

Effects of Prochloraz or Propylthiouracil on the Cross-Talk between the HPG, HPA, and HPT Axes in Zebrafish

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The objective of this study was to assess chemical-induced effects on cross-talk among the hypothalamic–pituitary–gonad (HPG), hypothalamic–pituitary–adrenal (HPA), and hypothalamic–pituitary–thyroid (HPT) axes of fish. Adult female zebrafish were exposed to 300 $\mu\text{g/L}$ prochloraz (PCZ) or 100 mg/L propylthiouracil (PTU), and the transcriptional profiles of the HPG, HPA, and HPT axes were examined. Exposure to PCZ decreased plasma testosterone (T) and 17β -estradiol (E2) concentrations and affected HPA and HPT axes by down-regulating corticotrophin-releasing hormone (CRH) after 12 and 48 h. By using correlation analyses, it was found that the decrease in E2 plasma concentrations caused by PCZ was correlated with the down-regulation of CRH mRNA expression. Exposure to PTU resulted in lesser concentrations of thyroxine (T4) and triiodothyronine (T3), greater concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) peptides, and increase in steroidogenic gene expression after 12 and 48 h. Concentrations of FSH and LH were negatively correlated with concentrations of T4 and T3. These results are consistent with the hypothesis that increased steroidogenic gene expression after PTU exposure resulted from a reduction in T4 and T3 concentrations, which resulted in greater secretion of FSH and LH.

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Introduction

A challenge in studying effects of chemicals on the endocrine system is to characterize and predict interactions among the major endocrine systems. In vertebrates, the reproductive, thyroid, and adrenal endocrine systems are controlled primarily by the hypothalamic–pituitary–gonad (HPG), hypothalamic–pituitary–thyroid (HPT), and hypothalamic–pituitary–adrenal (HPA) axes, respectively. These systems are responsible for regulating hormone dynamics by coordinating their synthesis, secretion, transport, and metabolism (1–3). Each endocrine axis interacts with the other endocrine axes to integrate bodily functions (1, 4, 5). For example, corticosteroids produced by tadpoles during periods of environmental stress are thought to synergize with thyroid hormone to accelerate metamorphosis (6). Thyroid hormones have been associated with the modulation of the expression of some genes along HPG axis in teleosts (4). Furthermore, exposure to the sex steroid E2 resulted in an increase in plasma concentrations of cortisol in juvenile Atlantic salmon (7). Therefore, chemical-induced changes along one endocrine axis are likely to lead to changes in the other endocrine axes.

Some chemicals that occur in the environment can simultaneously cause a variety of toxic effects along the thyroid, reproductive, and adrenal endocrine systems of vertebrates. For example, concentrations of thyroxine (T4) and triiodothyronine (T3) in the plasma of rats exposed to polychlorinated biphenyl (PCB) 126 decreased, while concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), and 17β -estradiol (E2) increased (8, 9). Exposure to perfluorooctanoic acid (PFOA) resulted in lesser concentrations of T3 and T4 and greater concentrations of testosterone (T), E2, and corticosterone in the plasma of mice and rats (10, 11). Fadrozole, an aromatase inhibitor, resulted in lesser concentrations of E2 in the plasma and production of eggs, and modulated thyroid hormone-related gene expression in fathead minnow (*Pimephales promelas*), Medaka (*Oryzias latipes*), and Western clawed frog (*Silurana tropicalis*) (12–14). Identification of interactions among these endocrine axes is useful in systematically predicting adverse effects caused by chemicals (15). However, the molecular mechanisms underlying chemical-induced effects on cross-talk among endocrine axes are largely unknown. Without such understanding it will be difficult to predict effects of different classes of compounds on aquatic animals, especially when they occur in mixtures.

The objective of the present study was to investigate the effects of exposure to two well-described endocrine-disrupting chemicals (EDCs), prochloraz (PCZ), and propylthiouracil (PTU), on the cascades of events among the different axes in zebrafish. The zebrafish has been suggested as an appropriate model to study effects of chemicals (16). PCZ is an imidazole fungicide which was designed to inhibit CYP51-mediated ergosterol biosynthesis. PCZ inhibits activities of the enzymes cytochrome P450 c17 α hydroxylase, 17,20-lyase (CYP17), and aromatase (CYP19) in mammals and fish (17, 18). Treatment with PCZ resulted in lesser concentrations of T and E2 both in vitro and in vivo (19, 20). PTU was designed to inhibit synthesis of T3 and T4 and to block conversion of T4 to T3 (21). Exposure to PTU results in significantly lesser concentrations of T3 and T4 in the plasma of fish and rats (22, 23). In this study, time-dependent transcriptional profiles of the HPG, HPT, and HPA axes and hormones were examined in zebrafish exposed to PTU or PCZ.

