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Phenylpyrazole Insecticide Fipronil Induces Male Infertility in the Estuarine Meiobenthic Crustacean *Amphiascus tenuiremis*

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Copepods are the most abundant arthropods on earth and are often the most important secondary producers in estuarine/marine food webs. The new GABA (γ-aminobutyric acid)-disrupting insecticide fipronil (FP) induces unique sex-specific reproductive dysfunction in male meiobenthic copepods, leading to trans-generational population depression at environmentally realistic concentrations (0.63 μg/L). Using a newly developed 96-well microplate lifecycle bioassay, more than 700 individual Stage-I juveniles were reared to adulthood in as short as 12 days in only 200 μL of control (CTL) or 0.63 μg-FP/L seawater solution. Individual virgin male: female pairs were then cross-mated for all possible combinations within and across rearing treatments and allowed to mate for an additional 12 days in CTL or 0.63 μg-FP/L solution. FP at 0.63 μg/L caused no significant lethality to any mating combinations but evoked 73% or 89% inhibition of reproduction when FP-reared *males* were mated with either a control- or FP-reared female in FP solution, respectively. In contrast, when *CTL-reared* males were mated with FP-reared females in FP solution, there was no difference in reproductive success compared to FP-free controls. When FP-reared males were mated with either female group in *FP-free* solution, these mating pairs displayed a 3-day delay in time to brood sac extrusion but ultimately did reproduce. As fipronil (1) has a high K_{ow}, (2) is persistent in sediments where meiobenthic copepods live, and (3) has been detected in estuarine waters >0.7 μg/L, it may pose high risk to copepod production in estuarine systems.

Introduction

In 1996, the U.S. Environmental Protection Agency (EPA) first approved use of the phenylpyrazole insecticide fipronil (FP) for control of rice water weevil (*Lissorhoptrus oryzophilus*), commercial turf-grass management, and Frontline Top Spot tick and flea control for pets (*1*). A recent U.S. EPA registration addendum further approved granular and bait applications of FP for broadcast control of domestic fire ants and termites (*2*). Fipronil—developed by Rhone Poulenc, Inc., in 1987—is a γ-aminobutyric acid (GABA)-disrupting insecticide similar in toxicity to classic cyclodienes such as dieldrin and endosulfan but with >500-fold selective toxicity to insects and low toxicity to mammals (*3*, *4*). FP acts by blocking the regulatory action of GABA-gated chloride channels in nerve cell membranes, leading to convulsions and eventual death (*5*, *6*). FP contains a unique trifluoromethylsulfinyl group that is not present in other similar organochlorine pesticides. Ultraviolet radiation induces photoextrusion of the sulfinyl group, resulting in a desulfinyl derivative that is as potent as the parent compound to insect GABA receptors but is 10-fold more potent than FP at mice chloride channels, resulting in a narrower window for selective toxicity between insects and mammals (*7*). Moderate persistence of fipronil (photodegradation in sediment (half-life): 34 days, (*1*)) coupled with equipotency of the photoderivative facilitate "single applications for long-term pest control efficacy" (manufacturer label, Chipco TopChoice, Bayer Crop Science). Fipronil has been measured as high as 5.29 μg/L adjacent to FP-treated rice fields in South Louisiana and 0.79 μg/L 20 miles downstream in estuarine surface waters. Although the parent compound was not detected in bedded Louisiana sediments, the reductive sulfide degradation product—which exerts greater acute toxicity than fipronil to estuarine invertebrates—was detected at concentrations ranging from 0.64 to 24.8 μg/kg (*8*). Fipronil was licensed by the U.S. EPA with data from one nontarget toxicological test model for estuarine and marine invertebrates, the demersal shrimp *Mysidopsis bahia* (*1*). Further research regarding fipronil effects on other crustaceans in estuarine ecosystems is necessary to obtain a comprehensive understanding of possible risks caused by widespread FP application and persistence in coastal settings.

Previously, it was found that FP affects development and reproduction of the estuarine, meiobenthic harpacticoid copepod *Amphiascus tenuiremis* (*9*). Specifically, FP caused significant delays in development from juvenile to adult at concentrations >0.22 μg/L and, more importantly, caused a 94% decrease in female egg production at 0.42 μg/L. Many studies have examined sublethal toxicant effects on *A. tenuiremis*' lifecycle and reproduction due to its culturability (*10*−*14*); but none have investigated toxicant effects on sex-specific or multigenerational reproductive success. A recently developed, 96-well microplate-based lifecycle bioassay technique was used here for controlled, trans-generational rearing and individually paired virgin male:female mating. This bioassay tested for sex-specific FP effects on growth rate, sexual development, fertility, teratogenesis, hatching success, reproductive output, and trans-generational population growth. Each microwell housed a single copepod as it developed from the egg, nauplius, or first copepodite stage (juvenile) to adult. After development into reproductively mature adults, monitored pairwise mating experiments and multigenerational data collection allowed for robust estimates of population level exposure effects.

