described by Villeneuve *et al*., (2009). There were 16 replicate tanks per treatment (48 tanks total), each containing four male and four female fathead minnows. All the fish from two of the 16 replicates were sampled 1, 2, 4, and 8 days after initiation of the prochloraz exposure (i.e., eight males and eight females per treatment at each time point). After sampling fish exposed to prochloraz for 8 days, toxicant delivery to the system was stopped and remaining fish were held in a constant flow of control water. On days 1, 2, 4, and 8 of this recovery phase, all the fish from two replicate tanks per treatment were sampled.

Fish were euthanized with a buffered solution of Finquel (MS-222; Argent, Redmond, WA). The animals were weighed, and blood was collected from the caudal vein/artery with a heparinized microhematocrit tube. Plasma was prepared by centrifugation and stored at −80°C until analyzed for T, E2, and VTG. Gonads, brains, and pituitaries were removed from the animals and gonad weights recorded for calculation of the gonadal-somatic index (GSI). Brain and a portion of the gonad were snap frozen in liquid nitrogen and stored until used for gene, protein, and/or metabolite analyses. The pituitary was placed in RNAlater (Sigma, St Louis, MO) and stored at −20°C. A second portion of the gonad was preserved in Davidson's fixative for possible histological analysis, and a third piece of the gonad was used immediately for an *ex vivo* steroid production assay.

Biochemical analyses. *Ex vivo* production of T (testis and ovary) and E2 (ovary) was determined with an adaptation of the method of McMaster *et al*. (1995), as described by Villeneuve *et al*. (2009), using radioimmunoassay (RIA) to measure steroids in the culture medium. Plasma concentrations of T and E2 in the fish also were determined by RIA (Jensen *et al*., 2001), and plasma concentrations of VTG were measured using an ELISA with a polyclonal fathead minnow antibody and fathead minnow VTG as a standard (Korte *et al*., 2000).

Gene expression assays. We determined relative expression of a number of genes involved in reproductive function using real-time (RT) quantitative polymerase chain reaction (QPCR). Transcripts chosen for analysis included those that code for protein targets of prochloraz as well as gene products that represent potential control points in the HPG axis (Ankley *et al*., 2005; Villeneuve *et al*., 2007b). Based on past studies with prochloraz, most of the gene expression work focused on females, as they appear to be more sensitive to endocrine-mediated effects of the fungicide than males (Ankley *et al*., 2005; Thorpe *et al*., 2007). However, we did measure transcripts of few genes in testis samples: *cyp17*, androgen receptor (*ar*), and a cytochrome P450 side-chain cleavage (*cyp11a*). Ovarian transcripts measured included *cyp11a*, *cyp17*, *cyp19a1a* (the isoform of aromatase expressed predominantly in the ovary), 17β-hydroxysteroid dehydrogenase (*hsd17b*), steroidogenic acute regulatory protein (*star*), estrogen receptor-α (*esr1*), VTG receptor (*vtgr*), gonadotropin-releasing hormone receptors 1 and 3 (*gnrhr1* and *gnrhr3*), and follicle-stimulating hormone receptor (*fshr*). Pituitary transcripts measured in females included lutenizing hormone β subunit (*lhb*) and follicle-stimulating hormone β subunit (*fshb*). Finally, we measured two transcripts in the brain of female fish: *cyp19a1b* (the isoform of aromatase predominantly expressed in the brain) and *cyp51*.

Assays for most of the genes noted above have been described elsewhere (Villeneuve *et al*., 2006, 2007a,b; Martinovic *et al*., 2008). However, QPCR assays for fathead minnow *gnrhr1*, *gnrhr3*, and *cyp51* are reported here for the first time. Partial complementary DNA (cDNA) sequences for fathead minnow *gnrhr1*, *gnrhr3*, and *cyp51* were identified using zebrafish (*Danio rerio*) nucleotide sequences as query sequences (Supplementary Table 1) for a nucleotide-nucleotide BLAST (BLASTn) search of the *P. promelas* expressed sequence tag database (National Center for Biotechnology Information). Identity of the putative fathead minnow sequences relative to the zebrafish query sequence was 87% for all genes, and expect values were close to zero, indicating good confidence in the sequence match (Supplementary Table 1). The QPCR primers were designed based on the partial cDNA sequences identified using PrimerExpress software (Applied Biosystems, Foster City, CA). All QPCR assays conducted were two-step assays. Samples were extracted, DNAse treated (DNA free; Applied Biosystems/Ambion, Austin,

TX), and then 250 ng total RNA was reverse transcribed to cDNA using methods described by Biales *et al.* (2007). Transcripts for *esr1*, *vtgr*, *gnrhr1*, *gnrhr3*, *cyp19a1b*, and *cyp51* were quantified using Power SYBR Green PCR Master Mix (Applied Biosystems). Template cDNA (2.0 µl) was combined with 200nM forward and reverse primers and 2× Master Mix in a 12.5-µl reaction. Amplification and quantification was conducted using a 7500 RT-PCR System (Applied Biosystems) using the following protocol: 95°C for 10 min, followed by 40 cycles of PCR (melt 95°C, 15 s; anneal and extend 60°C, 60 s). Following amplification, product specificity was verified by generating dissociation curves for all samples (dissociation, 95°C–60°C). Duplicate no-template controls were analyzed along with each set of samples to confirm that signals were not due to primer dimers. Relative transcript abundance was estimated based on a standard curve generated by analyzing multiple dilutions of a gene-specific DNA amplicon, without correction for amplification efficiency. Amplicons used as standards were amplified from fathead cDNA with the same gene-specific primers used for QPCR. Specificity of each amplicon (and the primer pair used to generate it) was verified using agarose gel electrophoresis. Dilution of the amplicon used as a standard was optimized to yield a standard curve over threshold cycle numbers ranging from ~15 to 35, as most experimental samples fell within that range.