Materials and Methods

Fish and Chemical Exposure. Adult female zebrafish (20-week old ovulating) were maintained and exposed using a previously described protocol (24). Prochloraz (PCZ) and propylthiouracil (PTU) were purchased from Sigma (St. Louis, MO). Stock solutions of chemicals were prepared in dimethyl sulfoxide (DMSO). Briefly, fish were exposed to 300 μg PCZ/L or 100 mg PTU/L or vehicle control (0.001% DMSO) at concentrations based on previous studies (20, 23). Fish were randomly sampled after 6, 12, or 48 h exposure and euthanized in 0.03% MS-222, and blood was collected for hormone analysis. Tissues from brain (including hypothalamus and pituitary), gonad, thyroid, and adrenal (anterior kidney) were collected and preserved in Trizol reagent for subsequent RNA isolation.

In Vitro Brain Explant Exposures. In vitro whole brain culture (including hypothalamus and pituitary) were performed using previously described methods (25), and the exposure was conducted in 12-well microplates (Falcon, Franklin Lakes, NJ). PCZ was dissolved in DMSO and diluted with DMEM/F12 medium (Sigma), and 500 μL of culture medium containing 300 μg /L PCZ was added to each well. Freshly excised intact brain tissue from female zebrafish was added, and samples of tissue were collected after 0, 3, 6, 12, and 48 h for determination of gene expression. Both control and treatment groups received 0.01% DMSO, and six samples were used in each treatment group.

Hormone Measurement. One replicate of 5 to 15 μL of plasma from each fish was used to determine hormone concentration. Concentrations of T, E2, T3, T4, cortisol, FSH, and LH were determined by use enzyme-linked immunosorbent assays (ELISA). The details can be found in Supporting Information. Concentrations of all hormones were expressed as the fold-change relative to the mean control response.

Quantitative Real-Time PCR Assay. Isolation, purification, and quantification of total RNA, first-strand cDNA synthesis, and quantitative real-time PCR were performed using previously described protocols (24, 26). The 18S rRNA was used as an internal control. Sequences of primers were designed with the Primer 3 software (<http://frodo.wi.mit.edu/>) (See Table S1, Supporting Information).

Statistical Analysis. Data was checked for homogeneity of variances by use of Levene's test. Differences were evaluated by ANOVA followed by Tukey's multiple range tests. Spearman correlation analysis with Bonferroni correction was used to examine the relationship between concentrations of E2 and T and CRF expression after PCZ exposure, or T3, T4 concentrations and FSH, LH concentrations after PTU exposure. Significant differences between treatments and corresponding control were identified by p -value < 0.05.

Results

Hormone Concentrations in Plasma. There were statistical significant differences among the different treatment groups and the controls for most of the hormone end points measured. Exposure to PCZ for 12 or 48 h resulted in significantly lesser plasma T (−1.97- and −4.15-fold) and E2 (−2.72- and −3.18-fold) concentrations, while no significant effects were observed after 6 h of exposure (Figure 1A). Treatment with PCZ for 48 h resulted in significantly less cortisol (−2.65-fold), but no significant effects were observed after other durations of exposure (6 or 12 h). Concentrations of T4 were significantly decreased after 48 h (−1.09-fold), while concentrations of T3 were unchanged after all durations of PCZ exposure.

