Environ. Sci. Technol. **2005**, *39,* 6079−6085

Common Pesticide Increases Costs of Antipredator Defenses in *Rana temporaria* Tadpoles

CÉLINE TEPLITSKY,† HENNA PIHA,*,‡ ANSSI LAURILA,† AND JUHA MERILÄ‡

Department of Population Biology, Evolutionary Biology Center, Uppsala University, Norbyvägen 18D, SE-752 36 Uppsala, Sweden, and Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, P.O. Box 65, University of Helsinki, FI-00014 Helsinki, Finland

Pesticides represent an important threat for natural populations. While their effects are assessed on short terms acute exposure, some of their harmful consequences may only become apparent when combined with other stressors, notably natural ones, such as predation. Here, we investigated in a laboratory experiment how exposure to a common fungicide (fenpropimorph) would affect the responses to predation in the common frog *Rana temporaria.* The concentrations of fungicide we used were comparable to those found in nature (0, 2, or 11 μg/L). The higher concentration of fungicide reduced tadpole activity late in the experiment, and only 7% of the tadpoles reached metamorphosis. In the lower concentration, the ability to respond adaptively to predator presence was not affected, but the costs (delayed metamorphosis, smaller relative body size) of this response were increased. Our results highlight the need to investigate sublethal effects of pesticides on organismal performance if assessment of pesticides real impact is to be obtained.

Introduction

Agricultural intensification has led to declines in many animal populations and resulted in the decrease of species richness in aquatic and terrestrial farmland communities (*1*−*3*). Pesticides are widely used in agriculture and can have adverse effects on nontarget populations through their negative effects on the behavior, growth, development, reproduction, and physiology of individuals (*4*−*6*). These effects can decrease the fitness of individuals and, if drastic enough, lead to population declines (*5*).

Pesticides occur in nature together with other abiotic and biotic stressors, such as fertilizers, pathogens, and predators, and these may alter the effects of pesticides on organisms (*7*−*10*). Spatially and temporally variable natural stressors (e.g. competition, predation, temperature, drought) often select for phenotypic plasticity as an adaptive response to environmental variability (*11*). Inducible defenses in aquatic organisms are an example of such responses and include alterations in both morphology and behavior (*12*−*14*). While the ability to respond to a predator is crucial for survival, the defenses often, but apparently not always, incur costs such as a decreased growth rate, delayed maturity, or reduced fecundity (*15*, *16*). As both defenses and pesticides can have negative effects on growth and development, the resulting energetic tradeoff may compromise induction of defenses under pesticide stress (i.e. cost of being insufficiently protected vs cost of the defense). In general, it is not known how pesticides affect these defenses (but see ref *17*).

Pesticides are likely to constitute a threat to the aquatic developmental stages of amphibians as they are typically applied at the time amphibians undergo their embryonic and larval development. Tadpoles also have permeable skin, which makes them highly susceptible to the chemical conditions of the aquatic environment (*18*). Indeed, pesticides are known to have various negative effects on tadpoles: decreased survival, delayed metamorphosis or decreased size at metamorphosis, increased abnormality rates (e.g. deformed tail, extra legs), lower activity levels and swimming abilities, and altered immune response (*19*−*23*). The few studies investigating the simultaneous impact of the presence of pesticides and predators have found that the stressors often act synergistically to decrease tadpole survival (*8*, *24*). However, pesticides may also affect the ability of amphibian larvae to respond adaptively to predator stress through nonlethal effects on antipredator defenses.

In this paper, we investigated the interacting effects of long-term exposure to fenpropimorph, a widely used agricultural fungicide, and predation risk (presence of caged dragonfly larvae) on common frog *Rana temporaria* larvae in a laboratory experiment. *R. temporaria* displays inducible defenses in behavior and morphology typical to many ranid frogs including modifications of behavior such as decreased activity levels and increased refuge use as well as morphological defenses such as deeper tails and shorter bodies (e.g. ref *14*). While behavioral changes increase survival by decreasing the risk of detection by the predator, the morphological changes increase survival during predator encounters, and, accordingly, induced morphology is a target of strong natural selection by predators (*12*, *25*). However, investment in the defenses is costly in terms of decreased competitive ability and reduced growth and development rates (*15*, *26*). We focused on the expression of inducible defenses in behavior and morphology, larval mass and developmental stage, and age and size at metamorphosis, the latter four traits reflecting the potential costs of antipredator responses. If exposure to a pesticide has negative effects on tadpoles' antipredator defenses, two outcomes are possible. First, the effects of the pesticide may impede the inducible (behavioral and morphological) responses of tadpoles to a predator. Second, the pesticide may increase the cost of responding to a predator in terms of reduced growth or development rates.

Methods

Study Organisms. *Rana temporaria* is a widespread anuran with a range extending from northern Spain up to the coast of the Arctic sea (*27*). It is the most numerous amphibian species in many areas of northern Europe and occurs also commonly in agricultural landscapes. Pesticides are applied particularly during the larval development of *R. temporaria*, but pesticide residues may be present in the environment throughout its aquatic development. As the common frog's breeding habitats (e.g. ditches) are often situated in agricultural areas, the most likely routes of pesticide transportation in these areas are runoffs and accidental applications.

We collected 10 freshly laid egg clutches of *R. temporaria* from a forest pond in central Sweden (Häggedal, Uppsala

* Corresponding author phone: +358-41-4416559; fax: +358-9-19157674; e-mail: henna.piha@helsinki.fi.
† Uppsala University.
‡ University of Helsinki.

10.1021/es050127u CCC: $30.25
Published on Web 07/06/2005

municipality 59°52′ N, 17°14′ E) in April 2003. The clutches were collected from a nonagricultural area to avoid potential genetic adaptation to pesticides (e.g. ref *28*). The clutches were transported to the laboratory in Uppsala and raised in 3 L vials (two vials per clutch) in 18 °C.

