The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae

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Fipronil is a phenylpyrazole insecticide designed to selectively inhibit insect gamma-aminobutyric acid (GABA) receptors. Although fipronil is often used in or near aquatic environments, few studies have assessed the effects of this neurotoxicant on aquatic vertebrates at sensitive life stages. We explored the toxicological effects of fipronil on embryos and larvae using the zebrafish (Danio rerio) experimental model system. Embryos exposed to fipronil at nominal concentrations at or above 0.7 \( \mu \text{M} \) (333 \( \mu \text{g/l} \)) displayed notochord degeneration, shortening along the rostral-caudal body axis, and ineffective tail flaps and uncoordinated muscle contractions along the body axis in response to touch. This phenotype closely resembles zebrafish locomotor mutants of the accordion class and is consistent with loss of reciprocal inhibitory neurotransmission by glycinergic commissural interneurons in the spinal cord. Consistent with the hypothesis that notochord degeneration may be due to abnormal mechanical stress from muscle tetany, the expression patterns of gene and protein markers specific to notochord development were unaffected by fipronil. Moreover, the degenerative effects of fipronil (1.1\( \mu \text{M} \)) were reversed by coexposure to the sodium channel blocker MS-222 (0.6mM). The notochord effects of fipronil were phenocopied by exposure to 70\( \mu \text{M} \) strychnine, a glycinergic receptor antagonist. In contrast, exposure to gabazine, a potent vertebrate GABA\(_A\) antagonist, resulted in a hyperactive touch response but did not cause notochord degeneration. Although specifically developed to target insect GABA receptors with low vertebrate toxicity, our results suggest that fipronil impairs the development of spinal locomotor pathways in fish by inhibiting a structurally related glycine receptor subtype. This represents an unanticipated and potentially novel mechanism for fipronil toxicity in vertebrates.

Key Words: zebrafish; fipronil; neurotoxicity; pesticide; GABA; glycine.

Fipronil is a phenylpyrazole insecticide that is widely used to control pests such as cockroaches, ants, termites, rice weevils, mole crickets, and fleas. Fipronil selectively binds to insect gamma-aminobutyric acid (GABA)-gated chloride channels, blocking the inhibitory action of GABA in the central nervous system (CNS). Physiologically, fipronil exposure produces hyperexcitation at low doses and convulsions and death at high doses. Insect GABA receptors are structurally similar to vertebrate GABA\(_A\) and GABA\(_C\) receptors (Hosie et al., 1997), which together with glycine receptors (GlyRs) are members of the ligand-gated chloride channel family (Jentsch et al., 2002). These receptors have structural features that are common among all members of the ligand-gated ion channel superfamily. Although GABA-gated chloride channels are expressed in the CNS of both vertebrates and invertebrates, fipronil has a considerably higher affinity for insect GABA receptors relative to vertebrate GABA\(_A\) receptors and little affinity for vertebrate GABA\(_C\) receptors. This property is thought to account for the low toxicity of fipronil in mammals relative to arthropods. (Chandler et al., 2004; Gant et al., 1998; Hainzl et al., 1998; Ratra et al., 2001, 2002; Tingle et al., 2003).

Recent investigations, primarily on crustaceans, have shown that fipronil can reach environmental levels that impact non-target aquatic life (Cary et al., 2004; Chandler et al., 2004; Key et al., 2003; Schlenk et al., 2001; Wirth et al., 2004). However, the toxicity of fipronil to fish and other vertebrates is not well characterized. This is particularly true for sublethal effects on sensitive life stages. For example, the potential neurotoxicological impact of fipronil on fish during early developmental stages is poorly understood.

GABA-mediated neurotransmission is involved in the modulation of several neural pathways that arise relatively early in fish development. For example, the circuitry underlying locomotor behaviors such as the escape response and rhythmic swimming is established soon after patterning of the hindbrain and spinal cord (Saint-Amant and Drapeau, 1998, 2000), respectively, and GABAergic interneurons are integral to these systems (Higashijima et al., 2004; Lee et al., 1993; Heggie et al., 1997; Triller et al., 1997). However, little is known about the potential effects of GABA receptor blockade during CNS development.