The objectives of this study were to determine (1) whether fipronil-induced reproductive depression in *A. tenuiremis* is sex-linked, (2) whether a rearing history in fipronil has carry-over effects on fertility when copepods are mated in FP-free seawater, (3) whether any negative effects of fipronil exposure can be remediated by copepod removal from fipronil, and (4) whether offspring of fipronil-exposed (reared and mated) parents exhibit differential sensitivity to fipronil.

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Experimental Methods

Test Organism. *A. tenuiremis* cf. Mielke (1974) is an infaunal sediment-dwelling harpacticoid copepod that is well suited for evaluating sublethal reproductive and developmental toxicity of sediment associated contaminants due to its moderate acute sensitivity, high chronic sensitivity, ease of culturing in sediments or seawater, 17-day lifecycle at 25 °C, and small size (0.4 μm) (*15*). Specifically, in 96-well microplates at 25 °C, Stage-I copepodites (juveniles) become reproductively mature in 7 days and male:female pairs produce hatched nauplii in 2−3 days. Additionally, harpacticoid copepods are usually the second most abundant meiofaunal taxon in sedimentary habitats of coastal oceans and are often the primary prey items for juvenile fishes in estuarine ecosystems worldwide (*16*).

Collection of Test Organisms. Juvenile *A. tenuiremis* (Stage-I copepodites) were collected directly from monoculture sediments by size exclusion and photoextraction (*17*) using a 90:75:63 μm stainless steel sieve series and incandescent fiber optic light pipes, respectively. The 63 and 75 μm retained fractions were transferred to separate 60-mm Petri dishes and illuminated on a dissection stereomicroscope. Stage-I copepodites—which are negatively phototactic and can swim—were separated from sediment particles and later-stage crawling nauplii using a silanized Pasteur pipet.

Preparation of Nominal Treatment Solutions. A 1 g/L parent fipronil stock dissolved in 100% acetone was made using technical-grade fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile; 98.0% pure) obtained from ChemService, Inc. (West Chester, PA). A diluted, working stock solution of 10 μg-FP/mL was used to spike nominal treatment solutions. An acetone carrier control (0.0 μg-FP/L) and a 0.6 μg-FP/mL nominal solution were made by adding the appropriate amount of acetone (6 μL) or fipronil stock (6 μL) to 100 L of aerated, 0.45 μm filtered synthetic seawater (30 ppt, Instant Ocean). All spiked solutions were vortexed for 4 h in the dark at 20 °C.

Rearing of F0 Juveniles. Rearing of Stage-I copepodites in microwells followed methods described in Chandler et al. (*9*). Briefly, 12 (6 per treatment) 96-well low-binding microplates (Corning Costar) were hydrated for 1 h with deionized water, emptied, and then re-loaded with one copepodite per testing microwell—either acetone carrier control ($n = 360$) or 0.60 μg-FP/L ($n = 348$). Excess transfer seawater was ~98% removed from each well using a gastight Hamilton microsyringe and reloaded with 200 μL of control or treatment solution. Microplates were placed in an incubator at 25 °C with a photoperiod of 12 h:12 h. Each copepodite was fed 2 μL of a fresh algae mixture (~10^7 cells of 1:1 *Isochrysis galbana*:*Dunaliella tertiolecta*) on day 0 of the bioassay and every 6 days thereafter over the experiment. Every 3 days, ~90% of the overlying treatment solution was removed and replaced with 200 μL of fresh treatment solution to ensure proper water quality and a consistent FP exposure. Isolated copepodites were allowed to develop in microwells for 12 days, and survival was checked daily. Dissolved oxygen, salinity, and pH were measured in fresh treatment solutions every 3 days to ensure suitable water quality (*18*). GC-ECD measured fipronil loss at 72 h from microplate binding and/or degradation was 15.8 ± 3.6%.