We used late-instar dragonfly larvae (*Aeshna sp.*) as predators, which were collected from a pond near Uppsala. They are voracious predators of tadpoles and common in the breeding ponds used by *R. temporaria*. When not in the experiment, the predators were maintained in individual 0.25 L vials in 18 °C and fed *R. temporaria* tadpoles every second day.

Pesticide. Fenpropimorph is a morpholine fungicide (chemical name (IUPAC): (±)-*cis*-4-[3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine) mainly used to control fungal diseases in cereals. It is widely used in Scandinavia (28.7 tons sold in Denmark in 1995 [http://www.itass.dk/1pesti.htm] and 27.7 tons sold in Sweden in 2003 [http://www.kemi.se/Kemi/Kategorier/Statistik/Stats/start.html]). In agricultural practices, typically 1−3 field applications are made at rates of 0.3−0.75 kg active ingredient/ha. Measured concentrations in nature usually range between 0 and 6 μg/L (*29*), but concentrations up to 12 μg/L have been found in streams in Norway (*30*). The half-life of fenpropimorph is above 64 days in water (at 50 °C) and approximately 54 days in the sediment (*31*).

Fenpropimorph is known to inhibit the synthesis of sterols in fungi, plants, and vertebrates (*32*). It is also known to affect uracil and cytosine uptake in *Saccharomyces cerevisiae* (*33*). The toxicity of fenpropimorph has not been tested for amphibians, but the 48 h LC_{50} value for carp is 3.2 mg/L and 9.5 mg/L for rainbow trout. We had no data on the sensitivity of dragonflies to fenpropimorph prior to our experiment. However, in the present study the survival and appetite of dragonflies was not affected by fenpropimorph. Similarly, in an experiment carried out the following year a 10-day exposure to fenpropimorph concentrations of 5 and 15 μg/L did not affect the ability of *Aeshna* dragonfly larvae to prey on *R. temporaria* tadpoles (Piha et al. unpublished).

Technical grade fenpropimorph was obtained from Sigma-Aldrich Co. (PESTANAL, analytical standard, purity 93.6%). A stock solution was made by dissolving 250 mg of fenpropimorph into 500 mL of acetone. The concentration of the stock solution was 460 mg/L, as determined by gas chromatography (GC) with a MS-detector at the Department of Environmental Assessment at the Swedish University of Agricultural Sciences. The same stock solution was used throughout the experiment. It was stored in a cold room (+4 °C) protected from light.

We prepared the test solutions by adding an appropriate amount of stock solution directly into experimental tanks filled with water. We used reconstituted soft water (RSW: deionized water and $NaHCO_3$ [48 mg/L], $CaSO_4 \times 2H_2O$ [30 mg/L], $MgSO_4 \times 7H_2O$ [61.4 mg/L], and KCl [2 mg/L] (*34*)) to avoid uncontrolled changes in water quality. To maintain a sufficient level of oxygenation, and also because of pesticide breakdown, test solutions in the experimental tanks were changed every fifth day.

Experimental Design. Our experimental design was a 3 × 2 factorial design with three pesticide concentrations (C0: 0, C1: 2, and C3: 11 μg/L in a chronic exposure) and two predator treatments (absence or presence of one *Aeshna* larva) as factors. Each treatment combination was replicated 8 times resulting in a total of 48 experimental units. No acetone controls were used, because earlier work found no effects of chronic exposure to acetone concentrations of 2 mL/L on *R. temporaria* tadpoles (Johansson et al., unpublished). Our acetone concentrations were much lower than this, and also below the maximum allowable ASTM standards <1.1% v/v (*35*), and 0.5 mL/L (*36*).

The experiment was conducted in a single laboratory room (constant 18 °C, D:L = 7:17) in opaque plastic tanks (38 × 28 × 13 cm) filled with 10 L of water. Because of a known temperature gradient in the laboratory room, we divided the experiment into three vertically ordered blocks; the mean temperature differences between blocks varied between 0.2 and 0.5 °C. Each block contained two to three randomly placed replicates of each treatment combination.

The experiment was started on May 2, 2003 (day 0), when tadpoles had reached stage 25 ((*37*) initial mass: mean ± SE: 15 ± 1 mg), and terminated after 57 days, at the time when all individuals from the control and lower pesticide treatment had metamorphosed. Each tank received 10 tadpoles (one from each family) and either one (predator present) or no (predator absent) dragonfly larva. The predator was placed in a cylindrical cage (diameter 11 cm, height 21 cm) made of transparent plastic film with a double net bottom (mesh size 1.5 mm) and plunged into the tank to a depth of 3 cm. Hence the predator was not able to catch the tadpoles, but the tadpoles were able to receive both visual and chemical cues from the predator. In the no-predator treatment the cage was left empty. Predators were fed *R. temporaria* tadpoles (ca. 300 mg) every other day. Tadpoles were fed every day ad libitum with chopped, boiled spinach.

Response Variables and Analyses

Tadpole Behavior. We recorded activity level over 3 days at three separate times: days 2−4 (time period 1), days 7−9 (time period 2), and days 17−19 (time period 3). During these 3-day periods, the number of tadpoles active in each tank was recorded three times a day (at 09:00 a.m., 10:30 a.m., and 12:00 p.m.). Activity level was estimated as the number of active tadpoles divided by the total number of tadpoles per tank. The nine activity measurements taken per time period were averaged into one value per tank, which was used as the response variable for each experimental unit. The data were arcsin-squareroot transformed before the analyses. To investigate the effect of different time periods on tadpole behavior, we first performed a factorial repeated measures ANOVA followed by factorial ANOVAs for each time period.