The zebrafish (Danio rerio) has become an important tool in developmental neurobiology, and simple behavioral screens have identified mutants defective in sensory and locomotor

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functions (Granato et al., 1996). Zebrafish mutant lines provide considerable insight into mechanisms of developmental toxicity, via the comparison of mutant phenotypes with those arising from exposure to contaminants or other small molecules (Hill et al., 2005; Incardona et al., 2004; Peterson et al., 2000). Genetic dissection of sensorimotor functions in zebrafish has converged with neurophysiological and neuropharmacological studies in larger fish species to provide a detailed picture of the cellular and molecular underpinnings of fundamental locomotor behaviors (Grillner, 2003; Kullander, 2005). Functional roles for both excitatory (glutamate) and inhibitory (glycine and GABA) neurotransmitters are thus well known in zebrafish.

Here we describe a novel neurotoxicological effect of fipronil during zebrafish development. Zebrafish embryos exposed to fipronil showed a dose-dependent loss of touch-induced escape response, a shortening of the longitudinal body axis, and notochord degeneration. The abnormal motility and morphological defects observed in fipronil-exposed larvae are very similar to accordion class zebrafish mutants, which are proposed to affect reciprocal inhibition in the spinal cord mediated by glycergic commissural interneurons (Granato et al., 1996; Hirata et al., 2005). Consistent with this, the effects of fipronil were mimicked by exposure to strychnine, a GlyR antagonist, but not by exposure to gabazine, a selective antagonist of vertebrate GABA<sub>A</sub> receptors. These findings suggest that rather than binding zebrafish GABA receptors, fipronil may inhibit a structurally related GlyR subtype expressed during development of spinal locomotor pathways. Alternatively, these data may implicate a pharmacologically novel GABA component of the developing spinal circuitry.

**MATERIALS AND METHODS**

**Zebrafish husbandry.** Wild-type zebrafish (AB strain) were maintained according to standard husbandry procedures (Westerfield, 2000) in a recirculating ZMod system (Marine Biotech, Inc., Beverly, MA) at 26°C on a light-dark (14:10 h) cycle. Artificial “system” water was prepared by passing municipal city water through a reverse osmosis system (Aqua Engineering and Equipment Inc., Winter Park, FL) with the subsequent addition of Instant Ocean salts (Aquatic Ecosystems Inc., Apopka, FL) to bring the water conductivity to 1500 µS/cm. System water was maintained at an average pH of 7.0–7.4. Adult male and female zebrafish were spawned using conventional procedures (Westerfield, 2000). Fertilized eggs were collected, cleaned, and staged (Kimmel et al., 1995) and then transferred to glass petri dishes containing fresh system water.

**Chemicals.** Fipronil (98.2% purity; Chem Services, Inc., West Chester, PA) was dissolved in acetone and stock solutions of 4.6µM or 11µM were stored in system water with a final acetone concentration of 0.2%. Stocks were stored at 4°C in the dark. Dilutions were prepared daily from stock solutions. A 10mM stock solution of gabazine (SR95531; Tocris Bioscience, Ellisville, MO) was prepared in sterile distilled water. A 6mM stock solution of strychnine (Sigma-Aldrich, St Louis, MO) was prepared in dimethyl sulfoxide, and a 20mM stock of MS-222 (tricaine methanesulfonate ethyl-3 amino-benzoate; Sigma-Aldrich) was prepared in system water.

**Exposures.** Waterborne exposures to fipronil, strychnine, and MS-222 were conducted in 60-mm glass petri dishes. Each exposure contained 15 embryos and was each exposure performed in triplicate. Exposures were static and solutions were renewed every 24 h. Exposures were initiated at 1–2 h postfertilization (hpf; 8–64 cell stage). Embryos were maintained in the dark at 28.5°C in a temperature-controlled incubator. For the dose-response experiment, embryos were exposed to fipronil continuously through 5 days postfertilization (dpf) at nominal concentrations of 0.007, 0.023, 0.07, 0.23, 0.7, 2.3, and 11µM (corresponding to 0.003, 0.01, 0.033, 0.1, 0.333, 1.0, and 5.0 mg/l, respectively).