Individualized Cross-Mating of Virgin Adults. By day 12 of the bioassay, 99.7% (CTL) and 98.4% (FP) of individual surviving copepodites had molted into reproductively mature adults. Virgin males and females were removed from both CTL and FP-exposed microplates and transferred within sex to four 50 mL crystallizing dishes containing their respective treatment solutions. A single male or female copepod was removed from crystallizing dishes and placed individually

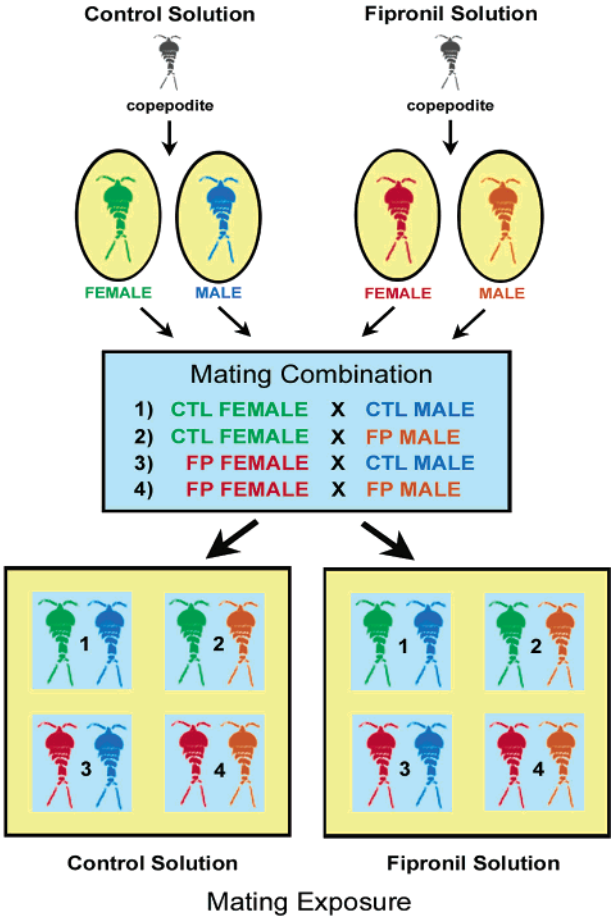

FIGURE 1. Flow diagram of the cross-mating experimental design used to test the importance of exposure rearing history and sex-linked effects on reproductive success. Individual Stage-I copepodites were reared to the adult stage in either control or fipronil solution for 12 days. The adults were then paired together in one of four described mating combinations and transferred to either fresh CTL or FP solution for 12 days.

pairwise into mating combination- and exposure-specific microwells (Figure 1). This was performed for each mating combination and condition ($n = 34$, 36, 34, or 22 pairs for mating combinations 1, 2, 3, or 4, respectively, in CTL solution and 31, 34, 25, or 17 pairs for mating combinations 1, 2, 3, or 4, respectively, in FP solution). Wells were loaded with 200 μL of fresh CTL or FP treatment solution, and each mating pair was fed 2 μL of algae mixture as described above. Using an inverted stereomicroscope, each mating well was visually monitored over 12 days at the same time each day for the following endpoints: survival, abnormal behaviors, reproductive success (percent of females producing a viable clutch), postmating days to female brood sac extrusion, days from brood sac extrusion to naupliar hatch, first clutch brood size (number of hatched nauplii/female), and percent hatching success (no. of hatched nauplii/ no. of hatched nauplii + no. of unhatched embryos).

F1 Transgenerational Fipronil Effects. Hatched nauplii from three mating pair combinations (*CTL-reared* mating pair in CTL solution and both the *CTL-reared female* × *FP-reared male* and *FP-reared* mating pair in FP solution) were allowed to develop into F_1 Stage-I copepodites in their respective treatments. Development time from hatch to Stage-I copepodite was ≤11 days in both the control and fipronil solutions. Sibling copepodite cohorts were collected from microwells, pooled/mixed together with other cohorts within their respective treatments in 50 mL crystallizing

dishes, and then transferred at random to fresh microwells containing their respective same-treatment solutions (i.e., CTL or 0.63 μg-FP/L). Once all F_1 Stage-I copepodites had developed into reproductively mature adults ($\leq$12 days), F_1 virgin males and females were paired randomly *within* treatments. Mating pairs were allowed to mate for an additional 12 days, and the above endpoints for the F_0 mating pairs were recorded for each F_1 mating pair.

Stage-Structured Population Growth Model. Fipronil effects on *A. tenuiremis* population growth were estimated using microplate lifecycle data fitted to a matriarchal stage-structured Leslie-matrix model (RAMAS EcoLab 2.0, Applied Biomathematics, Setauket, NY) (*19*, *20*, *21*). The 3-stage (copepodite to virgin-female to gravid-female) matrix model determined projected population abundances of females based on (a) stage-specific survival rates, (b) the proportion of copepodites developing into virgin females (i.e., capturing sex ratio shifts), (c) the proportion of females producing a viable clutch, and (d) fecundity (i.e., hatched nauplii/female). Our use of this model here conservatively assumes that all nauplii developed into copepodites (unlikely in laboratory and field settings); thus, final population projections should be compared relative to the "CTL-reared mating pair in CTL solution" rather than accepted as absolute field abundance projections under FP exposure. Lifecycle toxicity test data were used to derive instantaneous rates of population increase (λ) to predict population growth for each mating combination/exposure for each replicate plate. The model simulation was run 10 times using each replicate plate's lifecycle test data for a total of 30 simulations for each mating combination in each mating solution. Population growth was modeled through three generations beginning with 60 Stage-I copepodites. Model constraints included logistic density dependence, demographic stochasticity, an environmental carrying capacity of 10 000 individuals, and 50 computer replications of the Leslie-matrix model (*22*).