Tadpole Morphology, Mass, and Development. On day 20 of the experiment, we sampled approximately half of the tadpoles from each tank and preserved them in alcohol (70%) for later measurements. Because of differences in survival between tanks, we sampled 4−5 tadpoles from each tank so that five tadpoles remained in each tank. Adjustment of sample size was done in order to keep the density constant for the subsequent rearing. Tadpole morphology was measured with a digital caliper to the nearest 0.01 mm from preserved animals. Five variables involved in morphological defenses were measured: body length, maximum tail fin depth, maximum body depth, tail length, and maximum tail muscle depth. Size was estimated by individual scores on the first axis of a principal component analysis (PC1) run on these five variables. All traits loaded strongly positively on the first axis revealing this axis to be a size component. PC1 was then used as a covariate in all morphological analyses. We performed a MANCOVA on all the morphological traits followed by univariate ANCOVAs with pesticide concentration, predator presence, and block as fixed factors. The interactions between the covariate and the factors were not significant.

Wet mass of tadpoles was weighed to the nearest 0.1 mg and developmental stage was assessed according to Gosner (*37*). Both variables were analyzed first with a MANOVA and then separate ANOVAs. In initial analyses no significant block × treatment interactions were found, and these interactions were omitted from the final models.

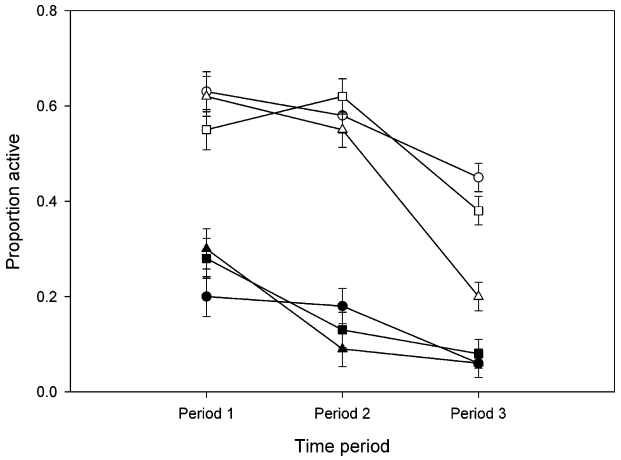

FIGURE 1. Tadpole activity level (least-squares means ± SE) in the pesticide and predator treatments during the three observation periods (early, middle, and late in the development). Circles: no pesticide; squares: 2 μg/L; triangles: 11 μg/L. Open symbols stand for the absence of predator, filled symbols for the presence of predator.

TABLE 1. Repeated Measures ANOVA Table for the Activity Level of *Rana temporaria* Tadpoles during Three Time Periods (Early, Middle, and Late in the Development) in the Pesticide and Predator Treatments

source	df	Wilks' λ	*F*	*P*
period	2, 39	0.243	60.91	<0.001
period × pesticide	4, 78	0.775	2.65	0.040
period × predator	2, 39	0.683	9.07	<0.001
period × block	4, 78	0.907	0.98	0.424
period × pesticide × predator	4, 78	0.736	3.24	0.016

Metamorphic Traits and Survival. Tadpoles remaining in the tanks were submitted to the same predation risk and pesticide exposure as they had experienced during the first part of the experiment. Metamorphs (appearance of at least one forelimb, stage 42 (*37*)) were checked daily. For each metamorph, date of metamorphosis was recorded, and body length and mass (to the nearest 0.1 mg) were measured at stage 42. The data on metamorphic traits were analyzed with factorial MANOVA followed by ANOVAs. Because survival and successful metamorphosis in the highest pesticide concentration were very low, this treatment was excluded from the analyses of metamorphic traits. To control for unequal survival among the experimental units, survival from day 20 onward was used as a covariate in the analysis of metamorphic traits. All the analyses of variance were conducted with the GLM procedure in SAS (version 8.02).

Tadpole mortality was analyzed with type III general linear models with a logit link function and binomial error structure as implemented in the GENMOD procedure of SAS (*38*). At the end of the experiment, the 12 tadpoles that had not reached metamorphosis in the highest pesticide treatment were very small and underdeveloped, and their future survival was more than unlikely. They were therefore considered as dead in the survival analyses. Removal of these 12 tadpoles from the analyses did not change the results. No significant block × treatment interactions were found in initial analyses, and these were omitted from the further analyses.

Results

Tadpole Behavior. Pesticide and predator treatments affected tadpole activity differently during the different time periods (Table 1, Figure 1).

While predator presence strongly decreased activity levels during all time periods, pesticide concentration affected activity levels only in the third observation period (Table 2). During this period, a significant pesticide × predator interaction arose because pesticide presence reduced activity levels only in the absence of predators (Figure 1).

Tadpole Morphology, Mass, and Developmental Stage. MANCOVA showed significant effects of predator presence, pesticide and their interaction on tadpole traits (Table 3 A). Predator presence affected tadpole morphology (Table 3, Figure 2a,b), decreasing their relative body size and increasing their tail depth, whereas there were no effects on tail muscle, tail length, and body depth (Table 3). Exposure to the pesticide decreased relative tail length, tail fin depth, and body length (Table 3, Figure 2a–c). Moreover, tadpole body length decreased at the lower pesticide concentration in the presence of predator, whereas there was no change in the absence of predator, resulting in a significant pesticide × predator interaction.

MANOVA revealed both significant effects of pesticide and predator presence on tadpole mass and developmental stage. Pesticide presence significantly decreased tadpole mass. The highest pesticide concentration strongly decreased tadpole mass and developmental stage (Table 4, Figure 2d,e).