PTU caused significantly lesser concentrations of T4 and T3 by −2.33- and −2.82-, and −1.37- and −1.49-fold at 12 and 48 h, respectively (Figure 1B). Significantly greater concentrations of FSH (1.19- and 1.50-fold) and of LH (1.33-

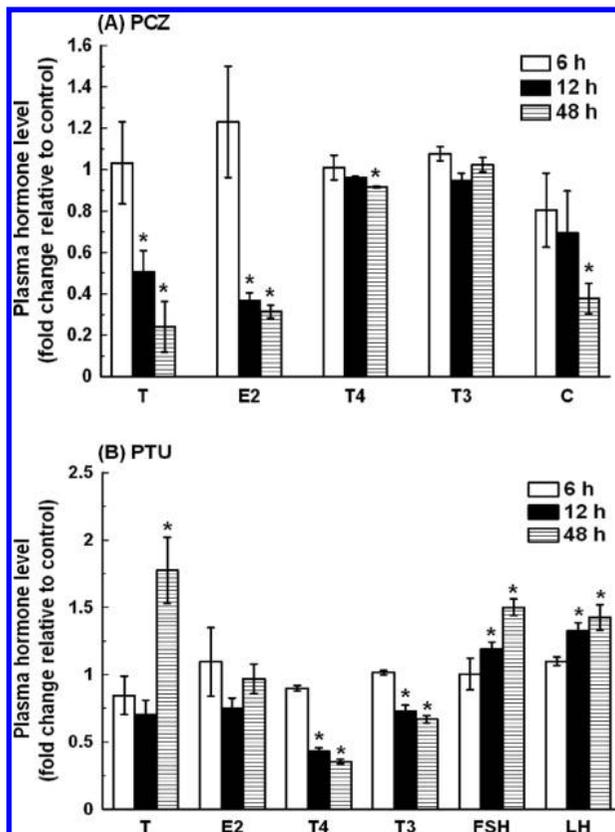


FIGURE 1. Time-dependent effects of (A) prochloraz (PCZ) and (B) propylthiouracil (PTU) on concentrations of hormones in plasma. Values represent mean \pm SE of six replicate fish. Significant differences between treatment group and the corresponding control group are indicated by * P < 0.05. T: testosterone; E2: 17 β -estradiol; C: cortisol; T4: thyroxine; T3: triiodothyronine; FSH: follicle stimulating hormone; LH: luteinizing hormone.

and 1.43-fold) were observed after PTU exposure for 12 or 48 h. Treatment with PTU for 48 h resulted in significantly greater concentration of T (1.78-fold), while concentrations of T after 6 or 12 h exposure were not significantly different. Concentrations of E2 were unchanged after exposure to PTU for 6, 12, or 48 h (Figure 1B).

Transcriptional Response to Chemicals. Changes in transcriptional profiles in animals exposed to PCZ were time-dependent (Figure 2; see Table S2, Supporting Information). Exposure for 6 h caused no significant change in expression of any HPG axis genes. However, cytochrome P450 19B (CYP19B) in brain was down-regulated, and gonadotropin-releasing hormone receptor 1 (GnRHR 1), GnRHR 2 and GnRHR 4 in brain, and luteinizing hormone receptor (LHR) and cytochrome P450 side-chain cleavage (CYP11A) in ovaries were up-regulated after 12 h of exposure. After 48 h, expression of GnRHR 1, GnRHR 2 and GnRHR 4 in brain, and follicle-stimulating hormone receptor (FSHR), LHR, hydroxymethylglutar-yl CoA reductase B (HMGRB), steroidogenic acute regulatory protein (StAR), 3 β -hydroxysteroid dehydrogenase (3 β HSD), cytochrome P450 17 (CYP17), 17 β -hydroxysteroid dehydrogenase (17 β HSD) and cytochrome P450 19A (CYP19A) in ovaries were up-regulated. Expression of CYP19B in brain was down-regulated. In the HPA axis, no significant alteration in expression of any of the genes investigated were observed after PCZ exposure for 6 h. However, corticotrophin-releasing hormone (CRH), CRH binding protein (CRHBP) and CRH receptor 2 (CRHR2) in brain were significantly down-regulated after 12 h of exposure. After 48 h, PCZ caused significant down-regulation in expression of CRH and proopiomelanocortin (POMC) in brain, and cytochrome P450 11B (CYP11B) in the adrenals.