Statistical Analyses of Fipronil Effects. Data were either arcsine square root or square root normalized and analyzed using a general linear model (GLM) mixed procedure with generation, mating exposure, mating combination, and plate replicates as fixed effects ($\alpha = 0.05$). Interactions among all four fixed effects were examined. Data sets with three or less observed data points—as a result of fipronil reproductive interruption—were removed from the statistical analysis in order to prevent invalidation of statistical assumptions for the following: days to brood sac extrusion, days from extrusion to naupliar hatch, brood size, and hatching success. Three of the mating combinations were removed from the statistical analysis due to insufficient reproductive success ($n \leq 3$) linked to FP exposure: the mating combinations of *FP-reared females* $\times$ *FP-reared males* mated in FP solution for both F_0 and F_1 and *CTL-reared females* $\times$ *FP-reared males* mated in FP solution for the F_1 generation only. Leslie life-stage matrix results were analyzed using ANOVA and Tukey multiple comparison procedures. Statistical significance was determined if $p < 0.05$.

Fipronil Analysis. Nominal FP spike concentrations were measured using a liquid:liquid methyl *tert*-butyl ether (MTBE) extraction technique. Briefly, fresh CTL and FP solutions were sampled in triplicate and collected (3 mL) into 20-mL amber vials at test initiation and every 3 days immediately prior to solution renewal. Due to the low fipronil concentrations used in the microplate bioassay, treatment samples were analyzed using a liquid 2:1 MTBE extraction. Fipronil was not measured directly from microplate wells due to limiting individual microwell volumes (0.2 mL); however, triplicate samples of eight pooled microwells (total volume = 1.5 mL) showed 84.2 $\pm$ 3.6% fipronil recovery efficiencies after 72 h in microwells. For each replicate sample, an internal standard (0.25 μl/L 4-bromoaminosole) was added to the seawater: MTBE mixture. Samples were vortexed for 1.5 min and sonicated for 5 min at 23 °C. The MTBE layers containing extractable analytes were transferred to 15-mm i.d. amber crimp-top GC vials and analyzed for fipronil analytes using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a ^{63}Ni electron capture detector (GC-ECD) (Hewlett-Packard, Palo Alto, CA) operated in splitless mode. The carrier gas was helium with injector and detector temperatures at 230 and 310 °C, respectively. A DB-5 30 m $\times$ 0.25 mm i.d. 0.25-μm film thickness column (Agilent Technologies, Palo Alto, CA) was operated at an initial temperature of 100 °C for 1 min with a 10°C/min increase to 270°C and a 10-min hold. Retention time for fipronil under these conditions was 12.8 min; extraction efficiencies for fipronil were 96.7 $\pm$ 1.5%.

Results

Chemistry Results. FP was not detected in any measured acetone carrier control solutions (detection limit = 0.1 μg-FP/L). The measured mean FP seawater concentration was consistent across 3-day samples at 0.63 $\pm$ 0.05 μg/L over the entire test matrix.

Bioassay Results. There were no filial FP effects on copepod survival ($P = 0.21$); mean percent survival for the parent (F_0) and F_1 generations were 98.5% and 95.5%, respectively. The combined F_0 and F_1 mean survival rates for CTL and 0.63 μg-FP/L were 99.5% and 96.2%, respectively. Similarly, there were no significant differences in mean survival among any of the mating combinations nested within mating exposure ($P = 0.61$). Mating combination mean percent survival across both mating exposures ranged from 95.3% to 100%.

There were no significant trans-generational differences in the proportion of mating pairs that produced viable offspring for the *CTL-reared* mating combination mated in CTL solution ($P = 1.00$). Within the 0.63 μg FP/L mating solution, there were no filial effects on viable offspring production for the *CTL-reared females* $\times$ *FP-reared males* mating combination ($P = 0.10$). However, the *FP-reared* mating combination did show statistically significant higher production of viable offspring in the parental generation compared to the F_1 generation ($P = 0.03$). This difference in reproductive success between the generations would not have a large differential impact on population growth as neither generation had higher than 20% of mated pairs produce viable clutches; such low offspring production would have similar negative implications for each generation. In FP mating solution, there was a significant decrease in mean reproductive success for *FP-reared males* mated to either *CTL-reared* ($P = 0.0006$; 73% decrease) or *FP-reared* ($P = 0.0001$; 88% decrease) females when referenced against CTL pairs in CTL solution (Figure 2). In contrast, there were no significant differences in reproductive success among any of the mating combinations when adult pairing was performed in *clean* seawater for 12 days; mean percent brood sac extrusion success in clean seawater ranged from 83.3% to 97.0% (P values all > 0.50).