Moreover, we found a significant pesticide × predator effect on developmental stage (Table 4), because predator presence had a highly significant effect only in the lower pesticide concentration (one-way ANOVA; predator effect in C0: $P = 0.066$, C1: $P = 0.008$, C2: $P = 0.987$; Figure 2e). Predator treatments did not affect tadpole body mass (Table 3, Figure 2d). The significant block effect (Table 4) reflected the slight temperature differences within the laboratory room (increased mass and stage at higher temperature).

Metamorphic Traits. MANOVA showed significant effect of predator presence and marginally significant effect of pesticide concentration (Table 5).

Age at metamorphosis was the most sensitive trait to the experimental treatments with both pesticide and predator presence interacting to increase the time to reach metamorphosis (Table 5, Figure 3a). The delaying effect of predator presence was roughly 4 days (or 10% of the larval period) longer when the tadpoles were exposed to the pesticide, suggesting a more than additive effect (Table 5, Figure 3a). Predator presence and pesticide also decreased body length

TABLE 2. Activity Level of Tadpoles in Response to Pesticide and Predator Treatments[a]

		period 1		period 2		period 3	
source	df	*F*	*P*	*F*	*P*	*F*	*P*
pesticide	2	0.77	0.469	1.82	0.175	6.86	0.003
predator	1	98.10	<0.001	207.45	<0.001	98.03	<0.001
block	2	0.44	0.649	0.78	0.463	0.66	0.521
pesticide × predator	2	1.77	0.183	0.70	0.502	6.58	0.003
error	40						

[a] Separate tests were conducted for each period (early, middle, and late development because of a significant interaction between period, pesticide, and predator treatments.

TABLE 3. (A) MANCOVA and (B) Univariate ANCOVA Results for Morphology of Tadpoles in the Pesticide and Predator Treatments

(A) MANCOVA

source	df	*F*	*P*
size	5, 35	162.33	<0.001
pesticide	10, 70	4.60	<0.001
predator	5, 35	14.76	<0.001
block	10, 70	1.15	0.339
pesticide × predator	10, 70	2.41	0.016

(B) Univariate ANCOVA

		body length		tail fin depth		body depth		tail length		tail muscle depth	
source	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
size	1, 39	68.09	<0.001	56.35	<0.001	111.19	<0.001	33.04	<0.001	38.19	<0.001
pesticide	2, 39	6.21	0.004	4.34	0.020	0.34	0.714	14.36	<0.001	0.24	0.786
predator	1, 39	17.17	<0.001	34.06	<0.001	0.43	0.515	2.45	0.125	0.52	0.476
block	2, 39	0.67	0.518	0.3	0.741	0.56	0.575	2.03	0.145	0.04	0.961
pesticide × predator	2, 39	4.30	0.021	2.08	0.139	1.16	0.323	1.76	0.186	0.89	0.421

TABLE 4. MANOVA and Univariate ANOVA Results for Mass and Developmental Stage of Tadpoles in the Pesticide and Predator Treatments

		MANOVA			mass		developmental stage	
source	df	*F*	*P*	df	*F*	*P*	*F*	*P*
pesticide	4, 78	40.70	<0.001	148.83	148.83	<0.001	85.01	<0.001
predator	2, 39	4.52	0.017	1.77	1.77	0.192	9.09	0.004
block	4, 78	3.25	0.016	6.72	6.72	0.003	4.13	0.024
pesticide × predator	4, 78	1.94	0.111	1.96	1.96	0.155	4.06	0.025

TABLE 5. ANOVAs Results for Age and Size (Body Length and Mass) of Tadpoles at Metamorphosis in the Pesticide and Predator Treatments

		MANOVA			age		body length		mass	
source	df	*F*	*P*	df	*F*	*P*	*F*	*P*	*F*	*P*
pesticide	3, 21	2.63	0.077	1, 23	5.86	0.039	3.69	0.067	5.19	0.032
predator	3, 21	11.67	<0.001	1, 23	36.28	<0.001	9.05	0.006	1.80	0.193
survival	3, 21	1.84	0.171	1,23	2.39	0.136	1.78	0.195	4.01	0.057
block	6, 42	2.35	0.048	2, 23	6.71	0.005	0.60	0.554	0.27	0.762
pesticide × predator	3, 21	1.77	0.183	1, 23	4.64	0.042	0.58	0.453	0.9	0.353

TABLE 6. Logistic Regressions of Tadpole Survival on Pesticide and Predator Treatments

	survival		
source	df	χ^2	*P*
pesticide	1, 38	171.66	<0.001
predator	1, 38	2.48	0.116
block	2, 38	0.13	0.937
pesticide × predator	1, 38	1.24	0.539

at metamorphosis (Table 5, Figure 3b), resulting in an additive negative effect of both stressors. Finally, pesticide decreased mass at metamorphosis (Table 5, Figure 3c).

Survival. Survival was strongly affected by the pesticide but not by predator treatment (Table 6, Figure 4). Only 7% of the individuals reached metamorphosis in the highest pesticide concentration. There was no survival difference between control (89%) and low pesticide concentration (87%).

Discussion

We found that a widely used pesticide decreased the survival, mass, and development of *R. temporaria* tadpoles. It also increased costs of the antipredator defense. Several previous studies have stressed the importance of studying the simultaneous impact of natural and anthropogenic stressors and revealed stronger negative impacts of pollutants on survival and life history traits than expected from studies using a single stressor (*8, 39*−*42*). Our study reinforces this view by highlighting that pesticides can increase the costs of inducible defenses.

