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# EFFECTS OF THE INSECTICIDE FIPRONIL ON REPRODUCTIVE ENDOCRINOLOGY IN THE FATHEAD MINNOW

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**Abstract:** Gamma-aminobutyric acid (GABA) and GABA receptors play an important role in neuroendocrine regulation in fish. Disruption of the GABAergic system by environmental contaminants could interfere with normal regulation of the hypothalamic-pituitary-gonadal axis, leading to impaired fish reproduction. The present study used a 21-d fathead minnow (*Pimephales promelas*) reproduction assay to investigate the reproductive toxicity of fipronil (FIP), a broad-spectrum phenylpyrazole insecticide that acts as a noncompetitive blocker of GABA receptor–gated chloride channels. Continuous exposure up to 5 μg FIP/L had no significant effect on most of the endpoints measured, including fecundity, secondary sexual characteristics, plasma steroid and vitellogenin concentrations, ex vivo steroid production, and targeted gene expression in gonads or brain. The gonad mass, gonadosomatic index, and histological stage of the gonad were all significantly different in females exposed to 0.5 μg FIP/L compared with those exposed to 5.0 μg FIP/L; however, there were no other significant effects on these measurements in the controls or any of the other treatments in either males and females. Overall, the results do not support a hypothesized adverse outcome pathway linking FIP antagonism of the GABA receptor(s) to reproductive impairment in fish. *Environ Toxicol Chem* 2013;32:1828–1834. © 2013 SETAC

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## INTRODUCTION

Gamma-aminobutyric acid (GABA) and its receptors play an important role in the neuroendocrine regulation of the hypothalamic–pituitary–gonadal (HPG) axis and, therefore, reproduction (reviewed for fish and mammals, respectively, by Trudeau [1] and Maffuci and Gore [2]). In conjunction with other neurohormones, GABA helps regulate the level of gonadotropin-releasing hormone (GnRH), which in turn controls the levels of gonadotropin I (GtH I; follicle-stimulating hormone [FSH]) and gonadotropin II (GtH II; luteinizing hormone [LH]) [1–5]. Gonadotropins regulate many aspects of gonadal function in both males and females, including steroidogenesis, spermiogenesis, and ovulation. While the GABA system is well conserved among vertebrates, a significant difference is that GABA generally has a stimulatory effect on GnRH levels in fish, whereas GABA is inhibitory in mammals [1,2,6]. The inhibitory activity of GABA on GnRH and GtH levels in most mammals appears to have been primarily replaced by dopamine and dopamine receptors in fish (reviewed by Popesku et al. [6] and Dufour et al. [7]).

Given the important role that GABA plays in neuroendocrine regulation of reproduction in fish, it is conceivable that environmental contaminants that interact with the GABA system could disrupt normal regulation of the HPG axis, leading to impaired fish reproduction. A number of environmental contaminants have been shown to affect different aspects of GABA signaling. For example, both pulp and paper mill extracts [8] and hardwood tree extracts [9] alter numerous neurotransmitter receptors and enzymes, including the dopamine 2 receptor and the GABA A receptor, in goldfish brain extracts. Similarly, a number of pesticides are known to target GABA signaling. One well-known example is fipronil (FIP).

Fipronil (CAS number 120068-37-3) is a broad-spectrum phenylpyrazole insecticide originally developed in the mid-1980s and first registered for use in the United States in 1996 [10]. With a wide range of applications, including agricultural, urban, and veterinary uses, FIP has been detected in agricultural [11,12] and urban runoff [13] waters at concentrations as high as 1 ug/L to 10 ug/L, although low nanogram-per-liter concentrations are more common. Fipronil interferes with normal GABA receptor–gated chloride channels [14] by acting as a noncompetitive blocker [15–17], leading to increased neuronal stimulation, hyperactivity, and eventually death in target insects [14]. Fipronil has a higher binding affinity for insect than mammalian GABA receptors [14,18,19], suggesting a level of safety for vertebrates. Nonetheless, FIP has been shown to be moderately to highly toxic to some nontarget birds [20–23] and fish [10,24,25]. We hypothesized that pesticides with GABA-modulating activities, such as FIP, may have the potential to impair fish reproduction at sublethal concentrations.

To date, evidence of the sublethal effects of FIP on fish is limited. Results from a few studies hinted at possible impacts on reproductive endpoints such as altered number and size or growth of larvae [25–29] and the expression of genes associated with egg production (vitellogenin [*vtg*]; zona pellucida glycoprotein 3 [*zp3*]) in larvae [30]. However, none of these previous studies directly measured effects of FIP on the HPG

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axis or reproduction. Furthermore, in general, relatively few studies have examined reproductive impacts of (potential) endocrine-active chemicals (EACs) that modulate neurotransmitters and/or their receptors. Villeneuve et al. [31] demonstrated that exposure to sublethal concentrations of haloperidol (up to 20 μg/L), a dopamine 2 receptor antagonist, did not affect fathead minnow (*Pimephales promelas*) reproduction, although it did appear to impact behavior [32]. However, most fish reproduction assays, including those conducted by our group, have focused on EACs that act on the estrogen receptor, androgen receptor, or steriodogenic enzymes [33–39].

The purpose of the present study was to utilize an adaptation of the 21-d fathead minnow reproduction assay developed for the US Environmental Protection Agency (USEPA) Endocrine Disruptor Screening Program [40,41] to examine the effects of FIP on numerous HPG axis and reproductive parameters in adult fathead minnows, including reproductive output, circulating steroids and vitellogenin, gonadal steroid production, gonadal histology, and targeted gene expression. These data provided the first test of a hypothesized adverse outcome pathway linking antagonism of the GABA receptor with reproductive impairments in fish. In addition, the present study provided an opportunity to characterize the utility of the 21-d fathead minnow test for detecting and diagnosing neuroendocrine disruption as a mode of reproductive endocrine disruption. Finally, the present study provides useful data concerning the potential hazards associated with environmentally relevant concentrations of a widely used pesticide frequently detected in the aquatic environment.