Although the percent of females extruding brood sacs in FP-free solution was not significantly different among mating combinations, there was a significant 3-day delay ($P < 0.0001$) from mating to brood sac extrusion when CTL- or FP-reared females were mated with FP-reared males in *CTL* solution (Figure 3a, $P < 0.0001$). Additionally, a similar 3-day delay was seen when CTL-reared females were mated with FP-reared males in *FP* solution ($P < 0.0001$). Although not included in the statistical analysis due to minimal reproductive success (i.e., low n), the *FP-reared females* $\times$ *FP-reared males* mating pairs in *FP* solution had a similar, strong delay in viable brood sac extrusion (3.3 days) as compared to controls (1.3 days) in CTL solution (Figure 3b). In contrast, when CTL- or FP-reared females were mated with CTL-reared

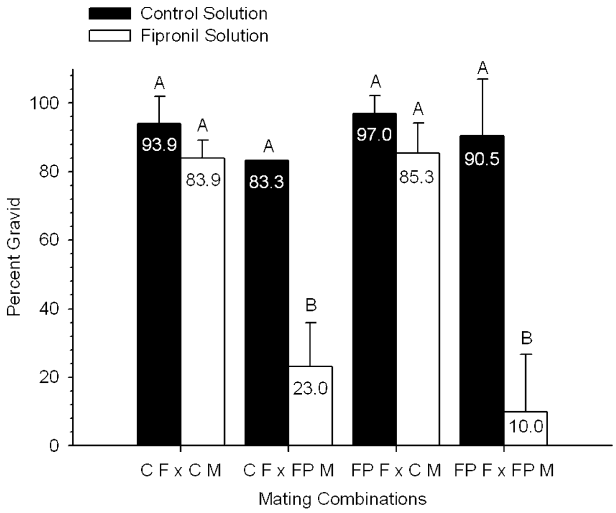

FIGURE 2. Reproductive success [measured as the mean percent of surviving females that produced viable offspring (±1 SD, $n = 3$ replicate microplates)] for each mating combination across generations mated in either CTL or 0.63 μg-FP/L solution. Mating combinations mated in FP solution containing a FP-reared male showed significant 73–88% decreases in reproductive success when compared to the control mating combination in CTL solution. Mating combination CF × FPM (Control Solution) has no error associated as each replicate microplate had 83.3% of its mating pairs produce viable brood sacs. Bars sharing any common letters are not significantly different ($p < 0.05$).

males in *CTL* solution, >72% of females extruded brood sacs within 1 day of pairing (Figure 3a). Likewise, there were no differences in days to brood sac extrusion when FP-reared females were mated with CTL-or FP-reared males in either treatment solution (Figure 3b, $P = 1.00$).

Days to naupliar hatch once brood sacs were extruded was not significantly different among mating combinations nested within CTL or FP mating exposures ($P = 0.667$). Mean naupliar hatch times ranged from 1.2 days for the *CTL-reared* mating pairs to 1.8 days for the *FP-reared* mating pairs (Figure 4a). Brood size (number of hatched nauplii) was variable over treatments and slightly, but significantly, elevated by FP (6.5 versus 5.4, $P = 0.015$; Figure 4b). There were no significant differences among any of the mating combinations nested within mating exposures (P values > 0.21, Figure 4b). Hatching success rates (no. of nauplii hatched/no. of embryos produced) ranged from 78% to 94% in *FP* solution to 86% to 98.0% in *CTL* solution (Figure 4c) and were not significantly different for any mating combinations nested within mating exposures (P values all >0.09).

Estimated instantaneous population growth rates (λ) based on Leslie-matrix stage-structured modeling of the two extreme mating combinations/exposures (*CTL-reared mating pairs in CTL solution* and *FP-reared mating pairs in FP solution*) were 1.427 and 1.172, respectively. After three generations there were significant differences in projected population sizes—154 individuals versus 38—a 75% FP-linked decrease (Table 1). This decline in population abundance was primarily driven by the high reproductive failure rates when FP-reared male *A. tenuiremis* were mated in FP.