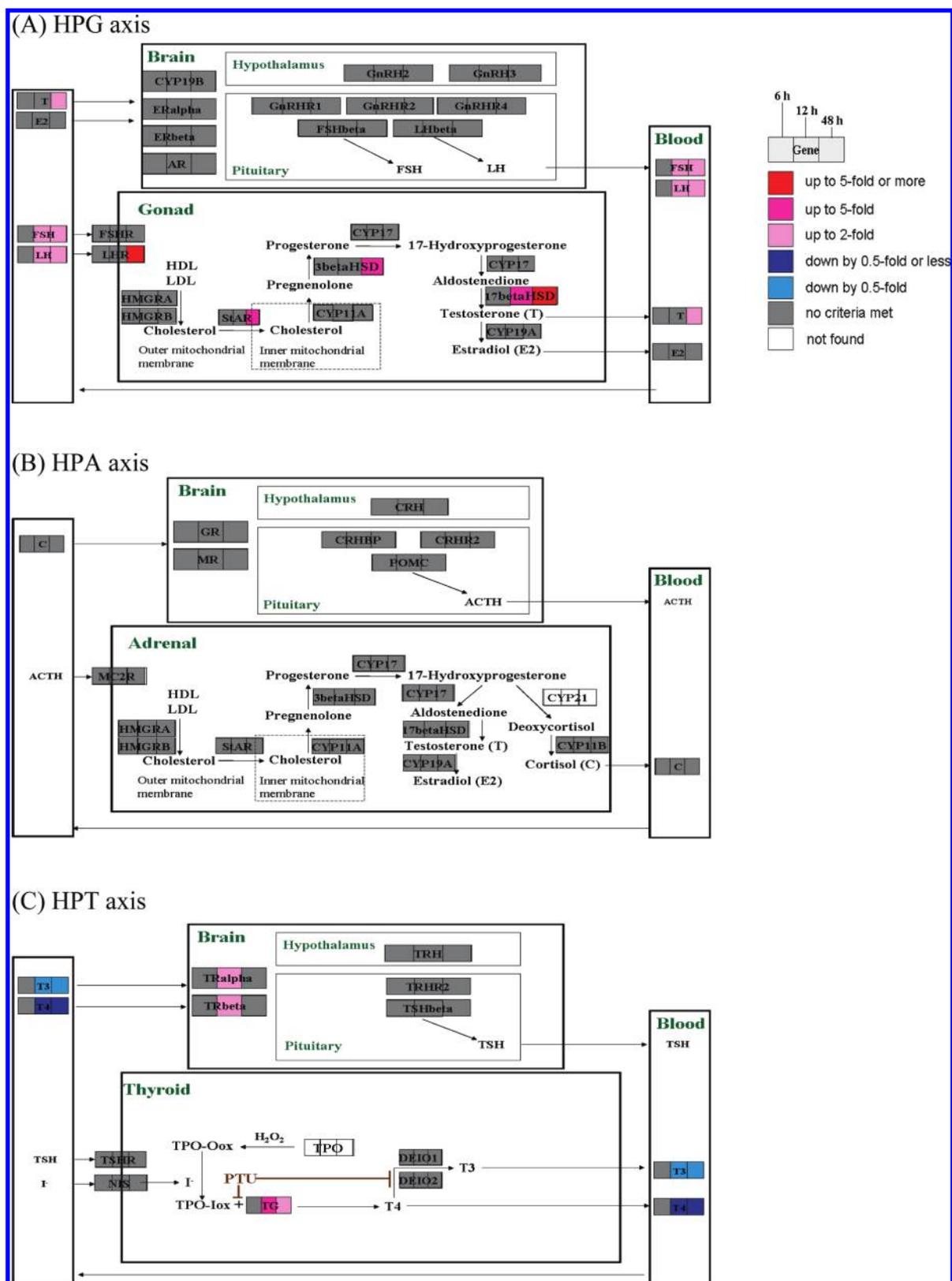


FIGURE 3. Time-dependent effects and proposed mode of action by propylthiouracil (PTU) on (A) hypothalamic–pituitary–gonad (HPG), (B) hypothalamic–pituitary–adrenal (HPA), and (C) hypothalamic–pituitary–thyroid (HPT) axes of female zebrafish. Gene acronyms are defined in Table S4, Supporting Information.

brain were observed. After 48 h, PTU exposure up-regulated TG expression in thyroid, while the other genes of the HPT axis were not affected. After 6 h, no significant alterations in gene expression were observed in HPG genes. After 12 h, only 17 β HSD was significantly up-regulated, while other genes were not changed. After 48 h, PTU caused significant

up-regulations in expression of LHR, StAR, 3 β HSD, and 17 β HSD in ovaries. No significant alterations in expression of any of HPA axis genes were observed at any of the durations (6, 12, or 48 h) of exposure to PTU.

