

ACUTE, SUBLETHAL EXPOSURE TO A PYRETHROID INSECTICIDE ALTERS BEHAVIOR, GROWTH, AND PREDATION RISK IN LARVAE OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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(Received 13 August 2007; Accepted 25 February 2008)

Abstract—The present study determined the effects of environmentally relevant, short-term (4-h) exposure to the pyrethroid insecticide esfenvalerate on mortality, food consumption, growth, swimming ability, and predation risk in larvae of the fathead minnow (*Pimephales promelas*). Acute effect concentrations were determined, and in subsequent experiments, fish were exposed to the following measured sublethal concentrations: 0.072, 0.455, and 1.142 $\mu\text{g/L}$ of esfenvalerate. To measure growth rates (% dry wt/d), 8-d-old fathead minnows were exposed to esfenvalerate for 4 h, then transferred to control water and held for 7 d. Food consumption and abnormal swimming behavior were recorded daily. Additional behavioral experiments were conducted to evaluate how esfenvalerate affects the optomotor response of the fish. To quantify predation risk, esfenvalerate-exposed fathead minnow larvae were transferred to 9.5-L aquaria, each containing one juvenile threespine stickleback (*Gasterosteus aculeatus*). Sticklebacks were allowed to feed for 45 min, after which the number of surviving minnows was recorded. No mortality occurred during 4-h exposures to esfenvalerate, even at nominal concentrations of greater than 20 $\mu\text{g/L}$. Delayed mortality (50%) was observed at 2 $\mu\text{g/L}$ after an additional 20 h in clean water. Fish exposed to 0.455 and 1.142 $\mu\text{g/L}$ of esfenvalerate exhibited impaired swimming and feeding ability as well as reduced growth compared to fish exposed to 0.072 $\mu\text{g/L}$ and controls. Predation risk also was significantly increased for larvae exposed to 0.455 and 1.142 $\mu\text{g/L}$ of esfenvalerate. These results demonstrate that larval fish experiencing acute exposures to sublethal concentrations of this insecticide exhibit significant behavioral impairment, leading to reduced growth and increased susceptibility to predation, with potentially severe consequences for their ecological fitness.

Keywords—Fish larvae Pyrethroid insecticide Swimming behavior Feeding Predation

INTRODUCTION

Pyrethroids are synthetic insecticides derived from natural pyrethrins, which are produced by a species of chrysanthemum. Pyrethroid use in agricultural and urban pest control has been increasing steadily because of the phasing out of organophosphate insecticides [1,2], the potential risk of which to aquatic systems has become a concern. Although pyrethroids are less detrimental than organophosphate insecticides to human health, they are highly toxic to fish and aquatic invertebrates. In fact, pyrethroids are several orders of magnitude more toxic to fish than are organophosphate insecticides [3]. Because of their lipophilic nature, pyrethroids are readily taken up by biological membranes and tissues. Once absorbed, these neurotoxins interfere with nerve cell function by interacting with voltage-dependent sodium channels. Pyrethroids prolong the sodium current, stimulating nerves to discharge repeatedly and resulting in hyperexcitability, tremors, convulsions, lethargy, and ultimately, paralysis in poisoned animals [4,5]. Studies have documented acute toxicity resulting from 0.23 to 1.0 $\mu\text{g/L}$ of commonly used pyrethroids, such as esfenvalerate in larvae and juveniles of multiple fish species, including the fathead minnow (*Pimephales promelas*) [6–8], Chinook salmon (*Oncorhynchus tshawytscha*) [9], Sacramento splittail

(*Pogonichthys macrolepidotus*) [7], and bluegill sunfish (*Lepomis macrochirus*) [10] (<http://www.cdpr.ca.gov/docs/pur/pur03rep/03chem.htm>). Few studies, however, have examined the sublethal effects of ecologically relevant, short-term exposure on important parameters, such as swimming and feeding ability, predator avoidance, and growth in larval fish, and have considered their potential population-level consequences.

Putting toxicological findings into a broader, temporal and spatial, ecological context is important for assessing the ecological effects of contaminants such as pyrethroid insecticides. In many agricultural and urban areas, the periods of peak pesticide application coincide with the spawning season [11] of multiple fish species. Thus, fish are likely to be exposed to pyrethroid insecticides as larvae and juveniles, when they are believed to be most vulnerable to contaminants [12]. Known sublethal effects of pyrethroids, such as the disruption of hormone-related functions [13], impairment of the immune response [14–16], inhibition of growth [2,17], and behavioral abnormalities (<http://www.epa.gov/ecotox/>) [18], are likely to reduce fish reproductive success and to increase predation risk and susceptibility to disease. Other sublethal effects shown in fish include altered stress protein expression [9], reduced neural function [19] and fecundity [14], and altered intraspecific interactions [19]. Sublethal effects may occur at concentrations far lower than those resulting in acute toxicity [17]; thus, studies linking pyrethroid-induced changes in physiology and behavior to survival can provide important information to managers involved in regulating pesticide application.

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Published on the Web 4/1/2008.