Transcripts for all other genes analyzed in this study were quantified using Taqman EZ RT-PCR kits (Applied Biosystems). Each 12-µl cDNA reaction mixture contained 150nM of a gene-specific Taqman probe (except *fshb* 300nM) and 200nM forward and reverse primers. Samples were amplified over 40 cycles (melt 94°C for 20 s, anneal and extend, 58°C for 60 s) using a 7500 RT-PCR System. As above, relative transcript abundance was estimated based on a standard curve generated by analyzing multiple dilutions of a gene-specific amplicon, without correction for amplification efficiency (for details on preparation of gene-specific standards for Taqman assays, see Villeneuve *et al.*, 2007a).

Data analysis Due to the large number of samples generated, it was not feasible to conduct all measurements for a given end point in any one assay set. Therefore, QPCR and RIA measurements were conducted in a manner such that, for any given sampling period (day), all treatment groups were analyzed in the same assay set. This helps limit the degree to which interassay variability might confound interpretation of treatment effects but could affect comparisons across time. Hence, initial statistical analyses were focused on comparisons within rather than between time points. Additional statistical analysis of some of the QPCR data used within treatment responses pooled across exposure (or recovery) phases of the test to help discern possible trends in up- or downregulation of specific genes. To facilitate presentation of results, QPCR data are presented as fold change (log 2) relative to control values.

Statistical analyses were conducted using SAS (SAS Institute, Cary, NC), Statistica 8 (StatSoft Inc., Tulsa, OK), and GraphPad Instat v. 3.01 (GraphPad Software, San Diego, CA). Data normality and homogeneity of variance were assessed using Kolmogorov-Smirnov and Levene's tests. Parametric data were analyzed using one-way ANOVA with chemical treatment as the independent variable. Duncan's multiple range or Dunnett's tests were used to determine differences between treatment groups. Data which did not meet parametric assumptions were either transformed (log 10) and analyzed via ANOVA, or analyzed using the Kruskal-Wallis test followed by Dunn's *post hoc* test. Results were considered significant at $p \leq 0.05$.

In addition to hypothesis testing, we used multivariate principal components analysis (PCA) to help identify associations between the different measurements made in the female fathead minnows over the exposure/recovery time course (van den Berg *et al.*, 2006). The 13 variables evaluated via PCA were plasma VTG and E2 concentrations, *ex vivo* T and E2 production, and levels of the nine ovarian transcripts measured on each sampling day (*cyp11a*, *cyp17*, *cyp19a1a*, *hsd17b*, *star*, *esr1*, *vtgr*, *gnrhr1*, and *fshr*). For the PCA, data for treatment groups were expressed relative to the mean corresponding control value (mean centered and scaled). The PCA was based on a total of 188 cases, and missing data points (due to technical errors during sample collection, extraction, or analysis) were handled through mean substitution (mean for that variable across all treatments and time points). A two-dimensional PCA loading plot was generated to show the relative influence of each variable on the variation represented by positions along factor 1 (x) and factor 2 (y). Mean (± SE) PCA scores for each treatment were plotted as a function of time. Because units were scaled to the control mean for each variable, mean scores of the control group at each time point are nearly identical and the error bars in the x (PC1) and y (PC2) factor plane represent the overall biological variation in the control group across all time points. For the prochloraz-treated groups, deviation from the control state (based on the 13 target end points) is reflected by a lack of overlap between the mean scores values (and associated x and y error bars) with the region representing biological variation within the control population over the course of the test.

RESULTS

Exposure Characterization

Measured prochloraz concentrations were slightly lower than the nominal (target) concentrations but were reasonably stable over the 8-day exposure period. Mean (SD, *n*) concentrations in the 30-µg/l prochloraz treatment group were 21 (1.1, 16), 22 (1.3, 14), 24 (1.1, 12), and 23 (1.2, 10) µg/l on test days 1, 2, 4, and 8, respectively. Mean (SD, *n*) concentrations in the 300-µg/l prochloraz treatment group were 269 (35.2, 16), 269 (8.2, 14), 300 (7.2, 12), and 296 (5.0, 10) µg/l on days 1, 2, 4, and 8, respectively. The first day after chemical exposure had been terminated, there was no prochloraz detected in the 30-µg/l treatment, while the mean concentration across the 300-µg/l treatment tanks was 14 µg/l (1.4, 8). By the next day (day 2 of the recovery phase), prochloraz in the high treatment tanks had diminished to 7.2 µg/l (0.75, 4), a concentration just above the analytical detection limit, and by day 4 of the recovery phase, no prochloraz was detected in any tank.

Biochemical Responses

There was no prochloraz-induced mortality of fish during either the 8-day exposure or the 8-day recovery phases of the test nor were there any observations of abnormal behavior. There were no significant effects of the fungicide on the GSI of either male or female fathead minnows (data not shown).