To determine the temporal window for fipronil-induced developmental defects, embryos were exposed to 1.1µM (0.5 mg/l) fipronil in 8-h pulses spanning the 0–to 8-, 8– to 16-, 16– to 24-, 24– to 32-, 32– to 40-, or 40– to 48-h interval postfertilization. Embryos were then transferred to clean system water and maintained until 72 hpf.

The effects of fipronil on functional, histological, and morphological aspects of zebrafish development were determined by continuously exposing embryos to 1.1µM fipronil beginning at 1–2 hpf. Animals were then observed or transferred to fixative at the appropriate developmental stage between 24 and 72 h. Embryos (n = 45) were similarly exposed to 70µM strychnine, a compound known to induce locomotor impairment and notochord degeneration in zebrafish (Granato et al., 1996; Hirata et al., 2004).

To overcome the hydrophobicity of gabazine, embryos at one to eight cells were microinjected with gabazine (n > 34 per dose) or sterile distilled water (n > 28) using a PLI-90 picoinjector (Harvard Apparatus, Holliston, MA) and borosilicate glass micropipettes (1.0-mm diameter) fabricated with a P-30 vertical puller (Sutter Instruments, Novato, CA). To determine dose response, 4 nl gabazine was injected at concentrations of 1.25, 2.5, 5, 10, and 20nM. Each concentration was injected with a separate micropipette, and injection volume was established by setting the PLI-90 to deliver a droplet of 100-µm diameter (measured with an eyepiece reticle) into heavy oil. Based on a wet weight estimate of 240 µg for zebrafish embryos (Hagedorn et al., 1997; Petersen and Kristensen, 1998), this resulted in doses of ~21, 42, 84, 167, and 334 nmol/g, respectively. Gabazine-injected embryos were subsequently reared in clean system water.

The potential contribution of tetanic muscle contractions to fipronil-induced spinal defects was evaluated by treating embryos with 1.1µM fipronil for the first 24 h of development and then coexposing embryos to fipronil and 0.6mM MS-222 from 24 to 48 hpf (Hirata et al., 2004; Teraoka et al., 2006).

**Morphological and behavioral analyses.** Embryos and larvae were examined with a Nikon SMZ 800 stereomicroscope and a Nikon Eclipse E600 compound microscope equipped for differential interference contrast (DIC) microscopy. Digital light micrographs were obtained with a Spot RT camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Animals were anesthetized with ~2mM MS-222 for imaging as necessary. Embryos were screened for anatomical defects daily. During pulse-chase experiments (8-h exposure windows), animals were observed every 8 h during the first 48 h of the experiment.

The lengths of individual animals were measured at the end of the dose-response experiment (5 dpf), the pulse-chase experiment (3 dpf), and the gabazine, strychnine, and fipronil/MS-222 coexposure experiments (48 hpf). Lengths were measured from digital micrographs (acquired using SPOT imaging software) as the distance from the anterior end of the mouth to the end of the caudal peduncle along the notochord.

The response to a mechanical stimulus (touch) was used as a measure of sensorimotor integration (Granato et al., 1996; Saint-Amant and Drapeau, 1998). Hatched or dechorionated fish were gently touched on the head with a probe (nylon monofilament inserted into a probe handle). Animals that swam away after one, two, or three repeated stimuli were scored as responders. All others were scored as nonresponders. Selected examples were recorded with digital video microscopy using a Nikon SMZ2800 stereomicroscope equipped with a Unibrain iFire 400 camera and BTV Carbon Pro software.