The objectives of the present study were to determine the effects of short-term exposure to sublethal concentrations of the pyrethroid esfenvalerate [(S)- α -cyano-3-phenoxybenzyl-(S)-2-(4-chlorophenyl)-3-methylbutyrate] on food consumption, growth, swimming behavior, and predation risk in larvae of the fathead minnow. This particular pyrethroid is a broad-spectrum insecticide, and it is applied to a wide variety of crops, such as cotton, vegetables, fruits, and nursery trees [19]. We addressed the need for studies linking sublethal effects to the population demography of fish by examining how esfenvalerate exposure may influence swimming behavior and food consumption and how these behavioral changes might alter growth rates and predation risk. Numerous toxicological studies regarding the acute effects of pyrethroids exist [20], but typical exposure periods are from 4 to 7 d. Because of the hydrophobic nature of pyrethroids, these chemicals tend to partition to particles in the water column and to sediment once dissolved in water, and waterborne exposures to these pesticides are believed to be relatively short [10,21]. Our experiments were designed to simulate the short-term exposures that fish are most likely to experience in nature. We used the fathead minnow for these experiments, both because larvae at a specified age can be readily obtained and because standard exposure protocols as well as toxicity information for esfenvalerate exist for this species.

We hypothesized that a brief (4-h) exposure to esfenvalerate, as typically would occur after rainfall or during irrigation events, would impair swimming and feeding ability in exposed fathead minnow larvae, resulting in decreased growth and elevated predation risk. We used three approaches to test these hypotheses: Growth experiments, which involved measurement of growth rates over a 7-d period and daily records of feeding rates and swimming abnormalities following esfenvalerate exposure; optomotor response experiments, which documented the ability of the fish to respond to external stimuli immediately following exposure to the insecticide; and predation experiments, which involved assessment of relative predation risk after exposure. The threespine stickleback (*Gasterosteus aculeatus*) was used as the predator in these experiments, both because it is common in ponds and creeks in the Sacramento–San Joaquin Delta (CA, USA) to which the fathead minnow has been introduced and because it is a voracious predator that feeds readily in the laboratory. We also conducted an initial acute toxicity experiment to determine short-term effect concentrations and to be able to compare sublethal effect concentrations to acute toxicity parameters. Different groups of fish were used for each of these experiments.

MATERIALS AND METHODS

Fish acclimation

Seven-day-old fathead minnow larvae were obtained from Aquatox (Hot Springs, AR, USA) and placed in a 38-L aquarium on arrival for a 24-h acclimation period. Water-quality measurements were taken for water in which fish were transported (temperature [T], $23.3 \pm 1.1^\circ\text{C}$; pH 7.4 ± 0.2 ; dissolved oxygen [DO], 11.7 ± 1.5 mg/L; electrical conductivity [EC], 484.8 ± 44.4 $\mu\text{S}/\text{cm}$) and for laboratory acclimation water (T, $20.1 \pm 0.8^\circ\text{C}$; pH 7.9 ± 0.1 ; DO, 8.5 ± 0.8 mg/L; EC, 748.1 ± 68.7 $\mu\text{S}/\text{cm}$). Fish were fed live brine shrimp (*Artemia nauplii*) ad libitum on the day of arrival. The acclimation tank was placed in an incubator set at 18°C with a 16:8-h light:dark photoperiod. Almost no mortality ($<0.1\%$) occurred during acclimation, and the fish fed and swam normally. Fish

were acclimated in this manner for all the experiments described below.

Esfenvalerate exposures

All exposures were initiated with 8-d-old larvae and were conducted in an incubator set at 18°C with a 16:8-h light:dark photoperiod. Local well water (hardness, 350 mg/L; total alkalinity, 400 mg/L; total dissolved solids, 470 mg/L) was used as control water. The well at the University of California–Davis (Davis, CA, USA) Center for Aquatic Biology and Aquaculture is approximately 60 m in depth, and water is passed through a packed-column aerator to oxygenate and remove excess nitrogen.

At test initiation, 10 to 12 randomly selected larvae were transferred using a glass pipette from the holding tank to each of 4 to 10 (for experiment-specific details, see below) replicate, 600-ml glass beakers containing 250 ml of control water. Test solutions were prepared by directly adding 100 μl of the respective stock solution (esfenvalerate [Asana[®]; ChemService, West Chester, PA, USA] dissolved in methanol) to each beaker and stirring with a glass rod to distribute the stock solution evenly. For the solvent control, 100 μl of methanol were added to 250 ml of control water. Water-quality measurements were taken before and after the 4-h exposure (T, $18.2\text{--}21.3^\circ\text{C}$; pH $7.7\text{--}8.6$; DO, $6.1\text{--}9.6$ mg/L; EC, $684\text{--}899$ $\mu\text{S}/\text{cm}$).

To determine 4-h acute toxicity (mortality and abnormal swimming), fish were exposed to the following treatments: Control, solvent control (0.04% [v/v] methanol), and 1, 3, 7, 10, and 20 $\mu\text{g}/\text{L}$ (nominal) of esfenvalerate. Nominal esfenvalerate concentrations of 0.1 $\mu\text{g}/\text{L}$ (low), 0.7 $\mu\text{g}/\text{L}$ (medium), and 1.5 $\mu\text{g}/\text{L}$ (high) were subsequently used in all experiments measuring sublethal endpoints (growth, food consumption, and swimming behavior; optomotor response; and predation risk). We measured actual concentrations in two sets of samples prepared on May 31, 2006, and August 14, 2006, respectively. Water samples for chemical analysis were prepared as described above, transferred to amber bottles, and transported on ice immediately to the California Department of Fish and Game Water Pollution Laboratory (Rancho Cordova, CA, USA). Water samples were analyzed using gas chromatography with mass spectrometry and ion-trap detection, with a reporting limit of 0.002 $\mu\text{g}/\text{L}$ (recovery, $91.2\% \pm 0.08\%$). Measured esfenvalerate concentrations were 0.072 ± 0.01 $\mu\text{g}/\text{L}$ (nominal, 0.1 $\mu\text{g}/\text{L}$), 0.455 ± 0.03 $\mu\text{g}/\text{L}$ (nominal, 0.7 $\mu\text{g}/\text{L}$), and 1.142 ± 0.19 $\mu\text{g}/\text{L}$ (nominal, 1.5 $\mu\text{g}/\text{L}$).