Prochloraz did not alter the low to nondetectable plasma VTG concentrations in male fathead minnows (data not shown); however, the fungicide caused significant effects on plasma VTG in the females (Fig. 1). The 30-µg/l treatment did not affect VTG; however, by day 2 of the exposure, VTG was significantly depressed in the 300-µg/l group and remained decreased until day 2 of the recovery phase of the test (Fig. 1).

Exposure to prochloraz caused modest effects on plasma T concentrations in males, with a significant concentration-dependent depression noted by day 8 of the exposure (Fig. 2a). Although not significant, mean plasma T concentrations were lower in males from the 300-µg/l treatment than in those from the control or 30-µg/l groups on exposure days 1, 2, and 4. During the recovery phase of the test, there were no significant differences in plasma T concentrations among the three treatment groups (Fig. 2a). *Ex vivo* production of T by testis

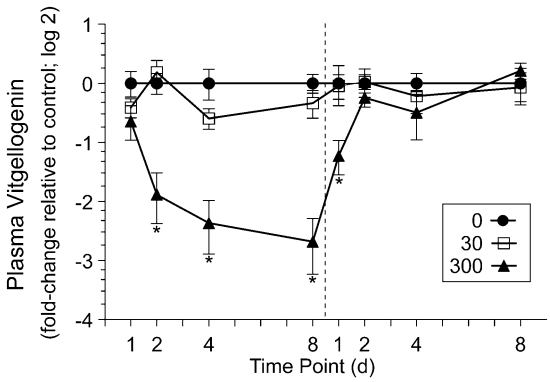

FIG. 1. Effects of 8 days of exposure to prochloraz, followed by 8 days of recovery, on plasma VTG concentrations in female fathead minnows. Data points indicate the mean (SE) fold change relative to controls and are expressed as log 2–transformed units. Asterisks indicate a significant treatment-related effect compared to controls for any given test day.

exhibited similarities to *in vivo* plasma concentrations of the androgen (Fig. 3a). Steroid production in the 300-µg/l treatment was consistently lowest during the 8-day exposure phase of the test (significantly so on day 4), and there was little difference between the three treatment groups during the 8-day recovery phase.

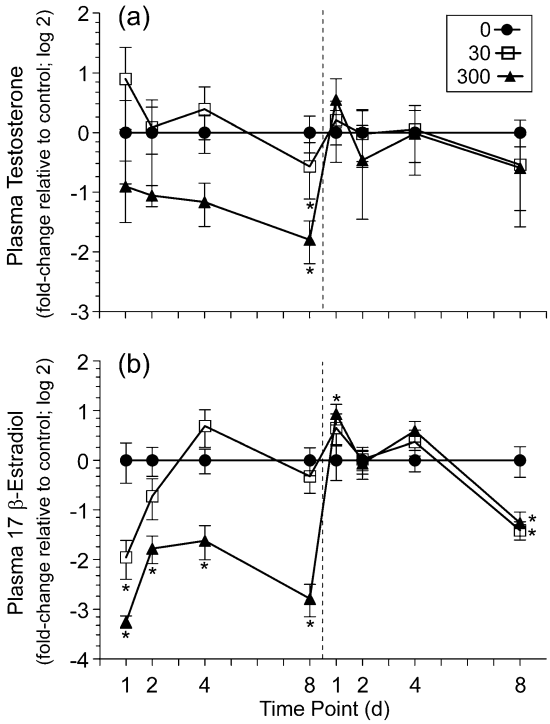

FIG. 2. Effects in fathead minnows of 8 days of exposure to prochloraz, followed by 8 days of recovery, on plasma concentrations of (a) T in males and (b) E2 in females. Data points indicate the mean (SE) fold change relative to controls and are expressed as log 2–transformed units. Asterisks indicate a significant treatment-related effect compared to controls for any given test day.

Due to sample volume limitations, plasma T concentrations were not measured in female fathead minnows. However, *ex vivo* ovarian production of T did not vary significantly between the treatment groups (Fig. 3b).

Prochloraz caused significant concentration- and time-dependent effects on E2 plasma concentrations and *ex vivo* production of E2 in the female fathead minnows (Figs. 2b and 3c). There was a concentration-dependent suppression of plasma E2 concentrations within 1 day of exposure to prochloraz (Fig. 2b). In females from the 30-µg/l treatment, plasma E2 concentrations exhibited a seemingly cyclical pattern, increasing to about 75% of control levels by day 2 of the exposure, exceeding control concentrations on day 4, and achieving concentrations comparable to controls by day 8 of the exposure phase (Fig. 2b). Over the course of the 8-day prochloraz exposure, plasma E2 concentrations in the 300-µg/l treatment group increased somewhat from the lowest point observed on day 1 but nonetheless remained significantly lower than control values on sampling days 2, 4, and 8 (Fig. 2b). On day 1 of the recovery phase of the experiment, plasma E2 concentrations in females from the 300-µg/l treatment were significantly higher than in control fish. Concentrations of E2 in fish from both prochloraz treatment groups were similar to controls on days 2 and 4 of the recovery phase, and both were significantly less than controls on day 8 (Fig. 2b).