Effects of Fenpropimorph. At 11 μg/L, fenpropimorph strongly increased the mortality of the tadpoles. This is important as the concentrations we used represent less than 1% of 48 h LC_{50} found in fish, the standard vertebrate model in aquatic toxicology. The concentrations we used are also ecologically relevant since concentrations up to 12 μg/L have been found in streams (*30*). Our results suggest that the 48 h LC_{50} may tell little about the toxicity of fenpropimorph for tadpoles, as much lower concentrations had sublethal to lethal effects in chronic exposure. Because fenpropimorph has been found in streams at detectable concentrations even 7 months after its application (*29*), it is possible that this pesticide may have negative impacts on natural populations. However, we are not aware of studies investigating the impact of fenpropimorph on multicellular organisms in the wild (but see refs *43* and *44* for unicellulars).

In contrast with other studies (*8*, *24*), we found no significant pesticide concentration × predator effect on survival. However, in the highest concentration, the mortality

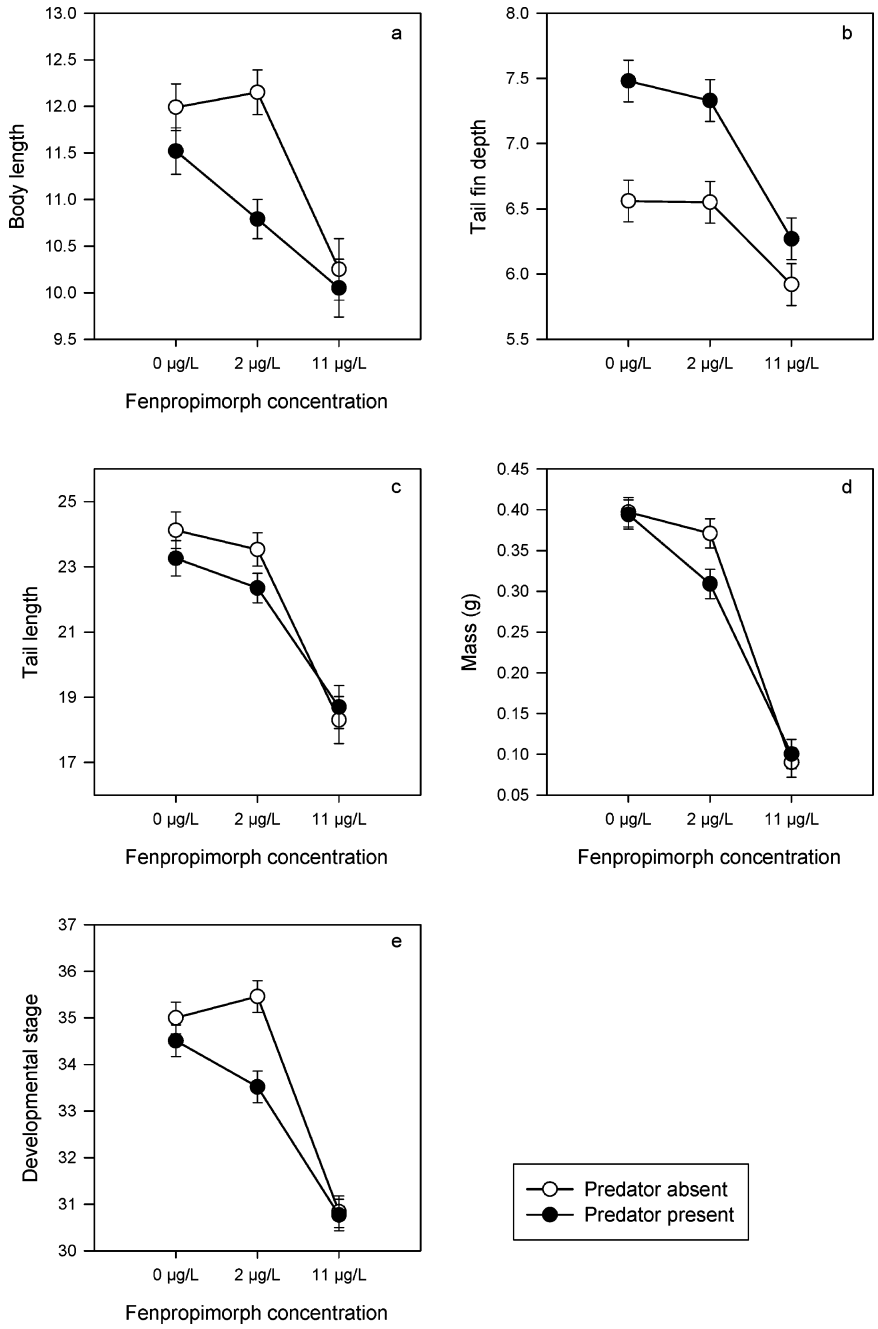

FIGURE 2. Tadpole morphology (a, b, c) and mass and developmental stage (d, e; least-squares means ± SE) as a function of pesticide and predator treatments. For statistical tests, see Table 3.

was 87%, whereas survival was unaffected in the lower concentration. Thus it seems that the effects of fenpropimorph can change dramatically within a relatively small concentration range (between 2 and 11 μg/L), and an additional intermediate concentration could perhaps have revealed such effects. Indeed, in a study by Relyea (*24*) the tadpoles were exposed to 0, 0.03, 0.3, 1.6, 3.2, and 6.5 mg/L of carbaryl. The effects of interactions between pesticide and predator presence were most evident in 1.6 mg/L.

Fenpropimorph exposure led to decreased mass and developmental stage of tadpoles, mainly due to higher pesticide concentration. Moreover, we found effects of the lower pesticide concentration (2 μg/L) on metamorphic traits (decreased size, increased time to metamorphosis). The clearly negative impacts of fenpropimorph on development suggest that fenpropimorph concentrations occurring in nature can have negative effects on tadpole fitness in the wild.

