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Effects of Fipronil and Chlorpyrifos on Endocrine-Related Endpoints in Female Grass Shrimp (*Palaemonetes pugio*)

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Organophosphate (OP) insecticides are widely used throughout the United States as agricultural and urban pesticides, and are among the highest priority pesticides for review under the 1996 Food Quality Protection Act. OPs exert their toxicity through inhibition of acetylcholinesterase (AChE), an enzyme involved in terminating nerve impulse transmission at cholinergic synapses (Kennedy 1991). Chlorpyrifos is a potent OP recently restricted to agricultural applications. It has a 96-hr LC_{50} (95% C.I.) of 0.37 μg/L (0.30-0.44) for the grass shrimp, *Palaemonetes pugio*, a dominant macropelagic invertebrate in estuarine tidal creek systems (Key and Fulton 1993).

Phenylpyrazole insecticides are rapidly gaining use throughout the United States and generally target γ-aminobutyric acid (GABA)-gated chloride channels in arthropods (Cole et al. 1993). As one of the first phenylpyrazoles introduced for pest control, fipronil has a high affinity for insect GABA-receptors (Gant et al. 1998) and is currently registered in the U.S. for residential fire ant control, turf grass management, and rice cultivation. Fipronil is moderately toxic to adult crayfish *Procambarus clarkii* (Schlenk et al. 2001) and highly toxic to grass shrimp *P. pugio* (Key et al. 2003) with a 96-hr LC_{50} (95% C.I.) of 63.7 (41.3-86.1) and 0.32 (0.15-0.49) μg/L, respectively.

The objectives of this study were to evaluate potential sublethal effects of chlorpyrifos and fipronil on endocrine-mediated processes in female grass shrimp, *Palaemonetes pugio*. The occurrence of gravid females, body weight and length, cholesterol, ecdysteroids, and vitellogenin in gravid females were determined to elucidate possible endocrine responses to chronic chlorpyrifos or fipronil exposure. *Palaemonetes pugio* is an ecologically important decapod species that inhabits estuaries from Nova Scotia to Texas (Williams and Wigley 1977) and plays a major role in nutrient cycling within salt marsh ecosystems (Welsh 1975).

MATERIALS AND METHODS

Chlorpyrifos and fipronil tests were performed in November 2000 and 2001, respectively. Chronic exposure tanks consisted of 70-L aquaria containing 20 ‰

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seawater and biological filtration systems driven by an Aquaclear 300™ filter. All treatment tanks [0 ng/L, 100 ng/L, and 200 ng/L nominal chlorpyrifos or fipronil concentrations (n=3 replicate tanks/treatment)] were pre-dosed for three weeks prior to the inclusion of shrimp in order to create a constant exposure system. After the acclimation period, shrimp were added to each replicate tank (12 males and 12 females for the chlorpyrifos test; 17 males and 17 females for the fipronil test). Adult shrimp used for both tests were from the same initial hatched cohort. Female and male *P. pugio* were exposed to 0 ng/L (acetone control), 100 ng/L, and 200 ng/L nominal chlorpyrifos or fipronil for 45 d. All treatments were subjected to white fluorescent lighting under a 12-hr light:12-hr dark cycle throughout the bioassay. Technical grade chlorpyrifos (99.8%) and fipronil (98.0%) were obtained from DowElanco Company (Indianapolis, IN, USA) and ChemService, Inc. (West Chester, PA, USA), respectively. A chlorpyrifos and fipronil stock solution (10 mg/L) was prepared in 100% acetone and the doses were administered to obtain an acetone concentration below the NOEC of 0.01% (Mayer 1987) in each treatment and control. Chlorpyrifos or fipronil stock solutions were used to dose the treatment tanks daily at a nominal concentration of 100 or 200 ng/L. Water quality parameters [salinity (‰), temperature (°C), dissolved oxygen (mg/L), and pH] were measured daily in the control tanks. Water quality parameters from chlorpyrifos control tanks averaged (± SD) 20.5 (0.7) ‰, 24.6 (0.6) °C, 8.1 (0.1), and 6.4 (0.6) mg/L, for salinity, temperature, pH, and dissolved oxygen, respectively. Water quality parameters from fipronil control tanks averaged (± SD) 20.3 (0.4) ‰, 23.2 (0.4) °C, 8.0 (0.2), and 6.9 (0.3) mg/L, for salinity, temperature, pH, and dissolved oxygen, respectively.