There was relatively greater variability in data from the *ex vivo* assay compared to *in vivo* steroid measurements, but trends in *ex vivo* production of E2 by ovarian tissue were similar to observed plasma concentrations of the estrogen (Fig. 3c). For example, there was a decrease in ovarian production of E2 on day 1 of the exposure, which was statistically significant in the 300-µg/l group. Although not significant, E2 production by ovarian tissue from fish from the 300-µg/l treatment remained lower than in the control or 30-µg/l treatments throughout the 8-day exposure (Fig. 3c). On day 4 of the exposure, *ex vivo* E2 production was greater in the 30-µg/l group than in controls, a pattern similar to that observed *in vivo*. Similarly, on day 1 of the recovery phase of the test, ovarian E2 production by fish from the 300-µg/l treatment was substantially higher than in controls (Fig. 3c). During the remainder of the recovery phase of the test, *ex vivo* E2 production did not vary significantly among the treatment groups, although there seemed to be greater production of E2 by prochloraz-exposed females than controls, particularly 2 days after exposure (Fig. 3c).

Gene Expression Results

The gene products measured in males, *ar*, *cyp17*, and *cyp11a*, displayed a relatively similar expression pattern in response to the prochloraz exposure (Figs. 4a–c). All three testicular transcripts appeared elevated to varying degrees during later portions of the chemical exposure and day 1 of the recovery phase of the test and returned to levels comparable to controls by the end of the assay. The *ar* and *cyp17* transcripts

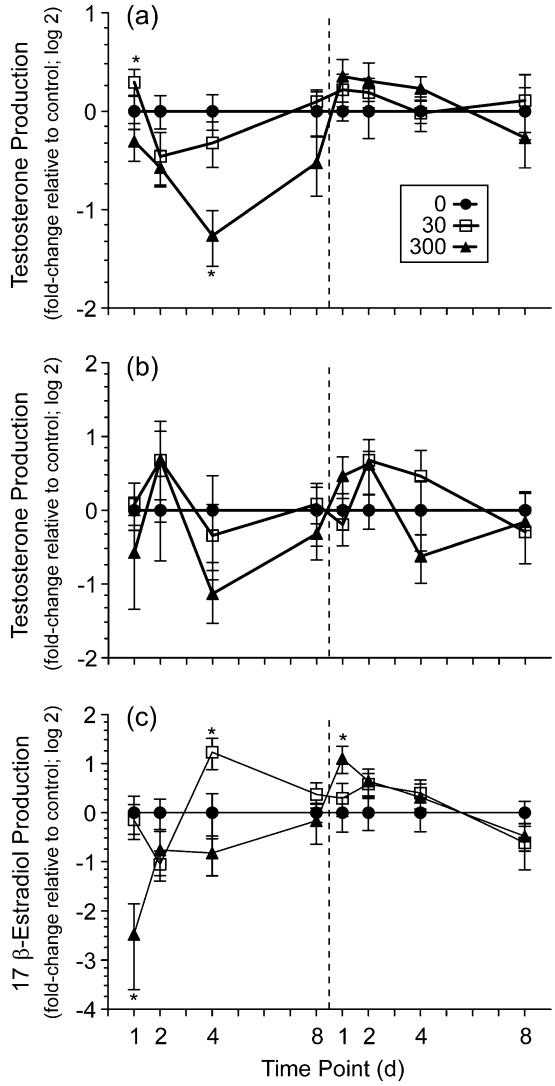

FIG. 3. Effects in fathead minnows of 8 days of exposure to prochloraz, followed by 8 days of recovery, on *ex vivo* production of (a) T in male testis, (b) T in female ovary, and (c) E2 in female ovary. Data points indicate the mean (SE) fold change relative to controls and are expressed as log 2–transformed units. Asterisks indicate a significant treatment-related effect compared to controls for any given test day.

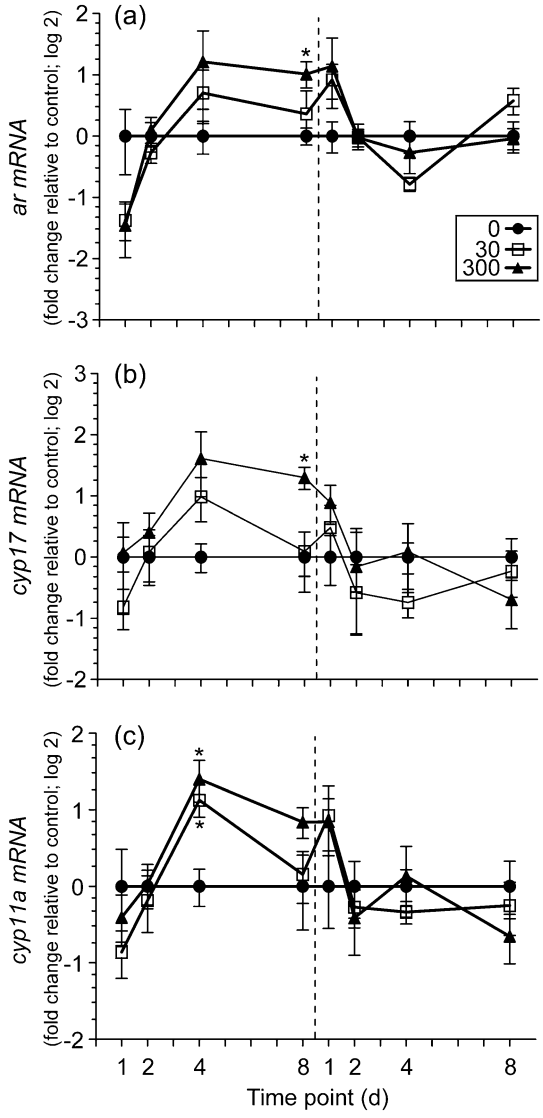

FIG. 4. Effects in male fathead minnows of 8 days of exposure to prochloraz, followed by 8 days of recovery, on testicular expression of transcripts of (a) *ar*, (b) *cyp17*, and (c) *cyp11a*. Data points indicate the mean (SE) fold change relative to controls and are expressed as log 2–transformed units. Asterisks indicate a significant treatment-related effect compared to controls for any given test day.