Discussion

In this study, copepod full lifecycle exposures to the phenylpyrazole insecticide fipronil at an environmentally realistic concentration resulted in male-specific reproductive depression. We previously found a 58% suppression in reproductive success when *A. tenuiremis* was exposed to fipronil concentrations as low as 0.16 μg/L (*9*). The cross-mating design used here allowed for delineation of sex-specific reproductive dysfunction due to a 0.63 μg-FP/L exposure. When CTL- or FP-reared females were mated with FP-reared males in fipronil solution, there was a 75–89% decrease in reproductive success (Figure 2). Carry-over effects (delayed fertilization success) of previous fipronil exposure were significant for males but not females reared in FP and then mated in *clean* seawater. Although FP-reared males mated in CTL solution with either a CTL- or FP-reared female displayed >83% reproductive success (measured as the production of viable offspring), this occurred only after a 3-day depuration in clean seawater. In contrast, FP-reared females mated in CTL solution with either a CTL- or FP-reared male did not display reproductive delays with >90% producing viable brood sacs as rapidly as the controls.

As fipronil-induced reproductive depression was remediated by copepod removal from fipronil, risk to field populations may be reduced if fipronil dissipates/degrades quickly. However, fipronil is moderately persistent in the field (*1*), which raises concerns regarding risks to nontarget crustaceans. Many sediment-dwelling crustaceans, like meiobenthic copepods, are deposit feeders and may experience particularly high risk of exposure due to fipronil's high affinity for sediment carbon (food) ($\log K_{ow} = 4.01$, (*1*)). The UV half-life of FP in water is only 3.6 h, but its toxic photodegradation product, fipronil desulfinyl, is stable, lipophilic ($K_{oc} = 1290$ (*8*)), and much more persistent in aqueous environments (*2*). In soils, FP has an aerobic half-life of 128 days, while the primary degradation products (desulfinyl, FP sulfide, and FP sulfone) have half-lives ranging from 693 to 700 days (*2*). In a salt marsh mesocosm 5 μg-FP/L one time dose, FP was measured at 0.11 μg/L and its toxic degradation products (fipronil desulfinyl, sulfone) at 0.22 and 0.50 μg/L, respectively, in seawater samples 28 days after the initial FP dose (*23*). This period covers 100% of most temperate copepod 3–4 week lifecycles (*24*). The FP-induced delays seen here for brood sac extrusion would also create a substantial setback for total lifetime production by an adult female *A. tenuiremis* given the average life expectancy is only 38 days (*25*). FP-induced infertility and/or delays in reproduction would yield strong negative implications for population growth and maintenance (up to 78% in only three generations based on the Leslie life-stage matrix). If this FP effect is common across species (presently unknown), FP could potentially reduce the ecologically important food value of meiobenthic copepods in estuarine food webs (*26*).

A. tenuiremis males were reproductively much more sensitive than females to the fipronil concentration tested here. This finding is congruent with other studies investigating sex-specific *lethal* sensitivities to other toxicants (*10, 27*). For example, DiPinto et al. (*28*) found that the Aroclor 1254 (polychlorinated biphenyl) LC_{50} for female *Microarthridion littorale* (estuarine harpacticoid copepod) was twice as high as the male LC_{50} and suggested that Aroclor was better eliminated by females via a lipovitellin:PCB shunt to egg masses (sensu 29). Sex-specific *reproductive* sensitivities are unreported/untested in other copepods but may be attributed to differences in neurohormonal control of testicular development relative to ovarian development. In higher order decapod crustaceans, hormonal control of male/female reproductive systems has been studied intensively (e.g., *30, 31*), but specific neurohormonal cascades are still not fully understood. However, in the fiddler crab, *Uca pugilator*, and crayfish, *Procambarus clarkii*, development of the androgenic gland is known to induce testicular maturation, and this maturation is controlled by 5-hydroxytryptamine (5-HT) induction of gonad-stimulating hormone (*30*). As copepods are phylogenetically and physiologically similar to macrocrustaceans, male-specific reproductive toxicity from FP should be evaluated in these more commercially important taxa as well. Only one study to date has evaluated FP acute

