Pakistan J. Zool., vol. 54(2), pp 791-800, 2022 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20181124041148

Effects of Four Individual Pesticides and their Pairwise Combinations on the Survival and Growth of the Tadpoles of Two Anuran Species

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Article Information
Received 24 November 2018
Revised 23 May 2019
Accepted 11 October 2019
Available online 08 October 2021 (early access)
Published 22 January 2022

Authors' Contribution
LW designed the experiment and wrote the manuscript under supervision of ZHL. WWS carried out most of the experimental work.

Key words
Survival, Growth, Pesticide, Toxicology, Anura, Tadpole

ABSTRACT

Environmental contaminants derived from pesticides could lead to a decline in wildlife populations and cause disruption of wildlife behavior, life history, and reproduction. Here, we have investigated the toxic effects of four pesticides, namely, chlorantraniliprole, penoxsulam, pymetrozine, and haloxyfop-P-methyl, on the survival and growth of the larvae of two anuran species, *Fejervarya limnocharis* and *Microhyla fissipes*. Our results showed that survival rates under most pesticide treatments (22/28) and growth under all pesticide treatments were lower than those under the control treatment. Mortality and growth reduction rates under treatment with pairwise combinations of pesticides were rarely higher than those under treatment with individual pesticides. At concentrations of 1 and 2 mg/L, the survival rates of *F. limnocharis* tadpoles did not drastically differ under treatment with all four individual pesticides. In contrast, the survival rates of *M. fissipes* showed significant differences under treatment with three of the four pesticides (except for pymetrozine). Our results suggested that individual pesticides and their combinations exerted different effects on organisms and implied the existence of pesticide- and species-specific toxicity effects.

INTRODUCTION

Amphibian populations have declined worldwide over the several past decades (Wake and Vredenburg, 2008; Aronzon *et al*., 2016). Pesticides are one of the major potential causes for this decline (Hayes *et al*., 2006; Sparling and Fellers, 2009; Smalling *et al*., 2015; Miko *et al*., 2017). Habitat loss (Collins and Storfer, 2003) and exposure to various pesticides are probably the direct factors that contribute to the decline in amphibian populations in agricultural areas (Hussain and Pandit, 2012; Mesléard *et al*., 2016). Subsequently, the redundancy and diversity of amphibians in agricultural areas have decreased compared to those in adjacent nonagricultural regions (Davidson *et al*., 2001; Relyea, 2009).

Most amphibians have a biphasic life cycle that comprises an aquatic phase and a terrestrial phase. Amphibians are highly sensitive to water pollution because of their association with aquatic habitats and permeable skin (Wright and Schindler, 1995; Hayes *et al*., 2006; Ezemonye and Tongo, 2009). Therefore, contaminants derived from pesticides and other chemicals have the potential to exert lethal and sublethal effects on amphibians. These effects include mortality (Denoël *et al*., 2012), reduced survival (Gahl *et al*., 2011; Bernabó *et al*., 2016), immunosuppression (Groner and Relyea, 2011), malformations (Denoël *et al*., 2012), and abnormal behaviors (Johnson *et al*., 2007; Denoël *et al*., 2012). For example, the herbicide atrazine can cause immunosuppression in adult northern leopard frogs (Brodkin *et al*., 2007) and impair the sexual development in male frogs (Hayes *et al*., 2003). In the wood frog *Lithobates sylvaticus*, the fungicide triphenyltin has drastic effects on survival, growth, and days to metamorphosis, as well as on the abundances of transcripts of genes of interest (Higley *et al*., 2013). *Rana dalmatina* tadpoles exposed to the insecticide endosulfan exhibit reduced growth, delayed development, increased malformations, and abnormalities in swimming patterns (Lavorato *et al*., 2013; Svartz *et al*., 2016). These findings indicate that various types of pesticides have severely toxic effects on the behavior, life history, and reproduction of amphibians (Hayes *et al*., 2002; Davidson, 2004; Vasconcelos *et al*., 2016). Although a number of studies have documented the toxicity of a given pesticide under different conditions, the majority of these works have only examined one pesticide at a time (Relyea, 2004). This approach contradicts the patterns of pesticide exposure in natural amphibian habitats, wherein pesticides exist in combination (McConnell *et al*., 1998; Sparling *et al*., 2001; Relyea, 2004; Krishnamurthy and Smith, 2011; Wei *et al*., 2014; Svartz *et al*., 2016). Although the impact of combined pesticides on other systems has

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0030-9923/2022/0002-0791 $ 9.00/0

received empirical attention (Hoagland *et al.*, 1993; Wood and Stark, 2002; Wei *et al.*, 2014), the effects of combined pesticides on amphibians remain incompletely understood (Gendron *et al.*, 2003; Relyea, 2004; Svartz *et al.*, 2016).

Pesticides are widely used and have caused water pollution in agricultural areas in China (Jiao *et al.*, 2007). The intensive utilization of pesticides and the low efficiency and limitations of pesticide wastewater treatment can contribute to the high total amount of pesticides present in the natural environment (Yan *et al.*, 2017). Given their complex structure, stable physical and chemical properties, and strong mobility, pesticides present in the natural environment can affect the ecological balance by posing a serious threat to the endocrine and reproductive systems of animals and humans (Yan *et al.*, 2017). Thus, the impact of agricultural pesticides on animal and human health has long been stressed and remains a high-priority issue (Kattwinkel *et al.*, 2011).