were significantly elevated in the 300-µg/l treatment group on day 8 of the exposure (Figs. 4a and 4b), and *cyp17* was significantly increased in this treatment group compared to controls when data from the exposure phase of the test were pooled for analysis. Both 30 and 300 µg/l prochloraz significantly increased *cyp11a* messenger RNA on day 4 of the exposure (Fig. 4c).

Prochloraz also affected gene expression in the female gonad. The largest fold change in expression was for *cyp19a1a*, which was consistently elevated in the 300-µg/l treatment group during the exposure phase of the test (Fig. 5a). Similar to the pattern seen in males, *cyp17* was significantly elevated in the 300-µg/l treatment group on day 4 of the exposure and significantly increased in this treatment group compared to controls when pooled data from the exposure phase of the test were analyzed (Fig. 5b). Ovarian *cyp11a* and *fshr* transcripts both were significantly increased in the 300-µg/l treatment 1 day into the recovery phase of the test and also were higher during the exposure phase of the assay when data pooled across time were analyzed (Figs. 5c and 5d). Although actual statistical results were not identical, qualitative expression profiles of *gnrhr1* and *star* were similar to those of *cyp11a* and *fshr* (Supplementary Figs. S1 and S2). Several other gene products measured in the ovary either were not significantly affected or exhibited no consistent pattern of expression

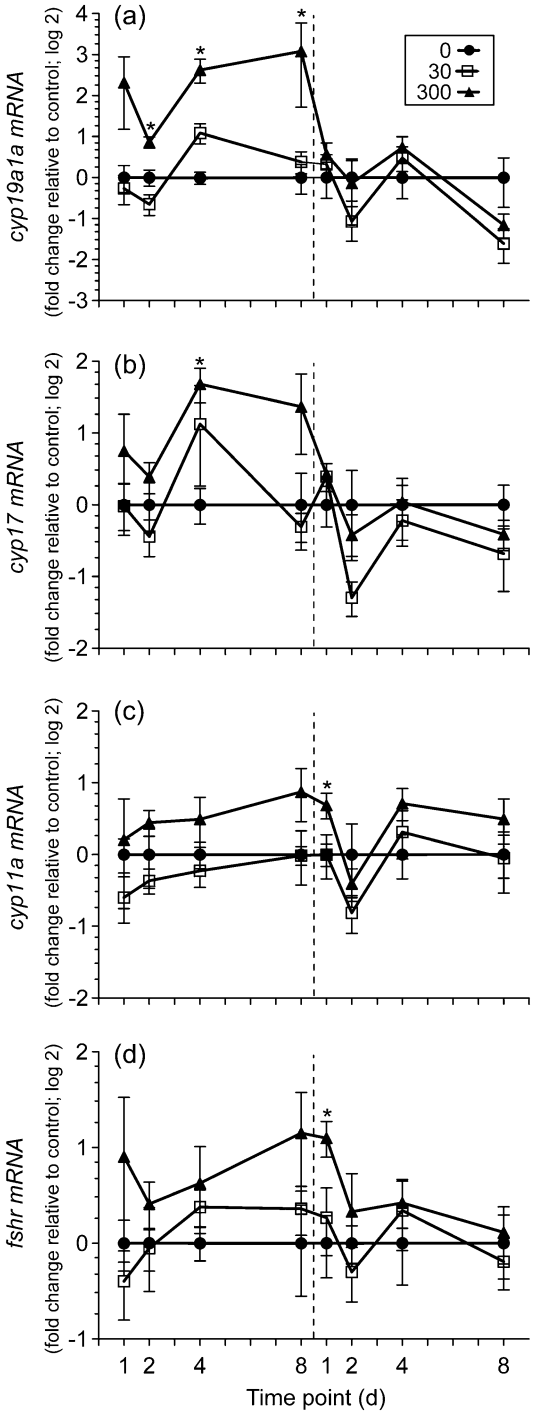

FIG. 5. Effects in female fathead minnows of 8 days of exposure to prochloraz, followed by 8 days of recovery, on ovarian expression of transcripts of (a) *cyp19a1a*, (b) *cyp17*, (c) *cyp11a*, and (d) *fshr*. Data points indicate the mean (SE) fold change relative to controls and are expressed as log 2–transformed units. Asterisks indicate a significant treatment-related effect compared to controls for any given test day.

relative to the exposure versus recovery phases of the test. These included *hsd17b*, *vtgr*, *gnrhr3*, and *esr1* (Supplementary Figs. S3–S6).

There were no marked changes caused by prochloraz in the expression of four other potentially sensitive gene products measured in the female pituitary (*lhb* and *fshb*) or brain (*cyp19a1b* and *cyp51*) (data not shown).