At the highest concentration and after 2 weeks of exposure, fenpropimorph decreased tadpole activity, which is a common effect of pesticides (*5*, *8*). This time lag is probably due to the slowly deteriorating condition of the tadpoles in the highest fenpropimorph concentration. Fenpropimorph did not interact with the tadpoles' ability to display decreased activity level in the presence of predators at the lower pesticide concentration. Predator presence induced a strong reduction in tadpole activity, a response that decreases encounter rates with predators and also makes tadpoles less conspicuous to predators that use visual or tactile cues (e.g. ref *45*). However, as predator presence strongly decreased tadpole activity, it is possible that an interaction may not have been detectable. Also, other components of the behavioral response that were not measured in the present study (e.g. escape behavior (*19*)) may be affected by pesticide treatments.

Effects of Pesticide × Predator Interaction. In the lower pesticide concentration, the tadpoles' ability to display an

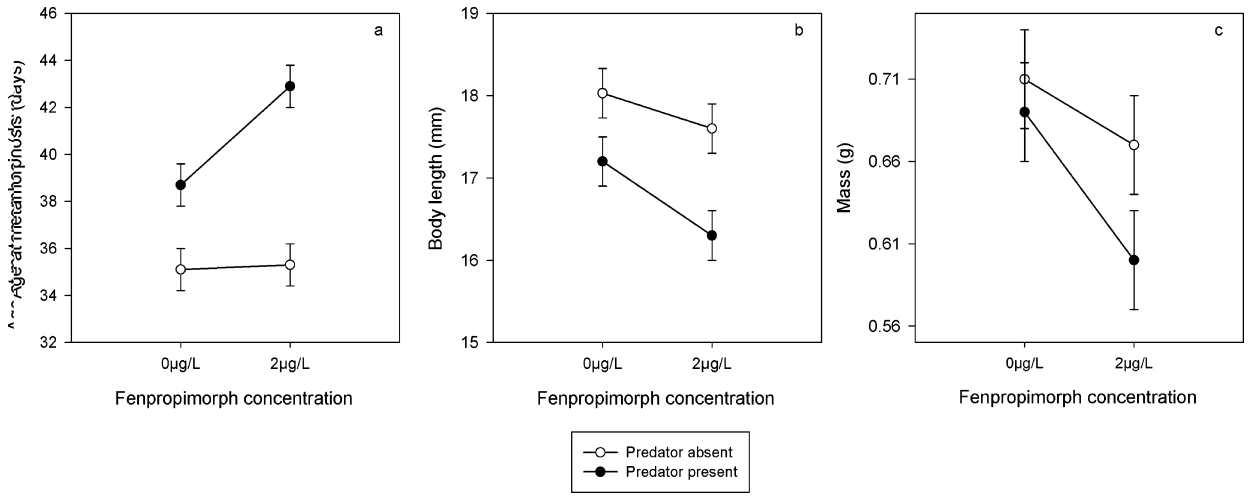

FIGURE 3. Tadpole age (a), body length (b), and mass (c) at metamorphosis according to the pesticide and predator treatments (least-squares means ± SE).

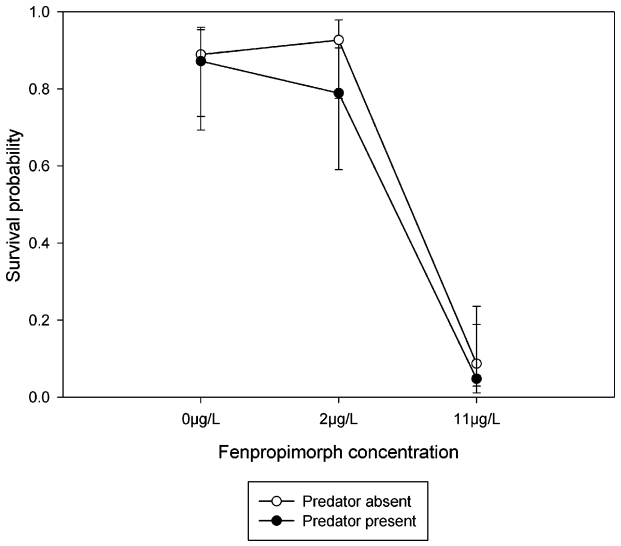

FIGURE 4. Tadpole survival until metamorphosis according to the pesticide and predator treatments. Values are estimates of survival probability obtained from logistic regression (estimate values, IDC 95%).

adaptive antipredator response was not affected, but the costs of antipredator defenses (i.e. decreased relative tadpole body size, increased time to metamorphosis) were increased compared to the no pesticide treatment. Decreased relative body size has been interpreted as a cost of inducible defenses (*46*). While this cost was not observable in the absence of pesticide, it appeared at the lower pesticide concentration.

More, predator presence delayed metamorphosis, and the delay was stronger in the lower pesticide concentration than in the control treatment indicating a synergistic effect between the two stressors. Delayed metamorphosis can have direct consequences on the survival of tadpoles developing in temporary ponds, which may dry up before tadpoles have metamorphosed (*47*, *48*). Delayed metamorphosis involves carryover effects such as decreased growth and survival in later life stages and lower fecundity (*49*−*51*). Through the effects on metamorphic life history traits sublethal concentrations of pesticides may have long-term effects on individual fitness and amphibian population dynamics.

Our results clearly show increased costs of developing a defense, but we did not detect a decrease investment in the defense. Two lines of evidence suggest it would be interesting to test the antipredator responses along a more detailed gradient of pesticide concentrations. First, although there are still very few studies on this subject, there is at least one study in *Daphnia* showing that the pesticide endosulfan can prevent the full expression of neckteeth protecting them from predation (*17*). Second, fenpropimorph decreased growth rate even at low concentrations, and recent work suggests that under low resource (and low growth) conditions tadpoles exhibit weak morphological induction by predators (*52*, *53*).