Short-term acute toxicity

To determine the median lethal concentration (LC50) for esfenvalerate after a 4-h exposure, fathead minnow larvae were transferred to four replicate beakers per treatment and exposed to from 1 to 20 $\mu\text{g}/\text{L}$ (nominal) of esfenvalerate as described previously. To assess delayed effects, larvae were then maintained in control water for an additional 20-h period. For transfer, exposed fish were removed from exposure beakers, gently rinsed with control water, and then moved to a clean, 600-ml beaker containing 250 ml of control water. Mortality and swimming abnormalities (defined by twitching, swimming erratically, or lying on one side) were recorded after the 4-h chemical exposure and then again after the additional 20-h period in control water.

Growth experiments

Growth. Growth was measured for a period of 7 d. To obtain initial dry weights for growth rate calculations, we allocated

10 unexposed fish to each of six replicate beakers per treatment and then killed the fish immediately using tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA). The fish were dried overnight on preweighed aluminum weighing pans in a drying oven set at approximately 100°C and then weighed to the closest 0.0001 g on a digital analytical balance (model AE 163; Mettler, Hightstown, NJ, USA). Final weights were obtained for larvae exposed for 4 h to sublethal esfenvalerate concentrations or control waters (as described previously) after a 7-d growth phase. Immediately following the exposure, fish were transferred to control water (in replicate, 600-ml Teflon® beakers containing 300 ml of water) as described above and then held for the duration of the experiment. Each day, fish were fed twice with live brine shrimp (morning, $n = 79$ shrimp/fish on average; afternoon, $n = 52$ shrimp/fish on average), and approximately 80% of the water was renewed. Water quality was monitored daily (T, $21.1 \pm 0.8^\circ\text{C}$; pH 8.4 ± 0.2 ; DO, 7.1 ± 0.6 mg/L; EC, 748.3 ± 9.3 $\mu\text{S/cm}$). Ammonia concentrations were measured on days 1 and 3 (total ammonia-nitrogen, 0.4 ± 0.1 mg/L) using the AmVer™ Low-Range Ammonia Test N Tube™ Reagent Set (Hach, Loveland, CO, USA). On day 7, fish were killed, dried overnight, and weighed. Specific growth rates were calculated using the following formula: $100 \cdot (\log_e \text{weight}_{\text{final}} - \log_e \text{weight}_{\text{initial}}) \div (\text{time}_{\text{final}} - \text{time}_{\text{initial}})$. This test was performed twice with different batches of larvae to achieve adequate replication. Because initial larval weights varied significantly between these groups, final weights for these experiments had a bimodal distribution, and this precluded the use of a parametric analysis of variance (ANOVA) in analyzing these data. As a result, we used growth rates, which had a normal distribution, as the endpoint of these experiments.

Food consumption. Before feeding the fish in the morning, the amount of food remaining in each beaker was scored as high (i.e., a dense covering of brine shrimp on the bottom of the beaker) or low (i.e., a sparse covering of brine shrimp on the bottom of the beaker). Food consumption was recorded once daily on days 1 (i.e., the day following exposure) to 6.

Swimming abnormalities. Two hours after feeding, the water in each beaker was replaced with fresh control water. Immediately before water changes, we recorded abnormal swimming behavior (defined above) in each beaker. The number of larvae swimming abnormally was recorded once daily on days 1 to 6.

Two growth experiments were performed (experiment 1: July 22–29, 2006; experiment 2: August 2–9, 2006). Replication was sixfold for growth rate calculations ($n = 3$ per experiment). Because we set up three extra replicates to allow for potential mortalities during the growth period, we had nine replicates for swimming behavior and remaining food data.

Optomotor response experiments

Immediately after 4-h exposures to sublethal esfenvalerate concentrations described above, individual fathead minnow larvae were subjected to a rotating square-wave stimulus to measure their optomotor response, or swimming response [22]. The square-wave stimulus used to elicit a swimming response in the fish consisted of black-and-white stripes of equal width (thickness, 1.9 cm) on the internal side of a cylinder made of card stock (diameter, 305 mm; height, 356 mm). When a square-wave stimulus is rotated around a fish, the fish typically will respond by swimming in the direction that the stimulus is moving [22]; swimming in the opposite direction or re-

maining motionless is an indicator of physiological impairment.

For each trial, we rinsed one larva from a randomly selected treatment with control water and immediately placed it in the experimental tank (a clear, cylindrical, Plexiglass tank; diameter, 152 mm; height, 305 mm) filled with control water (T, $20.5 \pm 0.3^\circ\text{C}$; pH 7.9 ± 0.1 ; DO, 9.0 ± 0.1 mg/L; EC, 730 ± 6.2 $\mu\text{S/cm}$) and surrounded by the square-wave stimulus. The stimulus was attached to a circular platform, which in turn was attached to a 7-rpm reversible gear motor, allowing us to rotate the stimulus clockwise or counterclockwise. We allowed the fish a 10-min acclimation period in the experimental tank before starting the experiments. The experiments consisted of a 10-min exposure to the rotating stimulus, starting with 2 min in the clockwise direction, then alternating the direction for each of six 1-min intervals, and ending with 2 min in the counterclockwise direction. We tested six fish larvae per treatment, and all experiments were filmed from above with a digital video camera (DCR-PC101 MiniDV Handycam®; Sony, Tokyo, Japan).