Fifty-mL water samples (pre- and post-dose) from each treatment were analyzed for chlorpyrifos (every 7 d during the bioassay) or fipronil (every 2 d for the first week) concentrations. Chlorpyrifos samples were immediately extracted onto C-18 solid phase extraction cartridges and eluted with pesticide-grade ethyl acetate. δ-Hexachlorocyclohexane (HCH) was added as an internal standard for quantification. Extracts were solvent exchanged into iso-octane under nitrogen. Fipronil samples were immediately processed by a liquid 5:1 methyl-tert butyl ether (MTBE) extraction. Hexachlorocyclopentadiene (HCCPD) was added as an internal standard for quantification. Chlorpyrifos and fipronil samples were both analyzed by gas chromatography with electron capture detection (GC-ECD). Total aqueous chlorpyrifos averaged (± SD) 117.36 ± 21.00 ng/L (100 ng/L nominal) and 193.09 ± 28.69 ng/L (200 ng/L nominal). Total aqueous fipronil averaged (± SD) 97.90 ± 20.84 ng/L (100 ng/L nominal) and 142.98 ± 40.52 ng/L (200 ng/L nominal). Chlorpyrifos (DL=0.3 ng/L) and fipronil (DL=10 ng/L) were not detected in the control tanks. Internal standard recovery efficiencies were >80% for both chlorpyrifos and fipronil.

Male and female grass shrimp survival was assessed daily, and observed mortalities were removed and recorded. Female *P. pugio* were immediately removed without replacement once identified as holding a newly extruded clutch

(<24-hr old) under the abdomen. Weight and length were recorded and a unique identifying number was assigned to each gravid female. The clutch from each female was gently teased from the abdomen, and females were stored at –70°C until further analysis. Whole *P. pugio* gravid females (without clutch) (2 females/replicate tank/treatment) were homogenized three times in cold (4°C) extraction buffer (0.05 M Tris-HCl, pH 7.5) and final homogenates were centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was filtered through a 0.45-μm HT Tuffryn® Membrane (Gelman Laboratory, Ann Harbor, MI, USA), transferred to a new 1.5 mL centrifuge tube, and stored at -70°C until further analysis.

Protein concentrations for all gravid female homogenates were determined using a fluorescent NanoOrange® protein reagent (NanoOrange® Protein Quantitation Kit, Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Samples were read against a bovine serum albumin (BSA) standard curve (range: 0.01-10 μg/mL; R^2=1.0000) using a BIO-TEK FL_x800 fluorescence microplate reader (485/528 nm filters; BIO-TEK Instruments, Winooski, VT, USA). Cholesterol concentrations in the gravid female homogenates were determined using a fluorescent Amplex™ Red cholesterol assay (Amplex™ Red Cholesterol Assay Kit; Molecular Probes) according to the manufacturer's instructions. Samples were read against a cholesterol standard curve (range: 0.005-10 μM; R^2=0.9959) using a BIO-TEK FL_x800 fluorescence microplate reader (560/590 nm filters; BIO-TEK Instruments).

An ecdysteroid ELISA was designed following Kingan and Adams (2000). ELISA plates (96-well, black with clear flat-bottom; Corning Costar, Corning, NY, USA) were coated overnight at room temperature with 90 μL/well F_c-specific goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:435 in coating buffer [10 mM phosphate-buffered saline (PBS), pH 7.5]. The coating solution was discarded and blocking buffer (25 mM PBS, pH 7.5; 1 mM EDTA; 0.1% BSA) was added to each well (300 μL/well). The plate was incubated for 1 hr at room temperature while shaking. After 3 washes with 0.05% Tween-20/PBS (PBST), blocking buffer was added to each well (100 μL/well) designated as blanks, and gravid female homogenate diluted in blocking buffer was added in triplicate to wells (50 μL/well) designated as samples. Polyclonal anti-ecdysteroid rabbit IgG diluted 1:1100 in blocking buffer was added to wells (50 μL/well) designated as samples. A 20-hydroxyecdysone (20HE)-horseradish peroxidase conjugate diluted 1:1100 in blocking buffer was added to all wells (50 μL/well), including blanks. The competition reaction was incubated overnight at 4°C. After 3 washes with PBST, QuantaBlu™ fluorogenic peroxidase substrate (Pierce, Rockford, IL, USA) was added to each well (100 μL/well) and developed for 2 hr at room temperature while shaking. After development, QuantaBlu™ stop solution (Pierce) was added to each well (100 μL/well) and immediately read against an ecdysteroid ELISA standard curve (range: 4-4000 pM; R^2=0.9995) using a BIO-TEK FL_x800 fluorescence microplate reader (360/460 nm filters; BIO-TEK Instruments).