## MATERIALS AND METHODS

### *Test organisms*

The fathead minnows used in these experiments were reproductively mature (approximately 6 mo old) and were obtained from the on-site culture facility at the USEPA laboratory in Duluth, Minnesota (USA). Water for culturing and exposures was obtained 200 m offshore from Lake Superior (MN, USA), at a depth of 20 m and was filtered (1 μm) and ultraviolet-treated prior to use. All exposure and laboratory procedures used in this experiment were approved by the laboratory's Institutional Animal Care and Use Committee.

### *Exposures*

The experimental design for the fathead minnow assay has been described in detail previously [40], including the modified paired spawning method [36]. Two pairs of 1 male and 1 female fathead minnow were randomly placed in 20-L aquaria containing 10 L of water. The pairs were separated by a water-permeable divider, and each pair had its own breeding substrate (10-cm section of polyvinyl chloride pipe cut in half). Fish were acclimated over a 14-d period, during which the number of eggs spawned (fecundity) and the number of eggs fertilized (fertility) were measured daily for each pair (data not shown). Following this acclimation period, exposures were initiated by delivering water only (control) or FIP (98% purity; ChemService) dissolved in water, without a solvent, at concentrations of 0.05, 0.5, or 5.0 μg/L (nominal). Five aquaria were used for each exposure condition, except for the control group, which had 6 aquaria. Throughout the acclimation and exposure periods, fish were held under a 16:8-h light:dark photoperiod and fed brine shrimp (*Artemia*) twice daily. Aquaria were maintained at 25 °C and received a continuous flow rate of 45 mL/min of water (with or without FIP).

Fecundity and fertility of each pair of fathead minnows were determined daily during the 21-d exposure. At the end of the exposure period, fish were euthanized with a buffered tricaine methanesulfonate solution (MS-222; Finquel); then they were weighed, and blood was collected immediately from the caudal vein using heparinized microhematocrit tubes. Plasma was isolated from blood cells by centrifugation and stored at −80 °C until analysis. Following blood collection, brains, pituitaries, and gonads were removed from fish using dissection tools cleaned with RNAseZap (Ambion), and secondary sexual characteristics (fatpad score and tubercle numbers) were determined for males [41,42]. Brains and pituitaries were stored separately in microcentrifuge tubes with RNAlater (Sigma-Aldrich) at −20 °C until analysis. Gonad samples were weighed to calculate the gonadosomatic index (GSI; gonad mass/fish mass × 100%) and divided into several subsamples. One gonad subsample was used immediately in an ex vivo steroid production assay, while 2 other subsamples were preserved in either Davidson's fixative for subsequent histological examination, or in microcentrifuge tubes with RNAlater at −20 °C until extraction and analysis.

### *Plasma steroids and vitellogenin*

Testosterone (T) and 17β-estradiol ($E_2$) concentrations in plasma samples were quantified by radioimmunoassay following liquid–liquid extraction with diethyl ether as described previously [41,42]. When volumes were limiting in some female plasma samples, only $E_2$ concentration was determined. Plasma vitellogenin (VTG) protein concentrations were quantified with an enzyme-linked immunosorbent assay using a polyclonal antibody to fathead minnow VTG, with purified fathead minnow VTG as a standard [41,43].

### *Ex vivo steroid production assay*

The methods for the ex vivo steroid production assay [37,38] were adapted from McMaster et al. [44]. Fresh gonad subsamples were stored on ice in individual wells of a 48-well Falcon 35-3078 microplate (Becton Dickinson) containing 500 μL of Medium 199 (Sigma-Aldrich) supplemented with 0.1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 1 μg/mL of 25-hydroxycholesterol (Sigma-Aldrich) until all fish were processed. These samples were subsequently incubated for 12 h at 25 °C, and the medium was removed from each well and stored in individual microcentrifuge tubes at −20 °C. Steroids were extracted from the media and analyzed as described earlier for the plasma samples.

### *Histology*

Gonad subsamples stored in Davidson's fixative were shipped to Experimental Pathology Laboratories (Herndon, VA, USA) and immediately transferred to 10% neutral buffered formalin. The gonads were embedded in paraffin and, after removal of approximately half of the tissue, longitudinally sectioned. Three sections at 50-μm intervals were cut, mounted on a single slide, and stained with hematoxylin and eosin. Gonad phenotype and stage were determined, and a variety of pathological findings were scored from 1 (minimal) to 4 (severe) for both testis and ovary samples [39,45].

### *Gene expression*

Total RNA was isolated from brain and gonad samples with Tri Reagent (Sigma-Aldrich) and from pituitary samples using RNeasy micro kits (Qiagen) following the manufacturers' protocols. The RNA quality and quantity were determined using

a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Total RNA concentrations were diluted to 10 ng/μL for brain and gonad samples or 1 ng/μL for pituitary samples for use in quantitative real-time polymerase chain reaction (QPCR) assays. The QPCR assays were performed using Taqman EZ RT-PCR kits (Applied Biosystems) following the manufacturer's protocol, using gene-specific mRNA standard curves to report relative transcript abundance as copies of mRNA/ng of total RNA [45,46]. The primers and dual-labeled probes were developed previously for FSH β subunit (*fshb*), LH β subunit (*lhb*), and GnRH (c, chicken-type isoform [*cgnrh*]) [31,45,46] or de novo in the present study for glutamate decarboxylase 65 and 67 (*gad65* and *gad67*) and cathepsin B (*catb*) using similar methods (Supplemental Data, Table S1). The gene expression methodology for *gad65* QPCR was as described in Supplemental Data, Table S1.