**Histology, immunohistochemistry, and in situ hybridization.** For general histology, embryos were fixed in 3% paraformaldehyde and 0.75%
glutaraldehyde in a sodium cacodylate buffer (pH 7.4; Electron Microscopy Sciences, Hatfield, PA) and embedded in Spun’s low-viscosity embedding medium (Pelco International, Redding, CA). Sections (∼ 1 μm) were cut with glass knives, adhered to glass slides, and stained with Richardson’s solution (2% methylene blue, 1% azo II, 2% sodium borate; Mallinckrodt Baker, Phillipsburg, NJ). Sections of the notochord were evaluated in 8–12 embryos for each treatment (1.1 μM fipronil, 0.2% acetone, or control) at 24, 30, and 48 hpf.

Whole-mount immunolocalization was carried out as described previously (Incardona et al., 2004). Primary antibodies included antiengrailed 4D9, antitoxin heavy chain mouse monoclonal F59 (both from Developmental Studies Hybridoma Bank, Iowa City, IA), and rabbit polyclonal antilaminin (Sigma-Aldrich). For laminin and engrailed immunocytochemistry, embryos were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1M sodium phosphate buffer at 24 and 30 hpf. For myosin (F59) immunofluorescence, embryos were fixed at 48 hpf in a graded methanol series followed by rehydration. Secondary antibodies were donkey anti-rabbit IgG (antilaminin) or donkey anti-mouse IgG (antiengrailed) conjugated to horseradish peroxidase (both from Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 488–conjugated goat anti-mouse IgG (F59; Molecular Probes/Invitrogen, Carlsbad, CA). Peroxidase-conjugated antibodies were visualized with 0.5 mg/ml diaminobenzidine and 0.003% hydrogen peroxide in tris-buffered saline. Immunoperoxidase-labeled embryos were imaged with DIC optics, and F59 immunofluorescence was imaged using a Zeiss LSM 5 Pascal confocal system (Carl Zeiss Advanced Imaging Microscopy, Jena, Germany). At least 10 embryos were examined for each time point and immunochemical marker.

For in situ hybridizations, embryos were fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer. Hybridization with digoxigenin-labeled riboprobes was carried out using standard protocols (Thisse et al., 1993; Westerfield, 2000). Genes analyzed included collagen 2α1 (coll2α1), tiggy winkle hedgehog (vhh), no tail (ntl), one-eyed pinhead (oep), and floating head (fh). Bound riboprobes were detected with alkaline phosphatase–conjugated anti-digoxigenin antibody (Roche Diagnostics, Indianapolis, IN) with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). Embryos were imaged using DIC microscopy and SPOT digital camera (Diagnostic Instruments) commercial software. Embryos were fixed from each control and fipronil treatment group (n = 12–15 animals) at 24 and 30 hpf for each in situ marker.

Statistics. Statistical analyses of individual fish lengths were performed with one-way ANOVA and Tukey’s honestly significant difference (HSD) post hoc test (Kaleidograph, Syngery Software Technologies, Inc., Essex Junction, VT). To evaluate the dose-dependent effects of fipronil on fish lengths, data were fit with a logistic regression using the formula:

\[ y = \frac{(m_l - (m_m - m_l))}{1 + e^{(r-x-m)}} \]

where \( m_l \) = minimum length, \( m_m \) = maximum length, \( m_s \) = slope, \( m_h \) = concentration halfway between minimum and maximum length, \( y \) = length, and \( x \) = exposure concentration. A nonparametric Kruskal-Wallis rank sum test (Kaleidograph) was used to compare touch response and the prevalence of anatomical abnormalities between exposed and control fish.