Our results show that even very low pesticide concentrations can be lethal in a chronic exposure and suggest that LC_{50} doses may tell only little about the chronic toxicity of a given compound and the risk it presents for natural populations. Furthermore, in combination with another stressor, very low pesticide concentrations can have negative effects on the fitness of nontarget organisms. This reinforces the view that synergistic effects of multiple stressors are important and suggest that pesticides may disrupt adaptive responses to natural stressors. Further studies in long-term pesticide-stress interactions in more natural settings are needed for evaluating the effects of pesticides on natural populations.

Acknowledgments

We thank Karin Carlsson for help with laboratory work. Beatrice Lindgren, Katja Räsänen, and Mikael Persson helped collecting the eggs and the dragonflies. We are also grateful to Katja Räsänen and Michelle Boone for their insightful comments. Our research was funded by the Swedish Research Council for Agriculture, Forestry and Spatial planning, the Swedish Institute, Fondation Singer-Polignac, Oskar Öflunds Stiftelse and Maj and Tor Nessling Foundation.

Literature Cited

(1) McLaughlin, A.; Mineau, P. The impact of agricultural practices on biodiversity. *Agric. Ecosyst. Environ.* **1995**, *55*, 201−212.
(2) Beja, P.; Alcazar, R. Conservation of Mediterranean temporary ponds under agricultural intensification: an evaluation using amphibians. *Biol. Conserv.* **2003**, *114*, 317−326.
(3) Donald, P. F.; Green, R. E.; Heath, M. F. Agricultural intensification and the collapse of Europe's farmland bird populations. *Proc. R. Soc. London, Ser. B: Biol. Sci.* **2001**, *268*, 25−29.
(4) Tew, T. E.; Macdonald, D. W.; Rands, M. R. W. Herbicide application affects microhabitat use by arable wood mice (*Apodemus sylvaticus*). *J. Appl. Ecol.* **1992**, *29*, 532−539.
(5) Walker, C. H.; Hopkin, S. P.; Sibly, R. M.; Peakall, D. B. *Principles of Ecotoxicology*, 2nd edition ed.; Bell & Bain Ltd.: Glasgow, U.K., 2001.
(6) Fordham, C. L.; Tessari, J. D.; Ramsdell, H. S.; Keefe, T. J. Effects of malathion on survival, growth, development, and equilibrium posture of bullfrog tadpoles (*Rana catesbeiana*). *Environ. Toxicol. Chem.* **2001**, *20*, 179−184.