Video analysis was conducted blind (i.e., without knowledge of the treatments), and the variables quantified were the amount of time that fish spent following the moving stripes and how much time fish remained stationary. From this point onward, the time that fish remained stationary will be referred to as the time spent nonresponsive to the stimulus.

Predation experiments

After 4-h exposure to sublethal esfenvalerate concentrations (see above), larvae were rinsed with control water and transferred immediately to 9.5-L aquaria containing 8.5 L of aerated control water (DO, 9.1 ± 0.0 mg/L) for predation experiments. Each aquarium was placed in a water bath inside a 76-L circular tank to maintain water temperature at $19.3 \pm 0.2^\circ\text{C}$. Before adding the fish, a clear Plexiglass divider was placed in the middle of each aquarium. Ten larvae from one replicate beaker of a single treatment were then placed on one side of the divider in each of five aquaria (corresponding to the five treatments), and artificial vegetation was then added to provide the larvae with shelter during the experiment. One juvenile threespine stickleback (total length, ~ 34 mm) was placed on the other side of the divider in each aquarium. Sticklebacks used in the present study were caught in local ponds and creeks in the Davis (CA, USA) area and then transported to the University of California–Davis Center for Aquatic Biology and Aquaculture, where they were held in 76-L circular tanks supplied with flow-through well water (T, $19.3 \pm 0.5^\circ\text{C}$; DO, 9.1 ± 0.1 mg/L). The sticklebacks were not fed for 24 h before the predation experiments. Each stickleback was used only once. After a 1-h acclimation period, the dividers were removed allowing the sticklebacks access to the minnow larvae. Experiments were run for 45 min, after which the sticklebacks were removed and the number of surviving minnow larvae recorded. We also performed control experiments without predator addition to determine if pesticide exposure and/or handling stress caused mortality. Ten replicate experiments were performed both with and without a predator.

Statistical analysis

We used the Comprehensive Environmental Toxicity Information System produced by Tidepool Scientific Software (McKinleyville, CA, USA) to calculate the following statistics for swimming and survival data collected during the LC50

experiment: 4-h swimming (no-observed-effect concentration [NOEC] and median effect concentration [EC50]), 4-h survival (NOEC and LC50), 24-h swimming (NOEC and EC50), and 24-h survival (NOEC and LC50). We calculated these endpoints both after the 4-h exposure and after holding the fish in control water for an additional 20 h to account for delayed effects of the 4-h exposure on swimming and survival.

For data regarding the amount of food consumed in the growth experiments, we used logistic regression to determine if an interaction existed between day and treatment. Because a significant interaction was found, we used the Cochran–Armitage test of a linear trend, a form of chi-square analysis that takes the order of the treatments into account [23,24], to evaluate differences in the amount of remaining food between treatments on each day. Data from optomotor experiments on the amount of time spent nonresponsive to the moving field were transformed into a categorical format because of the presence of many zeros in the data set. Trials in which the fish spent any amount of time nonresponsive were given a score of one, and those in which the fish did not spend any time nonresponsive were given a score of zero. After transformation, the Cochran–Armitage test was used to examine the tendency for fish to be nonresponsive. Subdivision of the chi-square tests allowed us to identify significant differences among treatments [25].

The Shapiro–Wilk normality test and the Levene test were used to evaluate whether quantitative data met the assumptions of the parametric ANOVA. Data regarding the percentage of fish swimming abnormally from the growth experiments did not meet the assumptions of normality and homogeneity of variances; however, with the strong signal that we observed and the sample sizes used, the ANOVA likely was robust to violations of these assumptions [26]. We ran additional analyses that did not depend on normality and variance homogeneity by transforming these data into a categorical format, and these analyses provided the same results as the parametric ANOVA. Here, we present results from the parametric ANOVA, because it allows greater ease of post hoc testing. We used repeated-measures ANOVA to determine if a significant interaction existed between day and treatment for swimming behavior data. Because a significant interaction was found, we looked at each day individually using one-way ANOVA with a Bonferroni correction [27]. One-way ANOVA also was used to compare growth rates, the amount of time fish spent following the moving stripes during optomotor response experiments, and predation risk among treatments. We used the Tukey honestly significant difference test to make multiple comparisons. The significance level was $p < 0.05$ for all statistical tests except the ANOVAs used to analyze abnormal swimming data for each day during the growth experiments; because a Bonferroni correction was used for each of these ANOVAs, the significance level for these analyses was $p < 0.008$.

RESULTS

Short-term acute toxicity

Both the NOEC and LC50 for a 4-h exposure to esfenvalerate were greater than 20 $\mu\text{g/L}$. The solubility of esfenvalerate, however, is reported to be only 2 $\mu\text{g/L}$ at 25°C [28]; thus, larvae likely were exposed to a maximum concentration of approximately 2 $\mu\text{g/L}$. Delayed mortality was observed after an additional 20-h period in control water; for delayed mortality, the NOEC and LC50 were 1 and 2.04 $\mu\text{g/L}$, respectively.