*Exposure verification*

Water samples were collected from each exposure aquarium ($n = 6$ control, $n = 5$ for each concentration of FIP) twice per week over the duration of the 21-d exposure and directly analyzed using liquid chromatography–mass spectrometry (model 1100 LC-MSD; Agilent) equipped with an electrospray interface (atmospheric pressure ionization–electrospray). An aliquot of sample (100 μL) was injected onto a Zorbax SB-C18 (Agilent) column (2.1 × 75 mm) and eluted isocratically at a flow rate of 0.2 mL/min. The mobile phase consisted of 68% acetonitrile, 10 mM ammonium acetate buffer, and 0.04% acetic acid. Fipronil concentrations were determined using masses 436 and 437 (selective ion monitoring, negative ion mode) with an external standard method of quantitation. Quality control samples such as procedural blanks, spiked recoveries, and duplicate analyses comprised 10% of the sample load. The agreement between duplicate analyses was 98 ± 2.1% (mean ± standard deviation [SD], $n = 18$) and the FIP recovery was 92 ± 1.2% (mean ± SD, $n = 12$). No FIP was detected in any control water sample over the course of the experiment (detection limit = 15 ng/L). The mean (± SD) measured FIP water concentrations were 0.049 ± 0.006 μg/L (0.05 μg/L nominal; $n = 36$), 0.57 ± 0.08 μg/L (0.5 μg/L nominal; $n = 36$), and 6.10 ± 0.59 μg/L (5.0 μg/L nominal; $n = 36$). Fipronil treatment concentrations are reported in text, tables, and figures as nominal values.

*Data analysis*

Data analyses were conducted as previously described [31]. Briefly, data that conformed to parametric assumptions (normality and homogeneity of variance) were analyzed by one-way analysis of variance; nonparametric data were analyzed using a Kruskal–Wallis test. A general linear models analysis of variance was used to analyze the ex vivo data, using both the mass of the gonad subsample and the FIP exposure concentration as independent variables. Tukey's or Bonnferoni post hoc tests were used to determine differences between treatment groups. Either SAS 9.0 (SAS Institute) or SYSTAT 11.0 (Systat Software) were used for all data analyses, with significance set at $p < 0.05$.

## RESULTS

*Fecundity and secondary sexual characteristics*

No mortality occurred during the acclimation or exposure phases of the test. Fipronil had no significant effect on mean cumulative fecundity in the fish. The highest egg production occurred in the 0.05 μg FIP/L group and the lowest egg production in the 5.0 μg FIP/L treatment group (Figure 1). The number of spawns per pair and the number of eggs produced per spawn over the 21-d exposure were also not affected by treatment (Supplemental Data, Figure S1). Similarly, FIP exposure did not impact fish mass, gonad mass, GSI, or secondary sexual characteristics of males (Table 1). While body mass was unaffected by FIP in females, significant differences were observed in the gonad mass and GSI in females exposed to 0.5 μg FIP/L compared with those exposed to 5.0 μg FIP/L. However, neither gonad mass nor GSI was significantly different between either of these 2 exposures and the control or 0.05 μg FIP/L exposure group in females (Table 1).

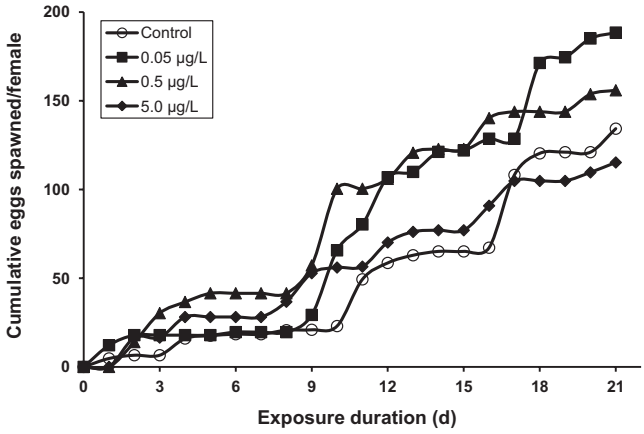


Figure 1. Cumulative egg production measured over the duration of the 21-d fipronil exposure. Data are mean, with $n = 10$ females per treatment, except control ($n = 12$).

*Steroids and vitellogenin*

Plasma T and $E_2$ were not significantly affected by fipronil exposure in either males or females (Figure 2A and B). Plasma VTG was unchanged following FIP exposure in males (data not shown) and females (Figure 2C). In addition, ex vivo steroid production was not significantly altered by any treatment in either sex (Supplemental Data, Figure S2).

*Histology*

No significant pathological effects due to FIP exposure at any concentration were observed following histological examination of gonads from both males and females (data not shown). In testes, there were numerous incidences of mineralization; in ovaries, several examples of increased oocyte atresia were noted. However, the degree and extent of these changes and other histological findings were minor and equally distributed across treatment groups, including controls. Fipronil exposure also had no significant effect on mean gonad stage in males. The gonad stage of ovaries from 0.5 μg FIP/L exposed females scored significantly different than the gonad stage of ovaries from 5.0 μg FIP/L exposed females. There were no other significant effects of exposure on gonad stage in females from the control or any FIP exposure group (Supplemental Data, Figure S3).

*Gene expression*

Fipronil exposure did not result in significantly different expression of any of the 6 mRNA transcripts examined in the present study (Figure 3A and B; Supplemental Data, Figures S4 and S5).

Table 1. Fish reproductive endpoints measured following 21-d fipronil exposure[a]

| Endpoint | Sex | Fipronil concentration (μg/L) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.05 | 0.5 | 5.0 |
| Body mass (g) | Male | 3.51 ± 0.19 | 3.40 ± 0.22 | 3.50 ± 0.21 | 3.46 ± 0.27 |
| | Female | 1.38 ± 0.08 | 1.29 ± 0.06 | 1.53 ± 0.07 | 1.36 ± 0.09 |
| Gonad mass (mg) | Male | 58 ± 4 | 47 ± 3 | 61 ± 7 | 57 ± 7 |
| | Female | 149 ± 22 A,B | 134 ± 12 A,B | 192 ± 20 A | 107 ± 10 B |
| GSI (%) | Male | 1.66 ± 0.08 | 1.41 ± 0.08 | 1.71 ± 0.14 | 1.63 ± 0.14 |
| | Female | 10.47 ± 1.32 A,B | 10.40 ± 0.74 A,B | 12.55 ± 1.14 A | 7.91 ± 0.66 B |
| Fatpad score | Male | 2.3 ± 0.2 | 2.0 ± 0.3 | 2.0 ± 0.2 | 2.0 ± 0.3 |
| Tubercle score | Male | 22.8 ± 0.9 | 22.8 ± 0.8 | 23.9 ± 1.8 | 20.1 ± 1.2 |

[a]Data are mean ± standard error of the mean; $n = 10$, except controls ($n = 12$) and 0.05 μg fipronil/L male gonad mass and GSI ($n = 9$). Within an endpoint, values that do not share the same uppercase letter are statistically different ($p < 0.05$).
GSI = gonadosomatic index.