Principal Components Analysis

PCA was used to help assess correlations among the different end points measured in females over time (Figs. 6a–c). A loading plot revealed that expression of several ovarian transcripts, especially *cyp11a*, *cyp17*, *cyp19a1a*, *star*, *fshr*, and *gnrhr1*, strongly influenced variation in the positive direction along factor 1, which explained 37.6% of the data variability (Fig. 6a). The expression of these genes was directly negatively correlated with plasma VTG concentrations in the females. Along the second factor (which explained 13.4% of the data variability), plasma E2 concentrations and *ex vivo* E2 production were closely associated with one another in a positive direction (Fig. 6a). Scores plots from the PCA revealed an interesting pattern in the data trajectories between the treatment groups relative to the controls. Specifically, the trajectory of changes in the lower (30 μg/l) treatment during both the exposure and the recovery phases of the experiment seemed to fluctuate in a cyclical manner in and around the response area defined by the control (Fig. 6b). Conversely, in the 300-μg/l prochloraz treatment, the trajectory of changes during the exposure did not overlap with controls, with data for animals from this group not returning to the control region until mid to later portions of the recovery phase of the test (Fig. 6c).

DISCUSSION

Inhibition of steroidogenic CYPs has been associated with many of the endocrine-disrupting properties of prochloraz in vertebrates (Ankley *et al*., 2005; Blystone *et al*., 2007). Results of the present study support this association. Exposure to prochloraz caused a profound and relatively rapid (within 1 day) depression of E2 in female fish, as indicated both by *ex vivo* production and by plasma concentrations of the steroid. Coincident with this, treatment with the fungicide also decreased plasma concentrations of the estrogen-inducible protein VTG in the females. Because *ex vivo* T production was not similarly affected, this suggests that in females ovarian aromatase (CYP19A) activity is a more sensitive target of prochloraz than other steroidogenic CYPs. The male fathead minnow data confirm that prochloraz can affect steroidogenic enzymes other than aromatase. Specifically, the fungicide depressed *ex vivo* T production and plasma T concentrations in the males. The enzymatic target for the inhibitory effect of prochloraz on T is less certain; however, there is evidence that lyase (CYP17) is involved (Blystone *et al*., 2007).

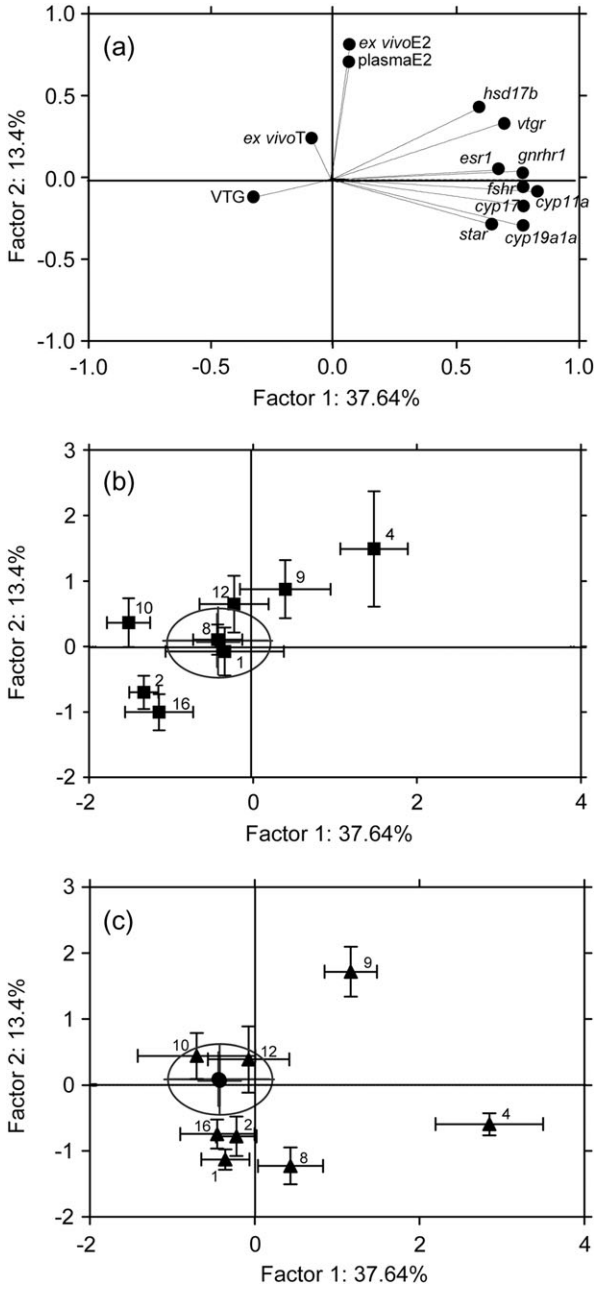

FIG. 6. PCA of alterations in the HPG axis of female fathead minnows exposed for 8 days to prochloraz, followed by 8 days of recovery. Panel (a) is a loading plot for the following data: plasma VTG and E2 concentrations; *ex vivo* (ovarian) production of T and E2; and ovarian transcripts of *cyp11a*, *cyp17*, *cyp19a1a*, *hsd17b*, *star*, *esr1*, *vtgr*, *gnrhr1*, and *fshr*. Panels (b) and (c) are scores trajectory plots for the data for, respectively, the 30- and 300-μg/l treatment groups plotted as a function of sampling day. Days 1, 2, 4, and 8 occur during the exposure portion of the test, and days 9, 10, 12, and 16 correspond to the recovery phase of the assay. The circles in the two panels indicate the control response area.