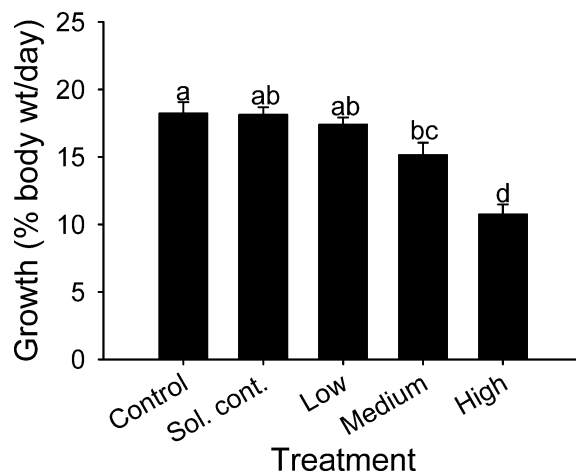


Fig. 1. Growth rate (% dry tissue wt/day, average \pm standard error) for fish from control, solvent control (sol. cont.; 0.04% methanol), and low (0.072 $\mu\text{g/L}$), medium (0.455 $\mu\text{g/L}$), and high (1.142 $\mu\text{g/L}$) treatments. Different letters (a, b, c, and d) indicate statistically significant groups ($p < 0.05$).

The NOEC and EC50 for swimming abnormalities calculated for a 4-h exposure and after the 20-h period in control water were both less than 1 $\mu\text{g/L}$.

Growth, food consumption, and swimming abnormalities

Growth. Growth rates declined with increasing pesticide concentration ($F_{4,25} = 19.03$, $p < 0.001$) (Fig. 1). Post hoc analyses indicated that fish exposed to the high pesticide treatment grew more slowly compared with those exposed to any of the other four treatments. Fish exposed to the medium treatment, however, only exhibited slower growth relative to the control treatment. No difference in growth was found among the control, the solvent control, and the low pesticide treatments. Mortality rates during the growth experiments were less than 4% for all treatments (Table 1).

Food consumption. Food consumption was impaired by exposure to esfenvalerate; however, fish exposed to esfenvalerate recovered during the course of the 7-d growth experiment. A highly significant difference was found among treatments on day 1 ($\chi^2 = 28.467$, $p < 0.001$, $df = 1$), with 100% of the beakers containing fish from the medium and high pesticide treatments receiving a high score for the amount of food remaining and less than 12% of beakers containing fish from the low treatment and controls receiving a high score. Ninety-nine percent of the variation in the statistical model was explained by the difference between the two highest pesticide treatments (i.e., the medium and high pesticide treatments) and the other three treatments on day 1. On day 2, 66% of the

Table 1. Percentage mortality for fathead minnow (*Pimephales promelas*) larvae exposed to five esfenvalerate treatments during growth, optomotor, and predation experiments

Treatment	Growth (%)	Optomotor (%)	Predation (%)
Control	0.9 \pm 0.9	0.0 \pm 0.0	0.0 \pm 0.0
Solvent control	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Esfenvalerate			
Low (0.072 $\mu\text{g/L}$)	1.9 \pm 1.2	0.0 \pm 0.0	0.0 \pm 0.0
Medium (0.455 $\mu\text{g/L}$)	3.7 \pm 1.5	0.0 \pm 0.0	0.0 \pm 0.0
High (1.142 $\mu\text{g/L}$)	2.8 \pm 1.4	0.0 \pm 0.0	7.0 \pm 2.8

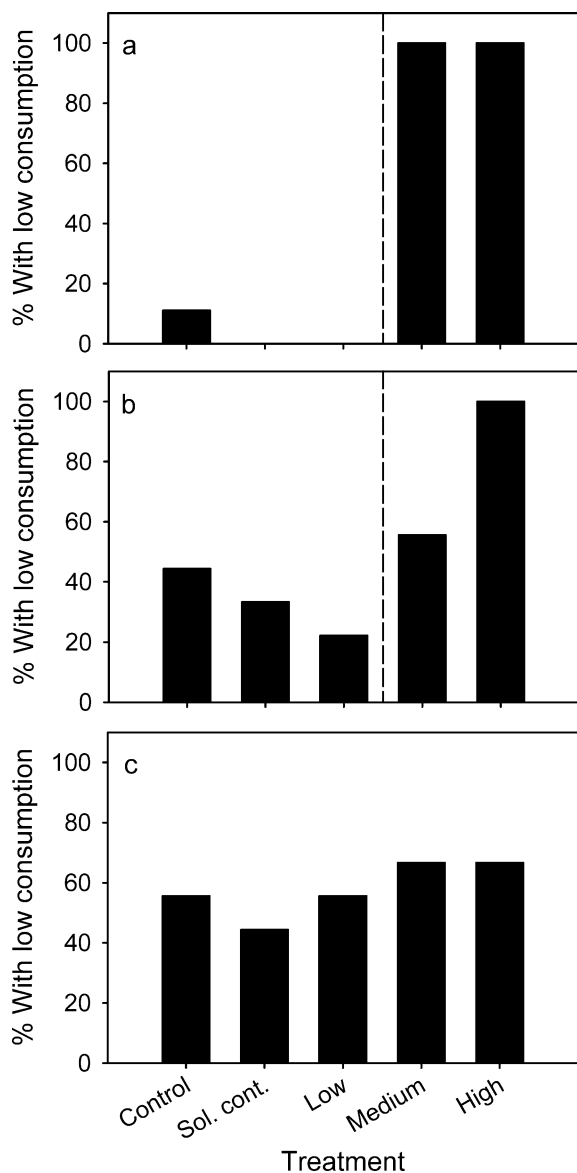


Fig. 2. Percentage of replicates (nine replicates = 100%) in which food consumption of fathead minnow (*Pimephales promelas*) larvae was low after exposure to control water, solvent control (0.04% methanol), and low (0.072 $\mu\text{g/L}$), medium (0.455 $\mu\text{g/L}$), and high (1.142 $\mu\text{g/L}$) esfenvalerate treatments on (a) day 1, (b) day 2, and (c) day 6 after exposure. Dashed lines indicate significant treatment groupings ($p < 0.01$).

variation in the amount of food consumption was explained by the difference between the two highest treatments and the other three treatments, and by day 6, a difference was no longer found among treatments (Fig. 2). Close examination of food consumption patterns over the 7-d period revealed that food consumption was higher in control beakers on day 1 than on days 2 and 6. This unexpected result may be attributed to the fact that the fish were not fed on the day before exposure (during shipment from Aquatox) but were then fed ad libitum twice daily starting on day 1 of the growth experiment. Thus, they likely were extremely hungry on day 1 relative to days 2 and 6.