RESULTS

Locomotor Defects in Fipronil-Exposed Embryos and Larvae

The locomotor behavior of zebrafish embryos develops in a stereotypical manner (Saint-Amant and Drapeau, 1998). At 17 hpf, embryos show spontaneous contractions of trunk muscles that are independent of sensory stimuli. At 21 hpf, the embryo responds to a touch on the head with rapid tail coils, and at 27 hpf the same mechanical stimulus produces bursts of swimming. The rate of swimming in response to touch becomes maximal by 36 hpf (Saint-Amant and Drapeau, 1998). Hatching-stage larvae (post 48 hpf) thus show a mature escape reflex in response to tactile stimulation, and this serves as a simple assay for sensorimotor integration. Unexposed fish showed a normal touch response at 30, 48, or 72 hpf as indicated by effective swimming movements of the tail (Fig. 1A). In contrast, embryos continuously exposed to fipronil \( \geq 0.7\mu M \) (0.333 mg/l) responded to a head touch with wavelike contractions of the body axis but were incapable of swimming (Fig. 1B and 1C and Supplemental Movie 1). The impairment of touch-induced locomotion was first evident at ~ 30 hpf and continued through to the end of the 5-day exposure. Impaired motor response was dose dependent (Fig. 2), and the prevalence of larvae with an abnormal touch response was significantly higher in fish exposed to \( \geq 0.7\mu M \) fipronil (p < 0.001, Kruskal–Wallis rank sum).

Pulsed fipronil exposures revealed a sensitive period of locomotor deficiency corresponding to the onset and maturation of the touch response. Embryos exposed to 1.1 μM fipronil in 8-h intervals prior to 24 hpf showed normal spontaneous movement at 24 hpf and a normal touch response at 48 and 72 hpf. By contrast, embryos exposed to the same concentration of fipronil at 24–32, 32–40, or 40–48 hpf were unresponsive to touch.

Fipronil-induced locomotor defects were reversible. After return to clean water, all animals exposed from 24 to 32 (n = 42) and 32 to 40 hpf (n = 44) and most of the fish exposed from 40 to 48 hpf (93%, n = 45) showed normal touch responses when tested subsequently at 72 hpf. This indicates that sensorimotor function generally recovers within 24 h.

Reduced Body Length, Notochord Degeneration, and Abnormal Axial Muscle Morphology

There were no indications of anatomical defects in zebrafish embryos exposed continuously to fipronil \( \geq 0.23\mu M \) until ~ 30 h of development. At this point, embryos began to show reduced body length (Fig. 3F), notochord degeneration (Fig. 3G and 3H), and abnormal axial muscle morphology (Fig. 3H–3J). Histology confirmed that notochord morphology was normal at 24 hpf and then degenerative at 30 (data not shown) and 48 hpf (Fig. 3H). Damage to the notochord was evident as a fragmentation of the notochord vacuoles and the presence of fibrous material (Fig. 3H). Myotomes in fipronil-exposed embryos were shorter (Fig. 3I and 3J), and muscle fibers were abnormally sinusoidal in shape (Fig. 3I). Immunofluorescent labeling with an antibody against myosin heavy chain (F59) revealed a disorganization of the superficial slow muscle fibers in fipronil-exposed embryos at 48 hpf (Fig. 3J).

Whereas the effects of fipronil on the embryonic touch response were reversible, degeneration of the notochord was irreversible. For both effects, however, the developmental window of sensitivity was similar. All embryos (n = 134) exposed to an 8-h pulse of fipronil (1.1 μM) prior to 24 hpf were normal in their morphology when examined at 24 hpf. The same
animals were unaffected at 48 hpf with the exception of a few fish that had small areas where one or two notochord vacuoles appeared abnormal. In contrast, all embryos exposed to a fipronil pulse from 24 to 32 hpf (n = 42) and > 75% of the animals exposed at 32–40 hpf (n = 44) or 40–48 hpf (n = 45) showed extensive notochord degeneration, shortened body length, and disrupted muscle morphology when examined at 48 hpf. When animals were transferred to clean water for 24 h and examined again at 72 hpf, notochord degeneration and shortened body length were still present. Shortened body length was dose dependent (Fig. 2), with an EC50 of 0.37 ± 0.037 (SE) μM. The longitudinal body axis of 5 dpf larvae continuously exposed to ≥ 0.23μM fipronil was significantly reduced relative to the controls (p < 0.001, Kruskal-Wallis rank sum). Logistic regression indicated a sigmoidal relationship between fipronil dose and body length (Fig. 2, r² = 0.996).

Finally, significant lethality was only observed at the highest concentration of fipronil (11μM). For this exposure, viability was 7% (n = 44) at 5 dpf (p < 0.001, Kruskal-Wallis rank sum), with most fish dying between 3 and 5 dpf. Survival at all other concentrations ranged from 73 to 100% and were not significantly different from controls (p > 0.05).