Vitellogenin concentrations in gravid females were determined using an anti-crustacean vitellogenin ELISA. ELISA plates (96-well, black with clear flat-bottom; Corning Costar) were coated with 100 µL/well coating buffer (2 µg/mL *P. pugio* vitellogenin diluted in 50 mM sodium carbonate, pH 9.6) overnight at room temperature while shaking. Coating buffer was discarded and blocking buffer (0.1% BSA/TBS) was added to each well (300 µL/well) and incubated for 1 hr at room temperature while shaking. After 3 washes with TTBS, 0.1% BSA/TBS was added to each well (150 µL/well) designated as blanks and gravid female (75 µL/well) homogenate diluted in 0.1% BSA/TBS was added in triplicate to wells designated as samples. Anti-*Leptocheirus plumulosus* (amphipod) vitellin IgG (~4 mg/ml; Volz et al. 2002a) diluted 1:2000 in 0.1% BSA/TBS was added to wells (75 µL/well) designated as samples. The competition reaction was allowed to incubate for 2 hr at room temperature while shaking. After 3 washes with TTBS, a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (BIO-RAD) in 0.1% BSA/TBS was added to all wells (150 µL/well), including blanks, and incubated for 1 hr at room temperature while shaking. After 2 washes with TTBS and 1 wash with TBS, the fluorescent substrate, 4-methylumbelliferyl phosphate (4-Methylumbelliferyl Phosphate Liquid Substrate System; Sigma-Aldrich, St. Louis, MO, USA), was added to each well (100 µL/well) and developed for 1 hr in the dark at room temperature while shaking. After development, 0.4 M NaOH (pH 13) was added to each well (100 µL/well) and immediately read against a *P. pugio* vitellogenin ELISA standard curve (range: 1-1000 ng/mL; R^2=0.9971) using a BIO-TEK FL_x800 fluorescence microplate reader (360/460 nm filters; BIO-TEK Instruments).

All statistical procedures were performed using SigmaStat® Statistical Software Version 2.0 (SPSS Science, Chicago, IL, USA). Data were pooled within each treatment after finding no significant differences among treatment replicates. One-way analysis of variance (ANOVA) and multiple comparison procedures were used to test for differences in gravid female production, and gravid female vitellogenin, cholesterol, and ecdysteroid titers ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Chlorpyrifos treatments were not significantly toxic (p=0.197) but fipronil significantly decreased (p=0.01) adult survival at 200 ng/L (19.6% decrease relative to controls). Gravid female body weight and length were not different across all chlorpyrifos (weight: p=0.47; length: p=0.63) and fipronil (weight: p=0.06; length: p=0.31) treatments. In addition, chlorpyrifos or fipronil at 100 or 200 ng/L did not significantly (p=0.98 and p=0.87, respectively) affect total gravid female production based on total survival over 45 d (Figure 1). Protein concentrations were not dose-dependent, and all biochemical endpoints were

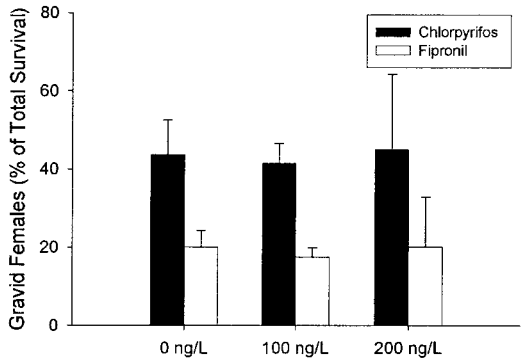

Figure 1. Percent of *Palaemonetes pugio* gravid females (<24-hr old clutch removed) after exposure to fipronil or chlorpyrifos for 45 d. Error bars = ± SD. There were no significant differences from treatment controls.