Swimming abnormalities. A significantly higher proportion of fish exposed to the medium (0.455 $\mu\text{g/L}$; 49.3% \pm 8.3%) and high (1.142 $\mu\text{g/L}$; 100% \pm 0.0%) esfenvalerate concentrations swam abnormally (i.e., twitched, swam erratically, or

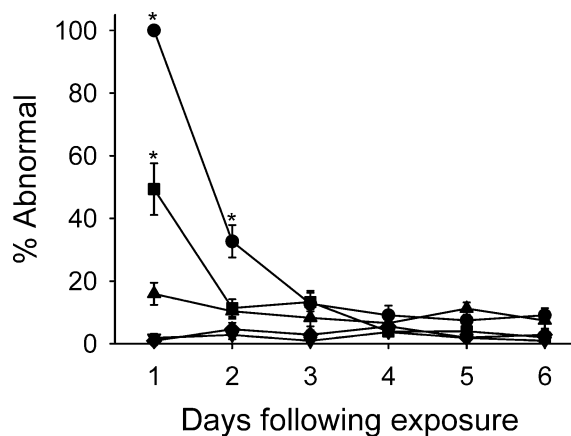


Fig. 3. Percentage of fish swimming abnormally (average \pm standard error, $n = 9$) after a 4-h exposure to control (\blacklozenge), solvent control (\blacktriangledown ; 0.04% methanol), and low (\blacktriangle ; 0.072 $\mu\text{g/L}$), medium (\blacksquare ; 0.455 $\mu\text{g/L}$), and high (\bullet ; 1.142 $\mu\text{g/L}$) esfenvalerate treatments. Measurements were recorded for 6 d postexposure. An asterisk indicates a significant difference ($p < 0.008$) from the solvent control and control.

were lying on one side) relative to fish exposed to the low concentration (0.072 $\mu\text{g/L}$; 15.9% \pm 3.5%) or the controls (solvent control, 1.9% \pm 1.2%; control, 0.9% \pm 0.9%) on day 1 of the growth experiment ($F_{4,40} = 105.89$, $p < 0.001$). Moreover, a higher proportion of fish exposed to the high concentration swam abnormally compared with fish exposed to the medium treatment. These results are supported by those from the acute toxicity test, which showed that the NOEC and EC50 were both less than 1 $\mu\text{g/L}$. As indicated by the significant day \times treatment interaction from the repeated-measures ANOVA ($p < 0.001$), however, this signal disappeared gradually during the 7-d growth experiment (Fig. 3).

Optomotor response

Data from the optomotor experiments obtained immediately after exposure to esfenvalerate corroborate our results on swimming abnormalities observed 20 h after 4-h exposures to esfenvalerate in the growth and acute toxicity experiments. Fish exposed to the medium and high concentrations were less likely to respond to the moving stimulus compared with fish exposed to the low concentration or the controls ($\chi^2 = 9.60$, $p = 0.002$, $df = 1$) (Fig. 4). Moreover, during the short periods of time when they were swimming, fish exposed to the medium and high esfenvalerate concentrations spent less time moving with the moving stimulus relative to fish exposed to the low concentration or the controls ($F_{4,24} = 14.94$, $p < 0.001$) (Fig. 4). Differences between the high treatment and the controls were highly significant (Tukey honestly significant difference test: $p = 0.001$), and those between the medium treatment and the controls were marginally significant ($p = 0.07$). Although fish exposed to the medium and high concentrations tended to swim faster compared with fish exposed to the low concentration and controls, this difference in velocity was not sufficient to explain the three- and fivefold difference in the amount of time spent swimming in the expected direction between the medium and high treatments and the other three treatments. No mortalities were observed during the course of the optomotor experiments (Table 1).

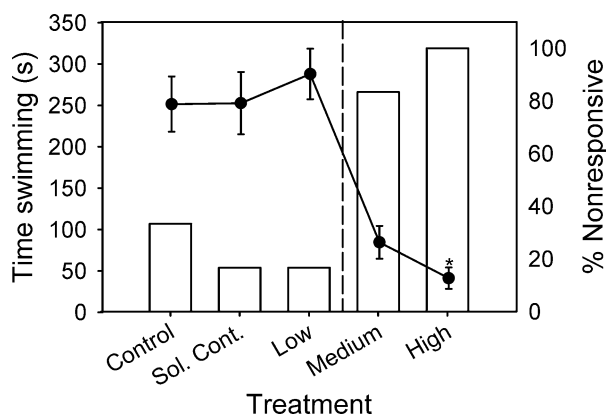


Fig. 4. Percentage of trials during which fish were scored as nonresponsive and amount of time spent swimming with the moving stimulus (mean \pm standard error, $n = 6$) for fish exposed for 4 h to control, solvent control (0.04% methanol), and low (0.072 $\mu\text{g/L}$), medium (0.455 $\mu\text{g/L}$), and high (1.142 $\mu\text{g/L}$) esfenvalerate treatments. The line plot illustrates time spent swimming with the stimulus, and bars correspond to the percentage of fish scored as nonresponsive for each treatment. An asterisk indicates a significant difference ($p < 0.05$) from the control and solvent control (Tukey honestly significant difference); dashed line indicates significant treatment groupings ($p < 0.001$).