## DISCUSSION

The present study contributes to an on-going effort to elucidate reproductive adverse outcome pathways associated with exposure to EACs acting through different molecular initiating events [47]. This integrated, multilaboratory project has been using both hypothesis- and discovery-driven approaches with fathead minnows and zebrafish to investigate the reproductive effects of known or hypothesized EACs and develop linkages between alterations in the HPG axis and adverse reproductive outcomes [48]. Among these studies, this was the first to examine the effect of a GABA-modulating chemical using a 21-d fathead minnow reproduction assay.

The results of the present study do not support the hypothesis that waterborne exposure to FIP, at concentrations up to 5 μg/L, poses a direct reproductive hazard to fish. Given the stimulatory role of the GABAergic system in fish [1], there was reason to believe that antagonism of the GABA receptor by FIP could lead to changes in spawning or fecundity. However, no such effects were observed. In contrast, a previous investigation exposing sheepshead minnows to comparable concentrations of FIP for

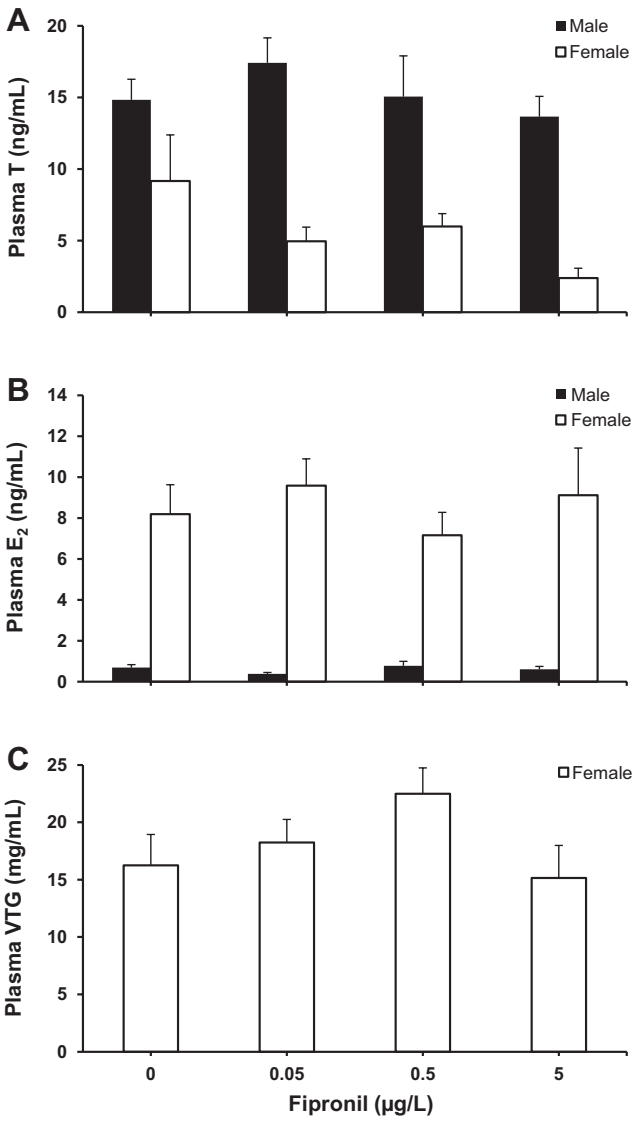


Figure 2. Effects of fipronil on male and female plasma components following the 21-d exposure: (**A**) testosterone (T), (**B**) 17β-estradiol ($E_2$), and (**C**; females only) vitellogenin (VTG). Data are mean ± standard error of the mean, with $n = 8$–12, except female plasma T ($n = 4$).

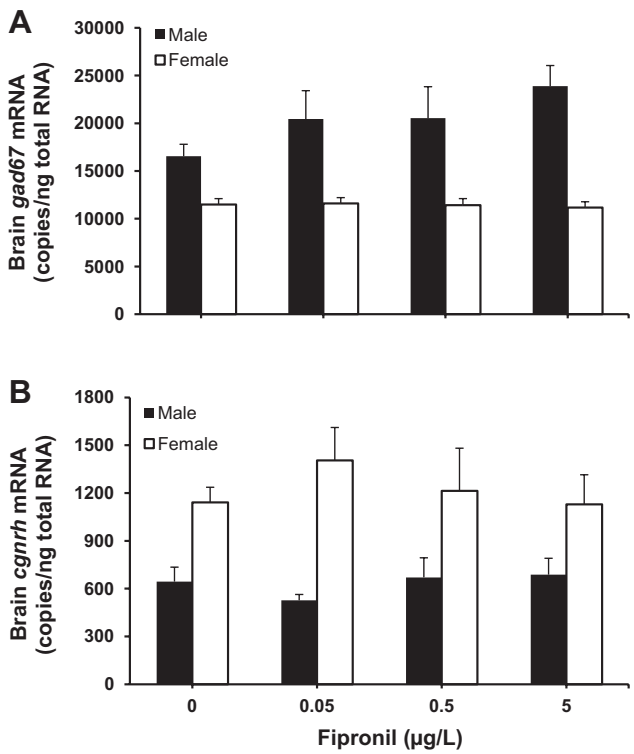


Figure 3. Transcript abundance of (**A**) *gad67* and (**B**) *cgnrh* mRNA in the brains of males and females exposed to fipronil for 21 d. Data are mean ± standard error of the mean, with $n = 8$–12, except 0.5 μg fipronil/L male ($n = 4$) for both transcripts.