Fipronil-Induced Morphological Defects Are Secondary to Abnormal Muscle Contraction

The notochord plays an important structural role that supports locomotion in larval fish. Moreover, during embryonic development, the notochord is the origin of signals involved in patterning axial structures such as the somites and overlying neural tube. Several genes involved in notochord formation and function are often altered by identified mutations in zebrafish.
To explore potential mechanisms underlying notochord defects due to fipronil exposure, we monitored the in situ expression patterns of several genes. Markers of notochord differentiation, including mRNAs for collagen 2a1 (col2a1), no tail (ntl), tiggy winkle hedgehog (twhh), one-eyed pinhead (oph), and laminin protein (data not shown) were unchanged in fish exposed to 1.1 \( \mu \text{M} \) fipronil at 24 and 30 hpf relative to controls. Similarly, fipronil-exposed embryos showed no changes in the number of engrailed-immunopositive muscle pioneer cells in the somites (data not shown), providing additional evidence for normal notochord development (Devoto et al., 1996). These data collectively indicate that fipronil-induced notochord lesions are unlikely to be related to the developmental processes that typically guide normal notochord differentiation.

To determine if notochord lesions and myotome shortening were related to abnormal muscle contraction, we evaluated the effects of pharmacologically inhibiting muscle contractions during fipronil exposure. The anesthetic MS-222, a voltage-gated Na\(^+\) channel blocker, has previously been used at low concentrations to chronically produce a flaccid paralysis in zebrafish embryos and larvae without affecting morphological development (Hirata et al., 2004; Teraoka et al., 2006). Notochord degeneration (Fig. 4C) and reduction of longitudinal body axis (data not shown) were prevented when embryos were coexposed to fipronil (1.1 \( \mu \text{M} \); continuous exposure beginning at 1–2 hpf) and MS-222 (0.6mM; 24–48 hpf). Combined with our other results, this phenotypic rescue indicates that fipronil-induced notochord lesions are likely a mechanical consequence of muscle hypercontractility and not due to a defect in notochord differentiation or intrinsic maintenance.
Fipronil Exposure Mimics the Effects of a Vertebrate Glycine Receptor Antagonist but Not a Potent GABA<sub>A</sub> Antagonist

To determine if the effects of fipronil on embryonic locomotor activity were due to blockade of GABA receptors, we tested the effects of a well-characterized vertebrate GABA antagonist. Of the vertebrate GABA receptor classes most likely to be antagonized by fipronil (GABA<sub>A</sub> and GABA<sub>C</sub>), GABA<sub>A</sub> receptors are known to be involved in spinal locomotor circuits (Grillner, 2003). Therefore, we exposed zebrafish embryos to the classical GABA<sub>A</sub> antagonist gabazine (Heaulme et al., 1986; Krogsgaard-Larsen et al., 2002; Mienville and Vicini, 1987). As observed with other pharmacologic agents (Milan et al., 2003) and consistent with the hydrophilicity of gabazine and presumed poor uptake by fish embryos, waterborne gabazine exposure had no apparent effect on zebrafish development (data not shown). However, microinjection of gabazine over doses ranging from ~21 to 334 nmol/g wet weight into fertilized eggs (n ≥ 34 embryos per dose) produced a marked change in locomotor behavior (the motor activity of embryos injected with water alone was indistinguishable from controls). Moreover, the effects of gabazine on zebrafish embryos were markedly different from the effects of fipronil exposure across the entire dosage range. Rather than showing a reduced touch response and “accordion”-like muscle contractions, gabazine-exposed larvae responded to mechanical stimulus with prolonged and rapid swimming activity when examined at 36–48 hpf. Video microscopy was used to monitor this behavioral response among embryos still within the chorion (Supplemental Movie 2). Notably, this hyperexcitation is consistent with known, seizure-inducing effects of gabazine (Heaulme et al., 1986). At doses of 334 and 167 nmol/g, 100% of exposed larvae exhibited seizures, 84% of larvae showed seizures at 84 and 42 nmol/g, and 70.6% showed seizures at 21 nmol/g. None of the morphological defects associated with fipronil exposure, including notochord degeneration (Fig. 4D), were observed in gabazine-injected embryos. In contrast, exposure to the GlyR antagonist strychnine (70 μM; 1–2 hpf through 48 hpf) resulted in the same suite of defects as fipronil, including impaired touch response, shortened body length, disorganization of muscle fibers (data not shown), and notochord degeneration (Fig. 4E).