Predation risk

Minnow larvae became more vulnerable to predation as pesticide concentration increased ($F_{4,45} = 6.44$, $p < 0.001$) (Fig. 5), and fish exposed to the high esfenvalerate concentration exhibited higher predation risk than those exposed to the low concentration or the controls. Fish exposed to the medium concentration also experienced relatively high mortality; however, predation risk for this group was only different from that of the solvent control. Experiments performed without a predator indicated that few of the mortalities that occurred during predation experiments resulted from pesticide exposure and/or handling stress. Mortalities were only observed in the high pesticide treatment during the experiments run without a predator, and relatively few fish died in this treatment (Table 1).

DISCUSSION

As pyrethroid use becomes more prevalent throughout the Central Valley of California and in other agricultural and urban areas worldwide, studies evaluating how these chemicals affect fish and invertebrate populations are becoming increasingly important. Previous studies have focused mainly on the acute and chronic toxicity of pyrethroids in standard 96-h exposure experiments, ignoring potential effects of short-term exposure to sublethal concentrations on ecologically important aspects of fish physiology, behavior, and fitness. In addition, information generally is lacking regarding the links between effects at multiple levels of organization. The present study fills some of these gaps in our knowledge, however, and it demonstrates that short-term exposure to sublethal concentrations of esfenvalerate can result in swimming abnormalities and increased predation risk as well as reduced foraging and growth rates in fish at a vulnerable stage of their life history.

The importance of examining the sublethal effects of esfenvalerate becomes apparent when reviewing LC50 data for environmentally relevant, short-term exposures and concentrations of esfenvalerate measured in the Central Valley of California. Environmental data regarding esfenvalerate con-

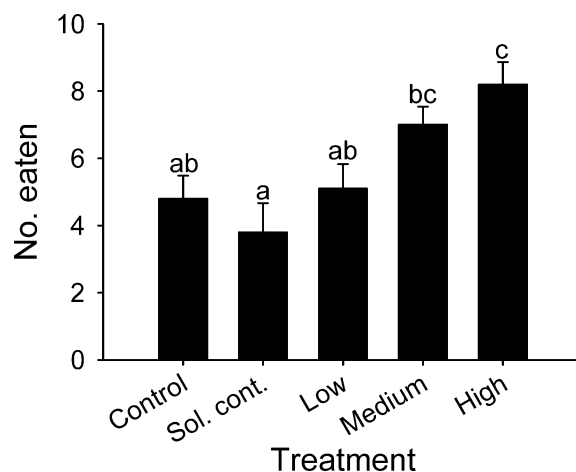


Fig. 5. Number of fathead minnow (*Pimephales promelas*) larvae consumed (mean \pm standard error, $n = 10$) by juvenile stickleback (*Gasterosteus aculeatus*) during 45-min predation experiments after 4-h exposure of fathead minnow to control, solvent control (0.04% methanol), and low (0.072 $\mu\text{g/L}$), medium (0.455 $\mu\text{g/L}$), and high (1.142 $\mu\text{g/L}$) esfenvalerate treatments. Different letters (a, b, and c) indicate statistically significant groups ($p < 0.05$).

centrations in surface waters are scarce, but whole-water concentrations as high as 3 and 5 $\mu\text{g/L}$ have been measured in edge-of-field and in-field storm water runoff samples from a prune orchard in Glenn County (CA, USA) (<http://www.cdpr.ca.gov/docs/sw/swmemos.htm>). It is unknown how long such high concentrations are present in surface waters in river systems such as the Sacramento–San Joaquin Delta, but the general assumption is that pyrethroids bind relatively quickly to particulates and become less bioavailable to aquatic organisms. For example, in mesocosm experiments conducted in Missouri (USA) [10], esfenvalerate had a dissipation half-life of approximately 10 h at water temperatures ranging from 27 to 30°C. Whole-water concentrations of esfenvalerate measured in Central Valley streams receiving winter storm runoff from fruit orchards range from trace to 94 ng/L [29].

Our data show that even though no mortality of fish larvae occurred within a 4-h exposure period at nominal esfenvalerate concentrations that exceeded the documented solubility maximum at 25°C [28], the delayed and sublethal toxic effects of such a short-term exposure to much lower concentrations are severe. We were unable to determine a LC50 for the 4-h exposure period, but the delayed LC50 after the 20-h recovery period was 2.04 $\mu\text{g/L}$. This delayed LC50 was approximately 10-fold higher than LC50s derived from standard 96-h exposure experiments conducted with fathead minnow larvae [6–8], suggesting that environmentally relevant, short-term exposures are more likely to result in sublethal effects than in mortality. We observed immediate behavioral abnormalities, reduced food intake and growth, as well as increased susceptibility to predation in larvae exposed to esfenvalerate at 0.455 $\mu\text{g/L}$ or greater. Neither the delayed LC50 nor the sublethal endpoints measured in the present study are routinely determined in standard bioassays required for the regulatory registration of pesticides and other chemicals, which may convey a false sense of safety with regard to the environmental effects of esfenvalerate and, possibly, other pyrethroid insecticides. Taken together, these data indicate that environmentally realistic exposures are more likely to result in delayed toxicity or sublethal effects on the physiology, behavior, and ultimately, environmental fitness of fish larvae.