28 d resulted in increased fish reproduction, as measured by the number of spawned fish [26]. As the study by Wirth et al. [26] was a mesocosm experiment, not specifically designed to measure reproductive endpoints, it is unclear whether altered predation and timing of spawning might have caused changes in the number and size of fish. In contrast, it does appear that FIP can affect reproduction in mammals. In a rat study, topical dosing with 70 mg FIP/kg or more caused a prolonged estrous cycle and a reduced pregnancy index [49], which would be analogous to delayed spawning and reduced fecundity, respectively, in fish. However, given the difference in dosing regime and the known differences in GABA signaling between mammals and fish [2], it is difficult to compare the effect concentrations with those employed in the present study.

Neither gonad size nor GSI was altered following exposure to FIP (relative to controls) in either male or female fathead minnows. In another investigation that examined gonad size following FIP exposure, the GSI in female rats also remained unchanged, although these exposures were associated with other reproductive impacts [49]. In the present study, FIP exposure had no influence on fathead minnow mass in either sex, similar to the results in common carp exposed to FIP [50]. In several other fish studies, FIP exposure had mixed effects on fish mass, decreasing both adult and larval body size ratios in medaka [28] and variously decreasing [25] or increasing [27] larval body mass in 2 different studies with fathead minnows. Overall, no consistent effects of FIP on fish growth have been observed across studies.

Plasma and ex vivo steroid levels were not significantly different following FIP exposure in the present study. The lack of a more definitive response may be partially explained by the complexity of GABA regulation of HPG function. In fish species examined to date, including goldfish, catfish, and croaker, GABAergic signaling varies seasonally with gonadal development, can be either stimulatory or inhibitory depending on life stage and tissue, and is modulated by circulating sex steroids [51–55].

In the present study, plasma VTG levels were unchanged in adult females by FIP and no induction of plasma VTG was evident in males. However, Beggel et al. [30] reported that FIP altered *vtg* expression in juvenile fathead minnows exposed to 31 μg/L of the pesticide for 24 h. Differences between the results of the present study and those of Beggel et al. [30] could be related to the life stage used, the different exposure regimes, or both.

Fipronil exposure has previously been shown to alter gene expression, including numerous *cyp* genes and *vtg*, in human hepatocytes and juvenile fathead minnows [30,56]. Moreover, micro RNAs were determined to be differentially expressed following exposure to FIP in adult zebrafish [57]. In the present study, we examined the effects of FIP exposure on 6 selected gene expression endpoints. Expression of *gad65* and *gad67* transcripts in brain were examined, as the enzymes produced from these transcripts are responsible for the generation of GABA from glutamate in all vertebrates, including fish [58–60]. While we hypothesized, based on the mode of action, that a potential adaptive response to FIP exposure might be elevated expression of *gad65* and *gad67*, no significant changes in *gad* gene expression occurred. Expression of brain *cgnrh*, whose peptide hormone is responsible for the release of FSH and LH, was similarly unaltered in both sexes, suggesting that FIP exposure had little effect on GABA regulation of GnRH levels. As both FSH and LH are part of the GABAergic signaling pathway, it was interesting to note that there were trends, albeit not significant, in pituitary *fshb* and *lhb* gene expression. In fact, the expression of *lhb* has been shown to be directly linked to GABA-regulated GnRH levels in goldfish [1], and therefore, the results in males were not altogether unexpected. Finally, we examined expression of *catb* in ovary. Cathepsin b is one of several lysosomal enzymes known to play an important role in egg production, and its activity varies over the course of follicle and oocyte maturation [61]. It was employed in the present study as a potential marker of disruption or alteration in follicle development; consistent with other reproductive and histological endpoints, however, no significant differences in *catb* expression were observed. We note, however, that more recent studies have suggested that regulation of cathepsin b activity may not be tightly coupled to its gene expression [62]. Overall, as a whole, the gene expression endpoints examined in the present study were consistent with apical endpoints, suggesting that FIP exposure had little or no impact on HPG axis–regulated reproductive function.

While evidence exists in the literature suggesting that exposure to FIP could affect normal reproductive function in mammals [49] and fish [26–30], the results of the present study were largely negative. One possible explanation for the across-study differences may be related to the FIP formulations and concentrations used. In the present study, pure FIP was used at concentrations approaching the maximum levels detected in environmental studies [11–13]. In contrast, in many other investigations, commercial formulations of FIP or higher concentrations (or both) were employed [24,27,30,50,63]. Differences in fish species and, perhaps more importantly, life stages are also possible sources of across-study variation.

Collectively, data from the present study provide no support for a hypothesized adverse outcome pathway linking antagonism of the GABA receptor to reproductive impairment in fish. Given that the molecular and biochemical endpoints examined in the present study were measured 21 d after the initial exposure to the chemical, we can only speculate as to whether FIP exposure perturbed HPG-axis function earlier in the exposure. However, given the lack of an adverse outcome (e.g., reduced fecundity) it would appear that those perturbations, if they occurred, did not exceed the adaptive capacities of the organisms under favorable laboratory conditions. This does not, however, rule out the possibility that exposure to higher concentrations of FIP could impair reproduction or that exposure to similar (low) concentrations of FIP may act through alternative pathways. For example, FIP appears to have developmental neurotoxic activity, as evidenced by results in both mammalian cells lines [64,65] and fish larvae [27,63]. These results are not surprising given the role of the GABAergic system in neurogenesis, including development of the central nervous system and neuronal pathway modulation, in mice, rats, and zebrafish [2,63,66]. Moreover, GABA and its receptors occur in a wide variety of non-neural tissues, such as smooth muscle and both male and female reproductive tracts [67–69]. Therefore, while FIP did not alter any known reproductive adverse outcome pathways in adult fathead minnows, it possible that FIP may affect fish early life stages [70].