DISCUSSION

The phenotypic effects of fipronil on zebrafish embryos include impaired motor response, notochord degeneration, continuously exposed to 1.1 μM fipronil. (C) Normal notochord in an embryo exposed to fipronil and then coexposed to the Na<sup>+</sup> channel blocker MS-222 to prevent muscle contraction. (D) Normal notochord in a gabazine-injected embryo. (E) Notochord degeneration in an embryo exposed to the GlyR antagonist strychnine. nc = notochord. Scale bar = 0.05 mm.
abnormal muscle fiber morphology, and shortened body length. These effects first appear at approximately 30 hpf and coincide with the normal development of both the touch-mediated escape response and the capacity for sustained swimming movements (Saint-Amant and Drapeau, 1998). The mode of action for fipronil appears to be primarily physiological and is characterized by reversible effects on sensorimotor function. This is in contrast to notochord distortions resulting from exposure to dithiocarbamate pesticides, which are dependent on normal muscle contractions, but are most likely due to a pesticide-induced structural defect in the notochord itself (Haendel et al., 2004; Teraoka et al., 2006). The reduced body length and abnormal muscle fiber morphology in fipronil-exposed larvae reflect sustained bilateral contractions of the axial muscles, and this in turn leads to notochord degeneration. The mechanical damage to the notochord is effectively blocked by a pharmacological paralytic (MS-222). While sensorimotor function recovered after presumptive depuration of fipronil, damage to the notochord was irreversible throughout the duration of the pulse experiment (3 dpf) and was still evident at the end of the continuous exposure (5 dpf). Although our analysis indicates that initial differentiation of the notochord and its early signaling functions are unaffected by fipronil, it is possible that fipronil-induced notochord lesions could affect the subsequent motor behavior through structural impact on notochord function.

Although the developmental defects caused by fipronil are consistent with effects on neuromuscular physiology, the precise targets are unclear. Fipronil is by design a selective antagonist of insect GABA receptors. However, treatment with the potent vertebrate GABA<sub>A</sub> receptor antagonist gabazine did not mimic the effects of fipronil in zebrafish embryos, but rather produced a seizure-like activity in response to touch, consistent with the previous observations in mammalian systems (Heaulme et al., 1986). Instead, the suite of fipronil-induced defects was almost identical to the pharmacological effects of the GlyR antagonist strychnine (Granato et al., 1996; Hirata et al., 2005). Moreover, exposure to fipronil or strychnine phenocopied zebrafish accordion class mutants, so named for the abnormal bilateral axial muscle contractions in response to touch (Granato et al., 1996). As with fipronil exposure, accordion class mutants have disorganized axial muscle fibers and notochord degeneration that are prevented by paralysis with MS-222 treatment. The glycineric reciprocal inhibitory pathway in the hindbrain and spinal cord is thought to be involved in these mutants, and the affected loci are therefore potential targets for fipronil. Of the seven originally identified accordion class mutants, three have been characterized at the molecular level. Consistent with the effects of strychnine, a mutation in the GlyR [beta] subunit underlies the defects in the bandoneon mutant (Hirata et al., 2005). However, the zieharmonica and accordion mutants were found to affect acetylcholinesterase (Downes and Granato, 2004) and the sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase 1 (SERCA1) (Gleason et al., 2004; Hirata et al., 2004), respectively. While the bandoneon mutants are unable to relax axial muscle due to loss of central glycineric inhibition of motor neurons, the zieharmonica and accordion mutants display sustained muscle contraction due to elevated acetylcholine levels at the neuromuscular junction and impaired uptake of intracellular Ca<sup>2+</sup> in skeletal myofibers, respectively (Gleason et al., 2004; Hirata et al., 2004).