The sublethal effects of esfenvalerate observed in the present study were largely reversible. Although larvae were impaired immediately after exposure to esfenvalerate at 0.455 $\mu\text{g/L}$ and above, recovery of swimming ability and feeding rates occurred within 1 to 2 d after exposure to esfenvalerate. Similar recovery of swimming ability was documented in juvenile bluegill after pulsed, 11-h exposures to esfenvalerate [19]. Teh et al. [17] documented recovery on the cellular level, showing that histopathological abnormalities observed in 7-d-old Sacramento splittail one week after 96-h exposure to orchard storm water runoff containing esfenvalerate were no longer present after a 90-d recovery period in control water. However, although recovery appears likely after short-term exposures to esfenvalerate, even brief cellular and behavioral disruptions can have important implications for growth and predation risk in the wild [19].

Growth is an extremely important factor for the success of larval fish in the wild, determining overall fitness through effects on reproductive success and survival with direct implications for the population. Despite the relatively rapid recovery of swimming and feeding ability observed in the present study, fish exposed to esfenvalerate at 0.455 $\mu\text{g/L}$ or greater exhibited reduced growth rates over a 7-d period. Disruption of feeding activity was observed within 1 day of exposure at concentrations that ultimately affected growth, indicating that feeding behavior is a rapid, sensitive, and predictive indicator of concentrations causing population-level effects. The direct consequences of esfenvalerate-induced effects on the nervous system, including body tremors and paralysis [4,5], may have led to impaired feeding ability and, ultimately, growth. It also is possible that esfenvalerate exposure negatively influenced growth through stress-induced changes in growth hormone levels [30] or mobilization of glycogen reserves [31].

The present results are corroborated by those of other studies that have documented inhibition of feeding behavior [32] and reduced growth [17] in fish exposed to sublethal concentrations of fenvalerate and esfenvalerate, respectively. In contrast, Little et al. [19] found that growth was not influenced in bluegill exposed continuously to a maximum esfenvalerate concentration of 0.2 $\mu\text{g/L}$ for 90 d. These conflicting results could be explained by the fact that Little et al. used juvenile bluegill (length, 41 ± 4 mm), which are likely to be less sensitive than larvae to pesticide exposure. The observed negative effects of esfenvalerate on larval growth may have important implications for the ecological fitness of the individual as well as the population, because recruitment and survival often are dependent on fish size. Larger fish are more likely to avoid predation [33] and are more fecund [34–36] than smaller individuals, indicating that esfenvalerate-induced inhibition of growth would likely have important population-level consequences.

We found that short-term disruption of normal behavior also can have significant implications for predation risk. The inability of larvae exposed to esfenvalerate at 0.455 $\mu\text{g/L}$ or greater to respond to a stimulus during the optomotor experiments, and the swimming abnormalities observed during the growth experiments, had significant implications for predator-induced mortality. Fish exposed to esfenvalerate at 0.455 $\mu\text{g/L}$ or greater experienced higher mortality rates during the predation experiments, likely because of their relative inability to avoid the stickleback predator. Similarly, Labenia et al. [37] found that acute exposure to the carbamate insecticide carbaryl reduced the swimming performance and predator avoidance

of cutthroat trout (*Oncorhynchus clarki clarki*), providing additional evidence that short-term exposure to insecticides can have ecologically relevant population-level effects.

The present study provides a conservative measure for the sublethal effects of esfenvalerate on larval fish. Fish in the wild may be subject to repeated pulse exposures as well as to mixtures of chemicals [8,38,39] (<http://tdcenvironmental.com/Pesticides.html>) and multiple stressors [16,40], the combined effects of which are largely unknown. Little et al. [19] showed that juvenile bluegill sunfish were not able to acclimate to pulse exposures of esfenvalerate. Thus, although fish larvae in the present study recovered their normal swimming behavior within 1 to 2 d, repeated exposures would likely affect fish in a cumulative manner, particularly with respect to growth impairment and predation risk. Esfenvalerate has been shown to exert synergistic effects with organophosphate pesticides, particularly chlorpyrifos [38] and diazinon [8], but little is known about their interaction with other chemicals or as part of complex contaminant mixtures. Similarly, information concerning the deleterious effects of esfenvalerate or other pyrethroids in combination with natural stressors is scarce. Clifford et al. [16], however, has documented dramatic increases in juvenile salmon mortality when fish were simultaneously exposed to low esfenvalerate concentrations and a common disease organism. These studies, along with the present results demonstrating the deleterious effects of sublethal esfenvalerate concentrations in larval fish, underscore the need for a more complete understanding of how pyrethroid insecticides can affect natural populations. Our findings of reduced growth, impaired swimming behavior, and increased predation risk following environmentally relevant exposures raise concern that these chemicals may exert negative effects on the reproductive success and survival of fish in natural ecosystems and, ultimately, lead to effects at the population level.

Acknowledgement—We would like to thank the staff of the University of California–Davis Aquatic Toxicology Laboratory at the Center for Aquatic Biology and Aquaculture for their assistance with exposure experiments and statistical analyses. We also thank C.M. Woodley, R. Kaufman, D. Deutschman, T.W. Anderson, and J.J. Cech, Jr. The present study was in partial fulfillment of the requirements for a doctoral degree at San Diego State University and the University of California–Davis. Funding for this project was provided to E.Y. Floyd by the Achievement Rewards for College Scientists Foundation, the Joint-Doctoral Program in Ecology at San Diego State University and the University of California–Davis, and the Aquatic Toxicology Program, University of California–Davis. J.P. Geist acknowledges financial support by the Bayerische Forschungsfoundation (Bavarian Research Foundation), Germany.

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