Effects of prochloraz on steroid and VTG status in female fathead minnows were both concentration and time dependent, with evidence of successful compensation of HPG axis function during exposure to the fungicide, particularly in animals from the 30-μg/l treatment group. Plasma E2 concentrations in females from this group were significantly depressed within 1 day of prochloraz treatment but had returned to control levels by the end of the 8-day exposure phase. Concentrations of plasma VTG in 30-μg/l treatment were never significantly decreased below control values. In the 300-μg/l treatment, plasma E2 concentrations also were decreased significantly on day 1 and increased slightly during the prochloraz exposure but did not return to control levels until the recovery phase of the test. Concentrations of plasma VTG in the females reflected those of E2, only with about a 1-day lag period. Specifically, VTG concentrations in the 300-μg/l group were not significantly decreased until day 2 of the exposure and did not recover to control levels until day 2 of the recovery portion of the test.

Based on studies in our laboratory and elsewhere with chemicals like prochloraz that depress vitellogenesis, it appears that the ability of fathead minnows to maintain egg production can be accurately reflected by plasma VTG concentrations in females (Miller *et al.*, 2007; Thorpe *et al.*, 2007). This relationship enables use of VTG status of fish from the present study as an indirect indicator of reproductive fitness (fecundity). Ankley *et al.* (2005) found that exposure of fathead minnows to 300 μg/l prochloraz in a 21-day test reduced plasma E2 and VTG concentrations in females, while a 30-μg/l exposure did not significantly affect these end points. Those observations are consistent with the present shorter term study. Ankley *et al.* (2005) also found that 300 μg/l of prochloraz significantly decreased egg production in the fish, but 30 μg/l did not. Hence, although in the present study 30 μg/l prochloraz caused at least transitory effects on plasma E2 concentrations, compensatory responses of the fish appear adequate to maintain normal VTG concentrations and successful reproduction, even in a longer term (21 days) continuous exposure to the pesticide.

The mechanistic nature of this compensatory response seems to involve upregulation of genes indirectly or directly involved in steroid synthesis in ovaries of treated fish. For example, prochloraz caused an increase in transcripts for FSHR, a receptor involved in feedback regulation of steroidogenesis in fish (Villeneuve *et al.*, 2007b). In addition, ovarian transcripts coding for three enzymes directly responsible for steroid synthesis—CYP11A (which catalyzes the first step in the conversion of cholesterol to steroids), CYP17, and CYP19A—were upregulated by prochloraz. The most upregulated gene (in terms of fold change of expression) in females from the 300-μg/l treatment codes for CYP19A, a putative target of the inhibitory effects of prochloraz on E2 production. The observed increase in *ex vivo* E2 production is consistent with the upregulation of key steroidogenic enzymes in the ovary. Notably, little was seen in terms of differential gene expression (or enhanced *ex vivo* steroid production) in the ovary after about day 1 of the recovery phase of the study.

While it is tempting to speculate that upregulation of one or more genes involved in E2 synthesis was solely responsible for observed compensation in the female fathead minnows, our analysis does not rule out the possibility that other adaptive strategies (e.g., decreased clearance of E2) contributed to the response.

Analogous to effects on E2 in females, prochloraz depressed *ex vivo* production and plasma concentrations of T in males, which is consistent with our previous work with the fungicide (Ankley *et al*., 2005). Prochloraz also affected expression of gene products in male fish in a manner suggestive of a compensatory response to decreased steroid production. For example, transcripts of *cyp11a* and *cyp17* were upregulated in testis. Testicular expression of *ar* also was upregulated by prochloraz. This could be in response to decreased T in the males (Villeneuve *et al*., 2007b), or it might be indicative of adaptation to a different mechanism of action of prochloraz. Specifically, in addition to inhibiting CYPs, it has been proposed that prochloraz can affect the vertebrate endocrine system through direct antagonism of the AR (Vinggaard *et al*., 2002, 2005); as such, upregulation of the gene coding for the receptor could be viewed as adaptive.

Zhang *et al*. (2008) used QPCR to examine expression of a number of the same genes we evaluated in various tissues from male and female Japanese medaka exposed to prochloraz. It is difficult to make direct comparisons between their study and ours not only due to species differences but also because Zhang *et al*. (2008) determined gene expression at only one time point (7 days) and did not measure concentrations of prochloraz in the exposure water. Nonetheless, there are several noteworthy similarities between the two studies. For example, Zhang *et al*. (2008) reported a significant downregulation of *vtg* expression in liver of female medaka treated with prochloraz, a finding that mirrors the decreased plasma concentrations of VTG protein in female fathead minnows exposed to the fungicide. Similar to our study, Zhang *et al*. (2008) observed upregulation of *cyp19a1a* and *cyp17* in ovaries of prochloraz-treated medaka. They also reported elevated transcription of *cyp11a* and *cyp17* in testis from the medaka, which was similar to what we observed in male fathead minnows exposed to prochloraz. There were, however, some differences between the two studies as well. For example, Zhang *et al*. (2008) reported that the *cyp19a1b* in brain of female medaka was upregulated by prochloraz, but we saw no evidence of this in female fathead minnows.