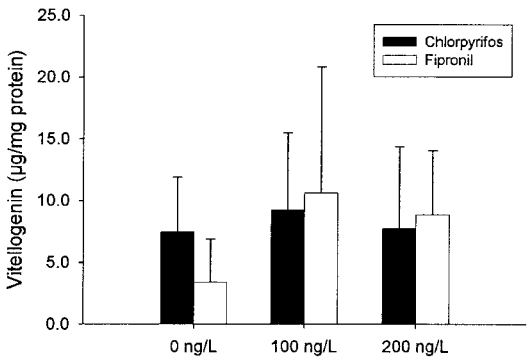

Figure 2. Vitellogenin concentrations in *Palaemonetes pugio* gravid females (<24-hr old clutch removed) after exposure to fipronil or chlorpyrifos for 45 d. Each treatment bar represents the mean of six gravid females. Error bars = ± SD. There were no significant differences from treatment controls.

normalized against protein. Chlorpyrifos or fipronil did not significantly increase or decrease gravid female vitellogenin concentrations (Figure 2). While chlorpyrifos or fipronil did not affect gravid female cholesterol titers ($p=0.82$ and $p=0.31$, respectively), there were significant ($p=0.04$) increases in ecdysteroid titers after exposure to 100 and 200 ng/L nominal chlorpyrifos (Figure 3). Enhanced ecdysteroid titers were not observed under fipronil exposure.

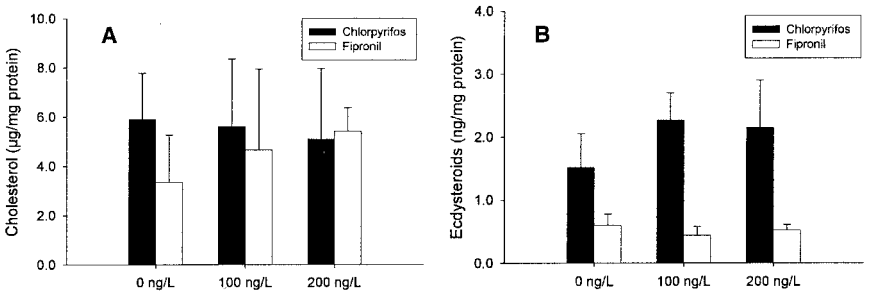

Figure 3. Cholesterol (A) and ecdysteroid (B) titers in *Palaemonetes pugio* gravid females (<24-hr old clutch removed) after exposure to fipronil or chlorpyrifos for 45 d. Each treatment bar represents the mean of six gravid females. Error bars = ± SD. *Significant difference from chlorpyrifos control ($p < 0.05$).

We observed no biologically-significant chlorpyrifos or fipronil effects on female grass shrimp egg production and related reproductive parameters (vitellogenin, cholesterol, and ecdysteroids). Although ecdysteroid titers were higher in gravid females exposed to chlorpyrifos, we observed no significant effects on body weight and length. Unfortunately, we did not track female molting stages and the corresponding ecdysteroid titers, limiting insight into chlorpyrifos-induced developmental effects. Thus, it appears as though—at least under cool fluorescent, laboratory light conditions—chlorpyrifos and fipronil do not interact with endocrine-related endpoints in female grass shrimp. However, we recently reported that endosulfan significantly affects *P. pugio* female egg production—and female cholesterol and vitellogenin—*only* in the presence of ultraviolet (UV-A) light (Volz et al. 2002b). As such, the reproductive effects of chlorpyrifos and fipronil should be investigated under UV exposure—an environmentally-realistic parameter that increases bioassay sensitivity. In addition, chlorpyrifos and fipronil have known environmental degradation products that may exhibit equal or greater chronic toxicity to grass shrimp (US EPA 1996; Nhan et al. 2002).

Lastly, while not initially interested in bioassay comparisons, we observed consistent differences between bioassay *controls* for all reproductive parameters. Although grass shrimp used for both tests were from the same hatched cohort, chlorpyrifos and fipronil tests were run in November 2000 and 2001, respectively. As grass shrimp fertility decreases with age (Bauer and Abdalla 2000), delayed breeding in the fipronil test probably caused lower control egg production. In addition, as cholesterol, ecdysteroids, and vitellogenin were all quantified simultaneously, endocrine-related differences were likely due to age-related effects and not analytical variation.

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the Agency's required peer and policy review and therefore does not necessarily reflect the views of the U.S. EPA and no endorsement should be inferred.

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