A final aspect of the present study that warrants discussion involves consideration of the utility of the 21-d fathead minnow assay, developed and currently being used for the USEPA Endocrine Disruptor Screening Program (http://www.epa.gov/endo/), for identifying EACs. While the negative responses in the present study do not provide compelling evidence that the assay will detect possible neuroendocrine-disrupting agents, the data

do address a concern that has been raised about the test itself, specifically, that it is likely to identify all chemicals as endocrine disruptors. The results of the present study clearly dispute that contention. None of the endpoints included in the standard guideline fathead minnow reproduction assay would have resulted in FIP, a widely distributed current-use pesticide, as being flagged as an EAC. In addition, even with a hypothesized neuroendocrine mode of action and the inclusion of additional diagnostic endpoints (e.g., changes in expression of potential target genes), the assay still produced a negative result. While this is admittedly just one example, it should serve to allay some fears that the current Endocrine Disruptor Screening Program fish assay is indiscriminant.

## SUPPLEMENTAL DATA

**Table S1.**
**Figures S1–S5.** (156 KB PDF).

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## REFERENCES

1. Trudeau VL. 1997. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. *Rev Reprod* 2:55–68.
2. Maffucci JA, Gore AC. 2009. Hypothalamic neural systems controlling the female reproductive life cycle: Gonadotropin-releasing hormone, glutamate, and GABA. *Int Rev Cell Mol Biol* 274:69–127.
3. Bieniarz K, Epler P. 1992. Advances in reproductive endocrinology of fish. *J Physiol Pharmacol* 43:215–222.
4. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. 2003. Regulation of fish gonadotropins. *Int Rev Cytol* 225:131–185.
5. Levavi-Sivan B, Bogerd J, Mañanós EL, Gómez A, Lareyre JJ. 2010. Perspectives on fish gonadotropins and their receptors. *Gen Comp Endocrinol* 165:412–437.
6. Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, Cossins AR, Trudeau VL. 2008. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Mol Cell Endocrinol* 293:43–56.
7. Dufour S, Weltzien FA, Sebert ME, Le Belle N, Vidal B, Vernier P, Pasqualini C. 2005. Dopaminergic inhibition of reproduction in teleost fishes: Ecophysiological and evolutionary implications. *Ann N Y Acad Sci* 1040:9–21.
8. Basu N, Ta CA, Waye A, Mao J, Hewitt M, Arnason JT, Trudeau VL. 2009. Pulp and paper mill effluents contain neuroactive substances that potentially disrupt neuroendocrine control of fish reproduction. *Environ Sci Technol* 43:1635–1641.
9. Basu N, Waye A, Trudeau VL, Arnason JT. 2012. Extracts from hardwood trees used in commercial paper mills contain biologically active neurochemical disruptors. *Sci Total Environ* 414:205–209.
10. US Environmental Protection Agency. 1996. New pesticide fact sheet. EPA 737/F-96/005. Washington, DC.
11. Demcheck DK, Skrobialowski SC. 2003. Fipronil and degradation products in the rice-producing areas of the Mermentau River Basin, Louisiana, February-September 2000. USGS Fact Sheet FS- 010-03. US Department of the Interior, US Geological Survey, National Water-Quality Assessment Program, Reston, VA.
12. Mize SV, Porter SD, Demcheck DK. 2008. Influence of fipronil compounds and rice-cultivation land-use intensity on macroinvertebrate communities in streams of southwestern Louisiana, USA. *Environ Pollut* 152:491–503.
13. Gan J, Bondarenko S, Oki L, Haver D, Li JK. 2012. Occurrence of fipronil and its biologically active derivatives in urban residential runoff. *Environ Sci Technol* 46:1489–1495.
14. Cole LM, Nicholson RA, Casida JE. 1993. Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pestic Biochem Physiol* 46:47–54.
15. Perret P, Sarda X, Wolff M, Wu T-T, Bushey D, Goeldner M. 1993. Interaction of non-competitive blockers within the γ-aminobutyric acid type A chloride channel using chemically reactive probes as chemical sensors for cysteine mutants. *J Biol Chem* 274:25350–25354.
16. Law RJ, Lightstone FC. 2008. GABA receptor insecticide non-competitive antagonists may bind at allosteric modulator sites. *Int J Neurosci* 118:705–734.
17. Charon S, Taly A, Rodrigo J, Perret P, Goeldner M. 2011. Binding modes of noncompetitive GABA-channel blockers revisited using engineered affinity-labeling reactions combined with new docking studies. *J Agric Food Chem* 59:2803–2807.
18. Hainzl D, Cole LM, Casida JE. 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem Res Toxicol* 11:1529–1535.
19. Ratra G, Casida JE. 2001. GABA receptor subunit composition relative to insecticide potency and selectivity. *Toxicol Lett* 122:215–222.
20. Goodyear JJ. 1994. 71-1A. Avian single dose oral LD50 test: Bobwhite quail (*Colinus virginianus*). MRID 429186-17. Data Evaluation Record. US Environmental Protection Agency, Washington, DC.
21. Stavola A. 1994. 71-1A. Avian single dose oral LD50 test: Red-legged partridge (*Alectoris rufa*). MRID 429186-14. Data Evaluation Record. US Environmental Protection Agency, Washington, DC.
22. Stavola A. 1994. 71-1A. Avian single dose oral LD50 test: Pheasant (*Phasianus colchicus*). MRID 429186-15. Data Evaluation Record. US Environmental Protection Agency, Washington, DC.
23. Kitulagodage M, Isanhart J, Buttemer WA, Hooper MJ, Astheimer LB. 2011. Fipronil toxicity in northern bobwhite quail *Colinus virginianus*: Reduced feeding behavior and sulfone metabolite formation. *Chemosphere* 83:524–530.
24. Nillos MG, Lin K, Gan J, Bondarenko S, Schlenk D. 2009. Enantioselectivity in fipronil aquatic toxicity and degradation. *Environ Toxicol Chem* 28:1825–1833.
25. Baird S, Garrison A, Jones J, Avants J, Bringolf R, Black M. 2013. Enantioselective toxicity and bioaccumulation of fipronil in fathead minnows (*Pimephales promelas*) following water and sediment exposures. *Environ Toxicol Chem* 32:222–227.
26. Wirth EF, Pennington PL, Lawton JC, DeLorenzo ME, Bearden D, Shaddrix B, Sivertsen S, Fulton MH. 2004. The effects of the contemporary-use insecticide (fipronil) in an estuarine mesocosm. *Environ Pollut* 131:365–371.
27. Beggel S, Werner I, Connon RE, Geist JP. 2010. Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval fathead minnow (*Pimephales promelas*). *Sci Total Environ* 408:3169–3175.
28. Hayasaka D, Korenaga T, Sánchez-Bayo F, Goka K. 2012. Differences in ecological impacts of systemic insecticides with different physiochemical properties on biocenosis of experimental paddy fields. *Ecotoxicology* 21:191–201.
29. Hayasaka D, Korenaga T, Suzuki K, Saito F, Sánchez-Bayo F, Goka K. 2012. Cumulative ecological impacts of two successive annual treatments of imidacloprid and fipronil on aquatic communities of paddy mesocosms. *Ecotoxicol Environ Saf* 80:355–362.
30. Beggel S, Werner I, Connon RE, Geist JP. 2012. Impacts of the phenylpyrazole insecticide fipronil on larval fish: Time-series gene transcription responses in fathead minnow (*Pimephales promelas*) following short-term exposure. *Sci Total Environ* 426:160–165.
31. Villeneuve DL, Garcia-Reyero N, Martinović D, Mueller ND, Cavallin JE, Durhan EJ, Makynen EA, Jensen KM, Kahl MD, Blake LS, Perkins EJ, Ankley GT. 2010.I. Effects of a dopamine receptor antagonist on fathead minnow, Pimephales promelas, reproduction. *Ecotoxicol Environ Saf* 73:472–477.
32. Villeneuve DL, Garcia-Reyero N, Martinović D, Mueller ND, Cavallin JE, Durhan EJ, Makynen EA, Jensen KM, Kahl MD, Blake LS, Perkins EJ, Ankley GT. 2010.II. Effects of a dopamine receptor antagonist on fathead minnow dominance behavior and ovarian gene expression in the fathead minnow and zebrafish. *Ecotoxicol Environ Saf* 73:478–485.
33. Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol Sci* 67:121–130.
34. Ankley GT, Jensen KM, Makynen EA, Kahl MD, Korte JJ, Hornung MW, Henry TR, Denny JS, Leino RL, Wilson VS, Cardon MC, Hartig PC, Gray LE. 2003. Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ Toxicol Chem* 22:1350–1360.