Correlation Analysis. After exposure to PCZ, expression of CRH in brain was positively correlated with concentrations

TABLE 1. Spearman Rank Correlation Coefficients (number) and Probabilities (*) between Parameters in Female Zebrafish (n = 36)^{a,b}

	T	E2	T4	T3
CRH	0.40	0.62*		
FSH			-0.54*	-0.51*
LH			-0.69*	-0.60*

^a Gene (CRH) and hormone (T4, T3, FSH, and LH) were expressed as the fold change relative to the corresponding controls. ^b Analysis was conducted separately with 36 female fish. **P* < 0.05 indicate significant correlation between parameters.

of E2 in the plasma, and concentrations of FSH and LH in the plasma were negatively correlated with concentrations of T4 and T3 in the plasma after PTU exposure (Table 1).

Discussion

PCZ Exposure. PCZ is a fungicide that inhibits CYP17 and CYP19 activities. Previously, PCZ has been shown to decrease concentrations of T and E2 in blood plasma and up-regulate expression of several genes associated with steroidogenesis in both mammals and fish (17, 18, 20). Our results are consistent with those findings. A previous in vitro study demonstrated that PCZ could slightly down-regulate several steroidogenic genes, (including StAR, CYP11A1, CYP17A1, 3 β HSD2, and CYP21) in H295R cells (27). Therefore, it is unlikely that up-regulations of steroidogenic genes in the present study are a direct effect of PCZ. The opposite trends between effects on plasma hormone concentrations (inhibition) and gene-expression profiles along the HPG axis (predominantly induction) were indicative of compensatory mechanisms. Furthermore, since PCZ down-regulated brain CYP19B expression and expression of CYP19B is regulated by E2 through binding to the estrogen responsive element (ERE) on its promoter in vitro and in vivo (28) and the concentration of E2 was significantly decreased, the lesser concentration of E2 in fish exposed to PCZ could be a primary reason for the observed down-regulation of CYP19B.

Interaction between the HPA and HPG axes were observed in PCZ exposed zebrafish in the present study. Promoter analysis by the AliBaba 2.1 program (<http://www.generegulation.com/>) demonstrated that several putative transcription regulation elements, including two ER and several activation protein 1 (AP-1), octamer binding factor 1 (Oct-1), and glucocorticoid receptor (GR) regulation elements, were found in the proximal promoter of zebrafish CRH. Similar results have been reported for human, chimp, sheep, mouse, rat, and African clawed frog (*Xenopus laevis*) (29). The fact that PCZ significantly down-regulated expression of CRH after 12 and 48 h, down-regulated POMC and CYP11B expression, and reduced the concentration of cortisol at 48 h but had no significant effect on concentrations of cortisol after 6 or 12 h exposure is consistent with down-regulation of CRH and not associated with plasma cortisol concentration. In addition, the results of the in vitro brain explant assay demonstrated that PCZ did not alter CRH expression, which is consistent with down-regulation of CRH expression in vivo being an indirect effect. Since expression of CRH was significantly correlated with concentrations of E2, it was hypothesized that down-regulation of CRH was resulted from reduction of the concentration of E2 through an ER-mediated mechanism. Under baseline physiological conditions in mammalian systems, functional interaction between the HPA and HPG axes through sex steroids and glucocorticoids has been demonstrated. Stress-induced activation of the HPA axis can disrupt secretion of LH into plasma, as well as sex steroid synthesis and release (30). In fact, the sex hormone

E2 can act and interact on/with both basal and stress-induced HPA function, influencing adrenocorticoid synthesis, stress-induced adrenocorticotrophic hormone (ACTH) and glucocorticoid release, and CRH synthesis by the hypothalamus (31). CRH is a primary neurohormone that activates the HPA axis and has been suggested to be one of the primary targets for E2, in which ER binds to the ERE on the CRH promoter in human (31, 32). Taken together, these results suggested that PCZ could down-regulate expression of some HPA axis genes and decrease concentrations of cortisol by reducing the concentration of E2.