Several of the responses observed in the present study were similar to those described by Villeneuve *et al*. (2009), who used the same basic experimental design to assess effects of fadrozole, a relatively specific inhibitor of aromatase, on the fathead minnow HPG axis. Like prochloraz, fadrozole (tested at 3 and 30 μg/l) caused a rapid (within 1 day) decrease in *ex vivo* production and plasma concentrations of E2 in females. Similarly, there was a compensatory response to fadrozole in terms of E2 production over the course of the exposure phase of the test such that by day 8 plasma concentrations of E2 in the 3-μg/l and control groups were similar (Villeneuve *et al*., 2009). There were also some notable differences between responses of female fathead minnows to fadrozole and prochloraz. For example, VTG concentrations in fadrozole-treated animals were significantly depressed in both the low and high treatment groups and never returned to control levels, even after an 8-day recovery phase. This could quite likely be due to differences in intrinsic potency of the two chemicals. Specifically, based on 21-day fathead minnow reproduction tests, the no-observable effect concentrations (NOECs) for reduced egg production for fadrozole and prochloraz are < 2 and 30 μg/l, respectively (Ankley *et al*., 2002, 2005). Hence, while the low test concentration for prochloraz in the present study (30 μg/l) is a NOEC (based on the 21-day test), the 3-μg/l fadrozole employed by Villeneuve *et al*. (2009) is not. So, even though a seemingly similar type of adaptive response occurred for both chemicals, the extent to which compensation "succeeded" (as judged by normal VTG production) appears to have been dependent on the degree of chemical potency.

Consistent with the present study, Villeneuve *et al*. (2009) found that compensatory responses of female fathead minnows to fadrozole-induced depressions in E2 production were associated with upregulation of ovarian genes involved in steroid synthesis. In general, gene expression changes caused by fadrozole were more pronounced (larger magnitude change, longer lasting, and occurring at both low and high test chemical concentrations) than for prochloraz. However, several of the transcripts measured in common between the two studies were altered in a similar manner. For example, the most consistent and largest relative changes in transcript abundance were for *cyp19a1a* in both studies. Similarly, both *fshr* and *cyp11a* were upregulated in the ovaries of female fathead minnows exposed to prochloraz (present study) and fadrozole (Villeneuve *et al*., 2009). In terms of identifying biological indicators diagnostic of inhibition of steroidogenesis, the correspondence between the present study and results of Villeneuve *et al*. (2009) is encouraging.

As opposed to females, general responses of male fathead minnows to prochloraz versus fadrozole differed markedly, likely reflecting mechanisms of action of the fungicide in addition to inhibition of aromatase. For example, unlike prochloraz, fadrozole did not depress *ex vivo* production or plasma concentrations of T in the males (Villeneuve *et al*., 2009). This is consistent with inhibition by prochloraz of biosynthetic enzymes "upstream" of T production such as, perhaps, CYP17. Unfortunately, Villeneuve *et al*. (2009) did not evaluate testicular gene expression changes, so the effects of fadrozole versus prochloraz on transcript profiles in males cannot be made.

Data from the present study highlight the dynamic nature of the HPG axis, both in terms of compensation to a chemical stressor and recovery after termination of an exposure. A couple of points are especially notable in this regard. First, recovery of components of the system after cessation of prochloraz exposure

was quite rapid. For example, plasma VTG concentrations in females from the high (300 μg/l) prochloraz treatment returned to normal within 2 days of the recovery phase, after being depressed to as low as 10% of control values during the chemical exposure. Recovery of the overall system is further illustrated by the PCA scores plots for the high treatment group (Fig. 6c), which indicates return to a "normal" set of parameter values, again within 2 days of termination of chemical exposure. Observed rapid recovery of HPG axis function after the perturbation by prochloraz has practical implications for assessing the risk of possible fluctuating or transitory exposures to endocrine-active chemicals in the environment.

A second noteworthy characteristic relative to response of the HPG axis to chemical stress involves what appears to be a cyclical type of response to chemical perturbation characterized, perhaps, by some period of overcompensation prior to returning to baseline (control) levels. For example, *ex vivo* production and plasma concentrations of E2 in females from the 30-μg/l treatment group were initially depressed during the prochloraz exposure, but, within a couple of days, values for both parameters exceeded controls and, finally, returned to baseline levels by the end of the exposure phase of the test. Villeneuve *et al.* (2009) described a similar pattern of *ex vivo* production and plasma concentrations of E2 in female fathead minnows following cessation of exposure to fadrozole. The cyclical nature of response of the overall system to prochloraz also is illustrated in Figure 6b, which describes the trajectory of changes observed in the low (30 μg/l)-treatment group relative to controls, where the PCA scores representing 13 endocrine-related variables fluctuated in and out of the region of variability of the control population during and after the chemical exposure. Cyclical or oscillatory behavior of this type is entirely consistent with a system, such as the HPG axis, that maintains homeostasis through feedback mechanisms (Goldbeter, 2002; Hasty *et al.*, 2001). However, characterizing this type of behavior, either during or after a chemical exposure, is important to understanding impacts on HPG function, particularly in situations where testing includes molecular/biochemical responses collected during limited time points. These types of responses, although critical to understanding mechanism of action, need to be evaluated and interpreted in a broader systems context to support reliable predictions of the risk of endocrine-active chemicals (Ankley *et al.*, 2009).

SUPPLEMENTARY DATA

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

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