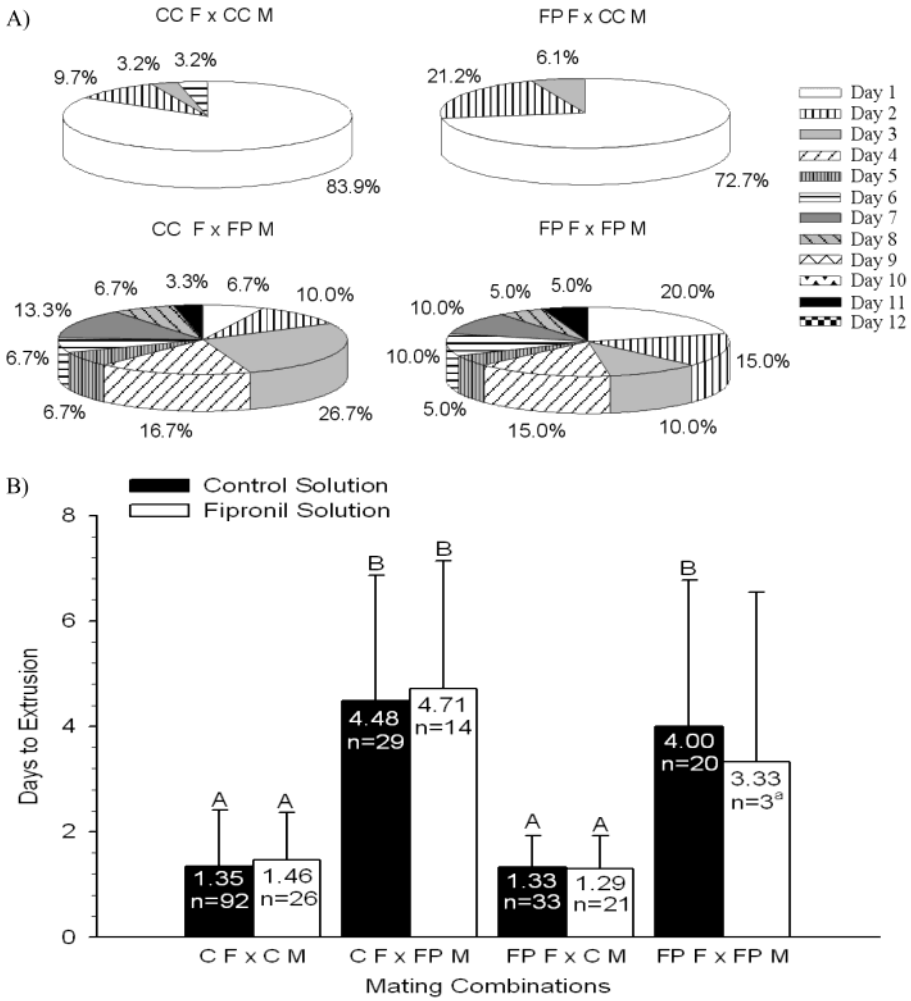

FIGURE 3. (a) Mean percent of females that extruded their first clutch each day throughout the 12-day mating period when mated in FP-free seawater. The CTL-reared mating pair reflects data collected for both generations. No copepodites in any treatment became adult on day 9 or 12 of the rearing period. (b) Mean number of days (±1 SD) from mating of adult males and females to brood sac extrusion for both mating exposures and across generations. Bars sharing any common letters are not significantly different ($p < 0.05$). [a]The FP-reared female × FP-reared male combination *mated in FP solution* yielded only 3 clutches in 37 trials and was removed from the statistical analysis.

toxicity to macrocrustaceans and reported that FP is very highly toxic to two species of nonmolting adult crayfish commonly co-cultured in southern Louisiana fipronil-treated rice fields (*32*).

A. tenuiremis has GABA receptors (Cary unpubl.) and likely has 5-HT due to its phylogenetic relatedness to higher level crustaceans (*33*). As such, *A. tenuiremis* may incur reproductive inhibition directly or indirectly via FP-induced disruption of neurohormonal cascades important to male gonadal development. Fipronil's unique trifluoromethylsulfinyl side group, which is believed to enhance its potency and insect-selective toxicity (*7*), may behave differently within male hormonal cascades. However, as fipronil is a GABA-disrupting compound and GABA receptors are not sex specific, there is no a priori reason FP should induce male-specific effects via its engineered GABA disruption pathway. A similar GABA disrupting insecticide, endosulfan, induced decreased molting rates in the microcrustacean *Daphnia magna* possibly due to disruption of the steroid molting hormone 20-hydroxyecdysone's binding to the ecdysteroid receptor (*34*). As ecdysone titers are intimately linked to crustacean sexual development and reproductive cycles (*35*−*37*), a male-specific FP:ecdysone disruption similarly may occur in *A. tenuiremis*.

Female copepods are under similar hormonal control of gonadal maturation as males, yet they were largely insensitive to sublethal fipronil exposure. One explanation for this insensitivity may be increased total lipid concentrations in female copepods compared to males (5.8−7.9% versus 3.3% (*38*)). Elevated lipid storage may provide a means for enhanced sequestration of lipophilic toxicants such as fipronil. McManus et al. (*29*) suggested lipid storage and subsequent egg deposition as the primary mechanism for elimination of Aroclor 1254 in female *Acartia tonsa*. Similarly, increased lipovitellin levels in pyrene-exposed female grass shrimp, coupled with a concomitant decrease in offspring survival from those females, was suggested as a pyrene shunting mechanism that minimized female endogenous exposure (*39*). Similarly, Volz and Chandler (In review, *40*) reported significantly increased lipovitellin levels in fipronil-reared (0.63 μg-FP/L) female *A. tenuiremis* compared to unexposed females and proposed increased lipovitellin production as a possible mode for FP sequestration. However, in our study, F_1 offspring of females from FP-exposed pairs showed no significantly different responses for any endpoints; thus, 0.63 μg-FP/L induced no differential, trans-generational sensitivities in the F_1 even for those offspring of the three only 'supermales' able to reproduce after rearing and mating in fipronil. A fipronil-related decrease in survival or reproductive success by F_1 offspring of FP-reared F_0 females would have supported an argument for maternal lipovitellin transfer