At the level of analysis performed here, we cannot distinguish between effects on spinal or other CNS circuitry and peripheral effects on the neuromuscular junction or axial musculature. However, the most likely explanation for our results is that fipronil acts as a GlyR antagonist in developing zebrafish. Sequence identity and structural similarity are high among all members of the ligand-gated ion channel superfamily (including GABA<sub>A</sub> and GlyRs), and cross-selectivity among both agonists and antagonists is common (Bueno and Leidenheimer, 1998; Machu, 1998; Sun and Machu, 2000; Yan et al., 1998). It should be noted, however, that the developing spinal and hindbrain circuitry involved in sensorimotor integration and swimming behavior in zebrafish have not been completely characterized, and the effects of fipronil could reflect a novel role for GABAergic signaling. This is unlikely, in part, because of the marked differences between the effects of fipronil and gabazine. Finally, fipronil could act on unidentified targets that are unrelated to neurotransmission.

Zebrafish are proving increasingly useful as a model system to identify novel mechanisms of developmental toxicity in vertebrates (Hill et al., 2005) and fish in particular (Hinton et al., 2005). This is due in part to the fact that patterning, organogenesis, and other developmental processes are very well understood in zebrafish relative to most other fish species. Moreover, as we have shown here, a comparison of the fipronil-induced phenotype to the zebrafish mutant bandoneon is an example of how a chemical-genetic approach can be used to reveal potential cellular targets for poorly characterized toxicants. These and other tools in zebrafish (i.e., reverse genetics) are making it increasingly possible to explore long-standing and difficult challenges in aquatic toxicology such as the mechanistic underpinnings of mixture toxicity (Incardona et al., 2005).

Finally, the aim of this study was to explore potential mechanisms of fipronil-induced developmental toxicity using the zebrafish model. Accordingly, more work is needed to establish the environmental relevance of these results for human health as well as for the health of wild fish that spawn in fipronil-contaminated habitats. Fipronil concentrations as high as 5.3 μg/l (Cary et al., 2004), 8 μg/l (Wirth et al., 2004), and 9 μg/l (Schlenk et al., 2001) have been detected in surface waters downstream of rice fields planted with fipronil-treated rice seed, and the insecticide has been previously been shown to be toxic to invertebrates at these levels. For example, caged crayfish placed in a pond receiving effluent from a field with treated rice containing 9 μg/l fipronil showed significantly higher mortality compared to crayfish similarly exposed to untreated rice field effluent (Schlenk et al., 2001). In the present study, we observed developmental defects in zebrafish...
at nominal concentrations beginning at 333 μg/l, which is well above levels that have previously been measured in the aquatic environment. However, we did not analytically determine dissolved-phase fipronil in our exposure solutions, and the actual concentrations of fipronil (vs. nominal) may have been much lower due to adsorption to the glass exposure chambers and the partitioning of the pesticide to other matrices (i.e., air). Also, native species may be more sensitive than zebrafish to the effects of fipronil, as has been previously shown for dioxins and salmonids (Henry et al., 1997). Irrespective, our findings indicate that fipronil is a developmental neurotoxicant in vertebrates and that future investigations should focus on axial muscle hyperexcitation as a toxicological end point for fish in aquatic habitats contaminated with this insecticide.

**SUPPLEMENTAL DATA**

Supplemental data includes two .mov (Quicktime) movie clips. Movie 1 (Fipronil touch response) illustrates the normal touch response behavior of untreated 48 hpf zebrafish embryos (dechorionated) compared to embryos exposed from 2 to 48 hpf to 1.1μM fipronil. Movie 2 (Gabazine touch response) illustrates the touch response behavior of 48 hpf embryos in the chorion injected with sterile distilled water compared to embryos injected with – 240μM gabazine. Supplemental data are available online at www.toxsci.oxfordjournals.org.

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**REFERENCES**