(7) Hanazato, T. Pesticide effects on freshwater zooplankton: an ecological perspective. *Environ. Pollut.* **2001**, *112*, 1−10.
(8) Relyea, R. A.; Mills, N. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2491−2496.
(9) Staton, J. L.; Schizas, N. V.; Klosterhaus, S. L.; Griffitt, R. J.; Chandler, G. T.; Coull, B. C. Effect of salinity variation and pesticide exposure on an estuarine harpacticoid copepod, *Microarthridion littorale* (Poppe), in the southeastern US. *J. Exp. Mar. Biol. Ecol.* **2002**, *278*, 101−110.
(10) Christin, M. S.; Gendron, A. D.; Brousseau, P.; Menard, L.; Marcogliese, D. J.; Cyr, D.; Ruby, S.; Fournier, M. Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. *Environ. Toxicol. Chem.* **2003**, *22*, 1127−1133.
(11) Pigliucci, M. *Phenotypic Plasticity: Beyond Nature and Nurture*; M. Johns Hopkins University Press: Baltimore, MD, U.S.A., 2001.
(12) Van Buskirk, J.; McCollum, S. A.; Werner, E. E. Natural selection for environmentally induced phenotypes in tadpoles. *Evolution* **1997**, *51*, 1983−1992.
(13) Tollrian, R.; Harvell, C. D. *The ecology and evolution of inducible defenses*; Princeton University Press: Princeton, NJ, U.S.A., 1999.
(14) Lardner, B. Morphological and life history response to predators in larvae of seven anurans. *Oikos* **2000**, *88*, 169−180.
(15) Van Buskirk, J. The costs of an inducible defense in anuran larvae. *Ecology* **2000**, *81*, 2813−2821.
(16) Dahl, J.; Peckarsky, B. L. Induced morphological defenses in the wild: predator effects on a mayfly, *Drunella coloradensis*. *Ecology* **2002**, *83*, 1620−1634.
(17) Barry, M. J. Effects of endosulfan on *Chaoborus*-induced life-history shifts and morphological defenses in *Daphnia pulex*. *J. Plankton Res.* **2000**, *22*, 1705−1718.
(18) Blaustein, A. R.; Wake, D. B. The puzzle of declining amphibian populations. *Sci. Am.* **1995**, *272*, 52−57.
(19) Bridges, C. M. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. *Environ. Toxicol. Chem.* **1997**, *16*, 1935−1939.
(20) Bridges, C. M. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). *Arch. Environ. Contam. Toxicol.* **2000**, *39*, 91−96.
(21) Broomhall, S. The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability to predation in *Litoria citropa* tadpoles. *Aquat. Toxicol.* **2002**, *61*, 243−250.
(22) Greulich, K.; Pflugmacher, S. Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquat. Toxicol.* **2003**, *65*, 329−336.
(23) Christin, M. S.; Menard, L.; Gendron, A. D.; Ruby, S.; Cyr, D.; Marcogliese, D. J.; Rollins-Smith, L.; Fournier, M. Effects of agricultural pesticides on the immune system of *Xenopus laevis* and *Rana pipiens*. *Aquat. Toxicol.* **2004**, *67*, 33−43.
(24) Relyea, R. A. Predator cues and pesticides: a double dose of danger for amphibians. *Ecol. Appl.* **2003**, *13*, 1515−1521.
(25) Van Buskirk, J.; Relyea, R. A. Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. *Biol. J. Linn. Soc.* **1998**, *65*, 301−328.
(26) McCollum, S. A.; Van Buskirk, J. Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution* **1996**, *50*, 583−593.
(27) Gasc, J. P.; Cabela, A.; Crnobrnja-Isailovic, J.; Dolmen, D.; Grossenbacher, K.; Haffner, P.; Lescure, J.; Martens, H.; Martinéz Rica, J. P.; Oliveira, M. E.; Sofianidou, T. S.; Veith, M.; Zuiderwijk, A. *Atlas of Amphibians ad Reptiles in Europe*; Societas Europeae Herpetologia and Museum National d'Histoire Naturelle (IEGB/SPN), Paris, 1997.
(28) Bridges, C. M.; Semlitsch, R. D. Genetic variation in insecticide tolerance in a population of southern leopard frogs (*Rana sphenocephala*): Implications for amphibian conservation. *Copeia* **2001**, 7−13.
(29) Kreuger, J. Pesticides in streamwater within an agricultural catchment in southern Sweden, 1990−1996. *Sci. Total Environ.* **1998**, *216*, 227−251.
(30) Ludvigsen, G. H.; Lode, O. O. *Oversikt over påviste pesticider i perioden 1995−2002 (An overview of found pesticides in 1995−2002)*; Report from the Norwegian Agricultural Environmental Program; Norwegian Centre for Soil and Environmental Research (in Norwegian), 2004.
(31) Tomlin, C. *The pesticide manual*; British Crop Protection Council: Farnham, Surrey, U.K., 1997.
(32) Corio-Costet, M. F.; Gerst, N.; Benveniste, P.; Schuber, F. Inhibition by the fungicide fenpropimorph of cholesterol-biosynthesis in 3t3 Fibroblasts. *Biochem. J.* **1988**, *256*, 829−834.
(33) Crowley, J. H.; Lorenz, R. T.; Parks, L. W. Fenpropimorph affects uptake of uracil and cytosine in *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **1994**, *38*, 1004−1007.
(34) *Standard methods for the examination of water and wastewater*, 16th ed. ed.; American Public Health Association: Washington, 1985.
(35) *Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (Fetax)*; E 1439-98; American Society for Testing and Materials: 2004.
(36) *Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians*; E 729-96; American Society for Testing and Materials: 2002.
(37) Gosner, K. L. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **1960**, *16*, 183−190.
(38) Allison, P. D. *Logistic regression using the SAS system. Theory and application*; SAS Institute Inc.: Cary, NC, U.S.A., 1999.
(39) Boone, M. D.; Semlitsch, R. D. Interactions of an insecticide with larval density and predation in experimental amphibian communities. *Cons. Biol.* **2001**, *15*, 228−238.
(40) Boone, M. D.; Semlitsch, R. D. Interactions of an insecticide with competition and pond drying in amphibian communities. *Ecol. Appl.* **2002**, *12*, 307−316.
(41) Pahkala, M.; Rasanen, K.; Laurila, A.; Johanson, U.; Bjorn, L. O.; Merila, J. Lethal and sublethal effects of UV-B/pH synergism on common frog embryos. *Cons. Biol.* **2002**, *16*, 1063−1073.
(42) Bridges, C. M.; Boone, M. D. The interactive effects of UV-B and insecticide exposure on tadpole survival, growth and development. *Biol. Conserv.* **2003**, *113*, 49−54.
(43) Svensson, J.; Leonardson, L. Effects of agricultural pesticides on denitrification in lake sediments. In *Environmental aspects of pesticide microbiology*; Anderson, J., Arnold, D. J., Lewis, F., Torstensson, L., Eds.; August 17−21, Sigtuna, Sweden, 1992; pp 55−60.
(44) Björnlund, L.; Ekelund, F.; Christensen, S.; Jacobsen, C. S.; Krogh, P. H.; Johnsen, K. Interactions between saprotrophic fungi, bacteria and protozoa on decomposing wheat roots in soil influenced by the fungicide fenpropimorph (Corbel): a field study. *Soil Biol. Biochem.* **2000**, *32*, 967−975.
(45) Anholt, B. R.; Werner, E. E. Interaction between food availability and predation mortality mediated by adaptive behavior. *Ecology* **1995**, *76*, 2230−2234.
(46) Smith, D. C.; Van Buskirk, J. Phenotypic design, plasticity, and ecological performance in two tadpoles species. *Am. Nat.* **1995**, *145*, 211−233.
(47) Laurila, A.; Kujasalo, J. Habitat duration, predation risk and phenotypic plasticity in common frog (*Rana temporaria*) tadpoles. *J. Anim. Ecol.* **1999**, *68*, 1123−1132.
(48) Loman, J. Temperature, genetic and hydroperiod effects on metamorphosis of brown frogs *Rana arvalis* and *R-temporaria* in the field. *J. Zool.* **2002**, *258*, 115−129.
(49) Smith, D. C. Adult recruitment in chorus frogs: Effects of size and date at metamorphosis. *Ecology* **1987**, *68*, 344−350.
(50) Berven, K. A. Factors affecting populations fluctuations in larval and adult stages of the woodfrog (*Rana sylvatica*). *Ecology* **1990**, *71*, 1599−1608.
(51) Altwegg, R.; Reyer, H. U. Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* **2003**, *57*, 872−882.
(52) Relyea, R. A.; Hoverman, J. T. The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. *Oecologia* **2003**, *134*, 596−604.
(53) Relyea, R. A. Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. *Ecology* **2004**, *85*, 172−179.

Received for review January 19, 2005. Revised manuscript received May 23, 2005. Accepted May 24, 2005.

ES050127U