Chlorantraniliprole, a novel pesticide that belongs to the anthranilic diamide group, demonstrates potent broad-spectrum activity against several insect orders (Wang *et al.*, 2010). This pesticide can dysregulate Ca^{2+} release from intracellular Ca stores. The dysregulation of Ca^{2+} release impairs the ability of insects to regulate muscle function and generates poisoning symptoms, such as rapid feeding cessation, lethargy, and muscle paralysis, and ultimately causes death (Wang *et al.*, 2010; Lahm *et al.*, 2009). Penoxsulam has been registered as an effective herbicide for use in rice production in 28 countries (Johnson *et al.*, 2009). The mechanism underlying the action of penoxsulam is the inhibition of the synthesis of acetyl lactic acid synthase (Hao *et al.*, 2014). Penoxsulam has severe toxic effects on aquatic animals. For example, it has adverse effects on the neural activity and brain and muscular tissue of *Cyprinus carpio* (Cattaneo *et al.*, 2011). Pymetrozine, a pyridine azomethine compound, is a whitefly control agent that is unrelated to neonicotinoids. Its unique mode of action disrupts feeding behavior and causes insects to die of starvation; nevertheless, its precise mode of action remains unclear (Castle *et al.*, 2009; Rao *et al.*, 2012). Haloxyfop-P-methyl is an aryloxyphenoxypropionate herbicide. It is widely used in many countries to control annual and perennial weeds in many crop species because of its advantages of high efficiency, broad-spectrum activity, and low mammalian toxicity (Bao *et al.*, 2010).

Although these four agrochemical pesticides are commonly used in cropping areas, information regarding their effects on aquatic organisms remains limited. Various pesticides remaining in the natural environment may exert highly toxic cumulative effects on surrounding organisms even if the pesticides themselves are present at low concentrations and are considered safe (Relyea, 2009). In this study, we examined the toxic effects of individual pesticides and their pairwise combinations on the survival and growth of two sympatric anuran species *Fejervarya limnocharis* and *Microhyla fissipes*. We focused on the pesticides chlorantraniliprole, penoxsulam, pymetrozine, and haloxyfop-P-methyl. We made the following predictions: (1) The four agrochemicals will have different toxic effects on the two experimental frog species. (2) Survival and growth rates under treatment with high concentrations of an individual agrochemical will be lower than those under treatment with low concentrations of the same agrochemical. (3) The survival and growth of tadpoles under treatment with two different pesticides will be lower than those under treatment with low concentrations of individual pesticides (1 mg/L) and higher than those under treatment with high concentrations of individual pesticides (2 mg/L).

MATERIALS AND METHODS

Animal collection

Egg clutches of *F. limnocharis* and *M. fissipes* were collected from ponds in Lishui, Zhejiang Province, China. The eggs were incubated in opaque plastic cages (60cm length × 40cm width × 30cm height) in 20cm deep dechlorinated tap water under natural conditions. Newly hatched tadpoles were reared on commercial fish food. Tadpoles that were considered to be in good health (swimming freely; with good reflexes; and with average weights of 0.0103±0.0009g (n=200) and 0.009±0.0011g (n=200) for *F. limnocharis* and *M. fissipes*, respectively) were selected for subsequent treatment. This research was approved by the Academic Committee of College of Ecology, Lishui University (STXY-AE-201401). Animals were handled in accordance with the current laws on animal welfare and research in China.

Experimental agrochemicals

Four widely available commercial pesticides were used in this study: chlorantraniliprole (suspension, 200g/L, American DuPont Co), penoxsulam (oil suspension, 25g/L, Dow Agrosciences Ltd., Indonesia), pymetrozine (wettable powders with 50% active ingredients, kesheng.com Inc., Jiangsu China), and haloxyfop-P-methyl (oil suspension, 108g/L, Dow Agrosciences Ltd., Indonesia).

Experimental design

Fifteen treatments were designed for *F. limnocharis* and *M. fissipes* as described by Relyea (2004), as follows: four low-concentration treatments containing 1 mg/L (active ingredient) chlorantraniliprole (A1), penoxsulam (B1), pymetrozine (C1), and haloxyfop-P-methyl (D1);

four high-concentration treatments containing 2 mg/L (active ingredient) chlorantraniliprole (A2), penoxsulam (B2), pymetrozine (C2), and haloxyfop-P-methyl (D2); six treatments (AB, AC, AD, BC, BD, and CD) based on six possible pairwise combinations of the four pesticides, wherein each pesticide was present at the concentration of 1 mg/L; and the control treatment, which comprised clean dechlorinated tap water. The treatments were replicated four times for each of the two anuran species for a total of 60 experimental units. Each experiment was performed by using a randomized block design with each block within the laboratory floor to remove directional effects. A total of 32.5μL of chlorantraniliprole, 260μL of penoxsulam, and 60μL of haloxyfop-P-methyl were added to 6.5L of clean dechlorinated tap water to prepare solutions with nominal concentrations of 1 mg/L. Volumes were doubled to prepare solutions with nominal concentrations of 2 mg/L. To prepare 1 mg/L solutions of pymetrozine, 0.09g of the chemical was dissolved in 3 mL of clean dechlorinated tap water prior to the addition of 433μL of the mixture to 6.5L of clean dechlorinated tap water. To prepare 2 mg/L solutions of pymetrozine, 866μL of the pymetrozine solution was added to 6.5L of tap water.