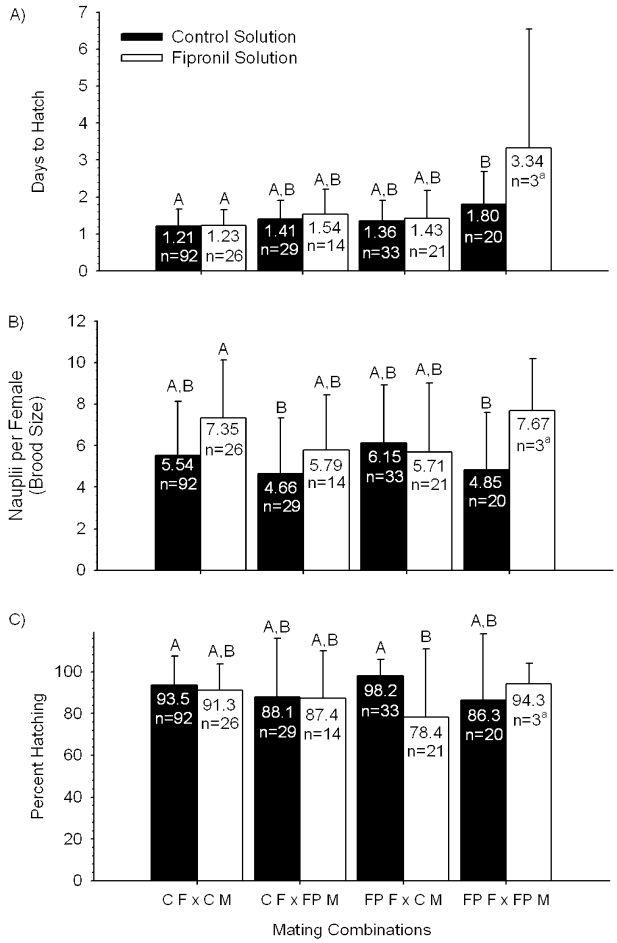

FIGURE 4. (a) Mean number of days (±1 SD) from brood sac extrusion to naupliar hatching for each mating combination and including both generations. (b) Mean brood sizes (i.e., hatched nauplii) (±1 SD) for each mating combination in CTL and FP solution. (c) Mean percent hatching success (±1 SD) for each mating combination in control or FP solution. Hatching success was calculated per female as the number of hatched nauplii divided by the total number of eggs in a clutch. [a]The FP-reared female × FP-reared male combination *mated in FP solution* yielded only 3 clutches in 37 trials and was removed from the statistical analysis.

TABLE 1. Leslie-Matrix-Projected Mean Abundances (±1 SD) and Mean Instantaneous Population Growth Rates (±1 SD) for Each Mating Combination in Either Mating Exposure after Three Generations

mating combination	mating exposure	mean abundance	mean lambda
C F × C M	control	153.6 (12.5)	1.427 (0.03)
C F × FP M	control	116.9 (18.2)[a]	1.309 (0.08)
FP F × C M	control	170.2 (28.5)	1.450 (0.09)
FP F × FP M	control	110.8 (48.7)[a]	1.265 (0.24)
C F × C M	fipronil	191.5 (12.3)[a]	1.505 (0.01)
C F × FP M	fipronil	52.6 (6.2)[a]	1.227 (0.05)
FP F × C M	fipronil	131.8 (14.9)[a]	1.384 (0.06)
FP F × FP M	fipronil	38.2 (3.0)[a]	1.172 (0.00)

[a] Mean abundances that are significantly different from the C F × C M mating pair in CTL solution. Means and SDs calculated from 10 model simulations per replicate microplate times three microplates per treatment.

of fipronil to offspring. However, F_1 offspring survived and reproduced as well as their respective FP-reared parents.

Further study is needed to determine the actual mechanism(s) for FP's strong sex-specific effects. It would be logistically difficult to test in such a small animal model, but FP may reduce or inhibit sperm production, viability, and/or mobility. Also, since the registration/use of fipronil has recently been expanded in the United States and FP is environmentally persistent, future studies should examine other invertebrate taxa over chronic low-level exposures to determine if sex-linked reproductive effects are multispecies or even multiphyletic among other ecologically important taxa.

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