Besides modulating the HPA axis, it has also been previously demonstrated that CRH acted as a TSH-stimulating factor in nonmammalian vertebrates (33). In fish, CRH is a more potent factor than thyrotropin-releasing hormone (TRH) for stimulating TSH release (33). Down-regulation of CRH could result in down-regulation of TSH in PCZ-exposed female zebrafish. Significant down-regulation of TSH expression was observed after 48 h of PCZ exposure, while expression of other HPT axis genes remained unchanged. TSH is responsible for regulating synthesis and release of T4 and T3 in vertebrates (3). Therefore, the lesser concentration of TSH would result in lesser thyroid hormone concentrations. In this study, PCZ resulted in significantly less T4, while the concentration of T3 remained unchanged. Previously, it has also been demonstrated in rats that exposure to 50 or 150 mg/kg PCZ from gestational day 7 to postnatal day 16 caused a decrease in concentration of T4, while T3 did not change (34). These results suggest that PCZ down-regulates CRH and TSH and results in lesser concentrations of T4 by reducing concentrations of E2. The indirect effects of PCZ on thyroid hormone biosynthesis could be sustained for prolonged periods.

PTU Exposure. PTU can inhibit synthesis of T3 and T4 and block conversion of T4 to T3. It has previously been reported that PTU decreased concentrations of T4 and T3 in the plasma of zebrafish (23). Thus, this observation is consistent with the results from the present study. Up-regulation of expression of some HPT axis genes caused by PTU is hypothesized to be a compensatory mechanism for lesser concentrations of T4 and T3 in zebrafish.

Effects of PTU on interactions between thyroid hormones and genes of the HPG axis in female zebrafish were primarily through the release of FSH and LH, which is supported by physiological study (35). The significantly greater concentrations of T, FSH, and LH, expression of steroidogenic genes (StAR, 3 β HSD, and 17 β HSD) along the HPG axis were observed after PTU exposure, while the expression of genes along the HPG axis in brain did not change. It is unlikely that induction in steroidogenesis is a direct effect since an in vitro study demonstrated that PTU inhibited steroidogenesis (including decreased CYP11A and StAR expression and T production) in rat leydig cells (36). Therefore, it is hypothesized that exposure to PTU resulted in increases in FSH and LH secretion, which up-regulated steroidogenic gene expression that in turn resulted in greater concentrations of T. Previously an increase in egg production by zebrafish was observed when fish were exposed to PTU for 21 d (23). FSH and LH are not only able to cause greater steroidogenesis but also able to promote growth and maturity of eggs (37). Therefore, it is hypothesized that the increase in egg production was due to greater concentrations of FSH and LH. In addition, greater egg production also suggested that the indirect effects, including greater steroidogenesis, could be sustained for prolonged periods of up to 21 days. Possible causes of the observed greater FSH and LH secretion were investigated. Clinical studies have demonstrated that boys with hypothyroidism also exhibited greater concentrations of FSH and LH due to greater secretions from the pituitary (38). Radioactive iodine therapy (RAI) of men with thyroid

cancer resulted in lesser concentrations of thyroid hormones, which produced dose-dependent greater concentrations of FSH and LH in plasma (38). These results are consistent with concentrations of FSH and LH being associated with lesser concentrations of thyroid hormone. The observation in the present study, that concentrations of FSH and LH were negatively correlated with additive concentrations of T4 and T3 taken together, suggest that the lesser concentrations of T4 and T3 caused by PTU resulted in greater secretion of FSH and LH, which in turn promoted steroidogenesis. It has previously been reported that several chemicals, such as PCB 126 and PFOA, could decrease concentrations of T4 and T3 and increase concentrations of FSH, LH, T, and E2 (8–11). Therefore, it was hypothesized that the greater steroidogenesis observed in the present study was due to lesser concentrations of T4 and T3, which in turn promoted secretion of FSH and LH.

The present study demonstrated that chemical-caused effects on one endocrine axis pathway will also indirectly affect the other endocrine axes in zebrafish. Our finding would be helpful to describe the basic physiology of the three axes in organisms exposed to single or mixtures of chemicals. In addition, caution should be exercised when extrapolation of chemical-induced endocrine-related effects is made from one simple in vitro study to an in vivo situation. Furthermore, the observation of changes in the initiating targets, including modulation of CRH by E2, and FSH and LH by T4 and T3 cross-talk, were identified. The information will be useful for characterizing effects of chemicals and predicting interactions among the major endocrine axes in animals after chemical exposure.

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Supporting Information Available

(1) Hormone measurement; (2) Western blot and ELISA detection of FSH and LH antibodies specification; (3) gene sequences of primers; (4) time-dependent transcriptional response of zebrafish to PCZ and PTU; (5) acronyms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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