35. Jensen KM, Kahl MD, Makynen EA, Korte JJ, Leino RL, Butterworth BC, Ankley GT. 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. *Aquat Toxicol* 70:99–110.
36. Ankley GT, Jensen KM, Durhan EJ, Makynen EA, Butterworth BC, Kahl MD, Villeneuve DL, Linnum A, Gray EL, Cardon M, Wilson VS. 2005. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (*Pimephales promelas*). *Toxicol Sci* 86:300–308.
37. Ankley GT, Jensen KM, Kahl MD, Makynen EA, Blake LS, Greene KJ, Johnson RD, Villeneuve DL. 2007. Ketoconazole in the fathead minnow (*Pimephales promelas*): Reproductive toxicity and biological compensation. *Environ Toxicol Chem* 26:1214–1223.
38. Martinović D, Blake LS, Durhan EJ, Greene KJ, Kahl MD, Jensen KM, Makynen EA, Villeneuve DL, Ankley GT. 2008. Reproductive toxicity of vinclozolin in the fathead minnow: Confirming an anti-androgenic mode of action. *Environ Toxicol Chem* 27:478–488.
39. Villeneuve DL, Blake LS, Brodin JD, Cavallin JE, Durhan EJ, Jensen KM, Kahl MD, Makynen EA, Martinović D, Mueller ND, Ankley GT. 2008. Effects of a 3β-hydroxysteroid dehydrogenase inhibitor, trilostane, on fathead minnow reproductive axis. *Toxicol Sci* 104:113–123.
40. Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1276–1290.
41. US Environmental Protection Agency. 2002. A short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (*Pimephales promelas*). EPA 600/R-01/067. Duluth, MN, USA.
42. Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comp Biochem Physiol C Toxicol Pharmacol* 128:127–141.
43. Parks LG, Cheek AO, Denslow ND, Heppell SA, McLachlan JA, LeBlan GA, Sullivan CV. 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: Purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. *Comp Biochem Physiol C* 123:113–125.
44. McMaster ME, Munkittrick KR, Jardine JJ, Robinson RD, Van Der Kraak GJ. 1995. Protocol for measuring *in vitro* steroid production by fish gonadal tissue. Canadian Technical Report of Fisheries and Aquatic Sciences 1961. Department of Fisheries and Oceans, Burlington, ON, Canada.
45. Villeneuve DL, Miracle AL, Jensen KM, Degitz SJ, Kahl MD, Korte JJ, Greene KJ, Blake LS, Linnum AL, Ankley GT. 2007. Development of quantitative real-time PCR assays for fathead minnow (*Pimephales promelas*) gonadotropin subunit β mRNAs to support endocrine disruptor research. *Comp Biochem Physiol C* 145:171–183.
46. Villeneuve DL, Blake LS, Brodin JD, Greene KJ, Knoebl I, Miracle AL, Martinović D, Ankley GT. Transcription of key genes regulating gonadal steroidogenesis in control and ketoconazole- or vinclozolin-exposed fathead minnows. *Toxicol Sci* 98:395–407.
47. Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Horning MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrano JA, Tietge JE, Villeneuve DL. 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicological research and risk assessment. *Environ Toxicol Chem* 29:730–741.
48. Ankley GT, Bencic DC, Breen MS, Collette TW, Conolly RB, Denslow ND, Edwards SW, Ekman DR, Garcia-Reyero N, Jensen KM, Lazorchak JM, Martinović D, Miller DH, Perkins EJ, Orlando EF, Villeneuve DL, Wang R-L, Watanabe KH. 2009. Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanism of action. *Aquat Toxicol* 92:168–178.
49. Ohi M, Dalsenter PR, Andrade AJM, Nascimento AJ. 2004. Reproductive adverse effects of fipronil in Wistar rats. *Toxicol Lett* 146:121–127.
50. Clasen B, Loro VL, Cattaneo R, Morase B, Lópes T, de Avila LA, Zanella R, Reimche GB, Baldisserotto B. 2012. Effects of the commercial formulation containing fipronil on the non-target organism *Cyprinus carpio*: Implications for rice-fish cultivation. *Ecotoxicol Environ Saf* 77:45–51.
51. Kah O, Trudeau VL, Sloley BD, Chang JP, Dubourg P, Yu KL, Peter RE. 1992. Influence of GABA on gonadotropin release in the goldfish. *Neuroendocrinology* 55:396–404.
52. Trudeau VL, Sloley BD, Peter RE. 1993. Testosterone enhances GABA and taurine but not N-methyl-D,L-aspartate stimulation of gonadotropin secretion in the goldfish: Possible sex steroid feedback mechanisms. *J Neuroendocrinol* 5:129–136.
53. Trudeau VL, Sloley BD, Peter RE. 1993. GABA stimulation of gonadotropin-II release in goldfish: Involvement of GABAA receptors, dopamine, and sex steroids. *Am J Physiol* 265:R348–R355.
54. Joy KP, Tharakan B, Goos HJ. 1999. Distribution of gamma-aminobutyric acid in catfish (*Heteropneustes fossilis*) forebrain in relation to season, ovariectomy, and E2 replacement, and effects of GABA administration on plasma gonadotropin-II level. *Comp Biochem Physiol A Mol Integr Physiol* 123:369–376.
55. Khan IA, Thomas P. 1999. GABA exerts stimulatory and inhinitory influences on gonadotropin II secretion in the Atlantic croaker (*Micropogonias undulatus*). *Neuroendocrinology* 69:261–268.
56. Das PC, Cao Y, Cherrington N, Hodgson E, Rose RL. 2006. Fipronil induces CYP isoforms and cytotoxicity in human hepatocytes. *Chem-Biol Interact* 164:200–214.
57. Wang X, Zhou S, Ding X, Zhu G, Guo J. 2010. Effect of triazophos, fipronil, and their mixture on miRNA expression in adult zebrafish. *J Environ Sci Health Part B Pestic Food Contam Agric Wastes* 45: 648–657.
58. Martin SC, Heinrich G, Sandell JH. 1998. Sequence and expression of glutamic acid decarboxylase isoforms in the developing zebrafish. *J Comp Neurol* 396:253–266.
59. Bosma PT, Blázquez M, Collins MA, Bishop JDD, Drouin G, Priede IG, Docherty K, Trudeau VL. 1999. Multiplicity of glutamic acid decarboxylase (GAD) in vertebrates: Molecular phylogeny and evidence for a new GAD paralog. *Mol Biol Evol* 16:397–404.
60. Trudeau VL, Spanswick D, Fraser EJ, Larivière K, Crump D, Chiu S, MacMillan M, Schulz RW. 2000. The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. *Biochem Cell Biol* 78:241–259.
61. Carnevali O, Cionna C, Tosti L, Lubzens E, Maradonna F. 2006. Role of cathepsins in ovarian follicle growth and maturation. *Gen Comp Endocrinol* 146:195–203.
62. Eykelbosh AJ, Van Der Kraak G. 2010. A role for the lysosomal protease cathepsin B in zebrafish follicular apoptosis. *Comp Biochem Physiol A Mol Integr Physiol* 156:218–223.
63. Stehr CM, Linbo TL, Incardona JP, Scholz NL. 2006. The developmental neurotoxicity of fipronil: Notochord degeneration and locomotor defects in zebrafish embryos and larvae. *Toxicol Sci* 92:270–278.
64. Lassiter TL, MacKillop EA, Ryde IT, Seidler FJ, Slotkin TA. 2009. Is fipronil safer than chlorpyrifos? Comparative developmental neurotoxicity modeled in PC12 cells. *Brain Res Bull* 78:313–322.
65. Sidiropoulou E, Sachan M, Flaskos J, Harris W, Hargreaves AJ, Woldehiwet Z. 2011. Fipronil interferes with the differentiation of mouse N2a neuroblastoma cells. *Toxicol Lett* 201:86–91.
66. Wullimann MF. 2009. Secondary neurogenesis and telencephalic organization in zebrafish and mice: A brief review. *Integr Zool* 4:123–133.
67. Ong J, Kerr DI. 1990. GABA-receptors in peripheral tissues. *Life Sci* 46:1489–1501.
68. Ritta MN, Calamera JC, Bas DE. 1998. Occurrence of GABA and GABA receptors in human spermatozoa. *Mol Hum Reprod* 4:769–773.
69. Kanbara K, Okamoto K, Nomura S, Kaneko T, Shigemotot R, Azuma H, Katsuoka Y, Watanabe M. 2005. Cellular localization of GABA and GABAB receptor subunit proteins during spermiogenesis in rat testis. *J Androl* 26:485–493.
70. Volz DC, Belanger S, Embry M, Padilla S, Sanderson H, Schirmer K, Scholz S, Villeneuve D. 2011. Adverse outcome pathways during early fish development: A conceptual framework for identification of chemical screening and prioritization strategies. *Toxicol Sci* 123:349–358.