Three randomly selected experimental tadpoles were housed in a round 1L plastic container with 800 mL of agrochemical solution for each treatment unit. Water temperature was maintained in the range of 28.1°C–29.5°C, and dissolved oxygen concentrations were maintained at 5.24–7.94ppm. Each treatment lasted for 20 days, and agrochemical solutions were changed every 3 days. Tadpoles were reared on commercial fish food (Shanghai Tech-bank feed industry Co. Ltd). At the end of each experiment, the final numbers of surviving individuals in each container were counted, and the body masses of the tadpoles were individually weighed to the nearest 0.001g by using an electric scale (Jinnuo Balance Instrument Co., Ltd., Jinhua, China). Total body length was measured to the nearest 0.1 mm by using a dial caliper (Shanghai Medical Laser Company).

Data analysis

All statistical analyses were conducted by using STATISCTICA 6.0. Prior to statistical analysis, all data were tested for normality through the Kolmogorov–Smirnov test and homogeneity of variance test (Wei *et al*., 2014). χ^2 tests were performed for the comparisons of survival rates between species. Multivariate analysis of variance (MANOVA) was performed to test the main effects of pesticides, experimental species, and agrochemical concentrations on growth. One-way ANOVA and Tukey's post hoc multiple comparisons test were used to evaluate the effects of each pesticide (individual and combined) on the growth traits of tadpoles. Three-factor analysis of variance was used to compare the effects of pesticides, species, and concentration on survival and growth. All results were expressed as mean±SD, with α=0.05 being taken as statistically significant.

RESULTS

Survival

The survival of *F. limnocharis* was significantly affected under all treatments (χ^2=309.9, *df*=10, *P*<0.001) (Fig. 1A). The survival rates of *F. limnocharis* decreased to ca. 83.0% under treatment with 1 mg/L chlorantraniliprole and 1 mg/L pymetrozine and to 91.0% under treatment with 1 mg/L haloxyfop-P-methyl (χ^2=32.510, *df*=9, *P*<0.001). Survival rates decreased to ca. 83% under treatment with 2 mg/L pymetrozine and 2 mg/L haloxyfop-P-methyl but not under treatment with 2 mg/L chlorantraniliprole and 2 mg/L penoxsulam (100% survival) (χ^2=20.207, *df*=9, *P*0.017). Survival rates reduced under treatment with chlorantraniliprole/penoxsulam, chlorantraniliprole/ haloxyfop-P-methyl, and penoxsulam/pymetrozine (χ^2=33.412, *df*=15, *P*=0.004). Survival rates did not show significant differences under treatment with the same pesticide at high or low concentrations: A1 vs A2 (χ^2=7.165, *df*=3, *P*=0.067), B1 vs B2 (χ^2=0.000, *df*=3, *P*=1.000), C1 vs C2 (χ^2=0.000, *df*=3, *P*=1.000), and D1 vs D2 (χ^2=4.978, *df*=3, *P*=0.173).

In contrast to those of *F. limnocharis,* the survival rates of *M. fissipes* were affected under individual treatment with all four pesticides at concentrations of 1 mg/L (χ^2=75.171, *df*=9, *P*<0.001) and 2 mg/L (χ^2=118.2, *df*=9, *P*<0.001) and under treatment with pairwise combinations of pesticides (χ^2=151.1, *df*=15, *P*<0.001) (Fig. 1B). Survival rates showed significant differences between low- and high-concentration pesticide treatments: A1 vs A2 (χ^2=18.079, *df*=3, *P*0.001), B1 vs B2 (χ^2=10.745, *df*=3, *P*=0.013), and D1 vs D2 (χ^2=8.647, *df*=3, *P*=0.034). Survival rates did not show significant differences under C1 vs C2 (χ^2=7.029, *df*=3, *P*=0.071).

Growth

The body mass and lengths of *F. limnocharis* under the control treatment were higher than those under the 1 mg/L (body mass: $F_{4,50}$=12.678, *P*<0.001; body length: $F_{4,50}$=7.317, *P*<0.001), 2 mg/L (body mass: $F_{4,51}$=28.343, *P*<0.001; body length: $F_{4,51}$=15.686, *P*<0.001), and combined (body mass: $F_{6,72}$=13.990, *P*<0.001; body length: $F_{6,72}$=6.060, *P*<0.001) pesticide treatments (Fig. 2). Significant differences were observed under individual treatment with all four pesticides at concentrations of 2 mg/L but not under individual treatment with all four pesticides at concentrations of 1 mg/L (Fig. 2). Body masses showed significant differences under low- and high-concentration pesticide treatments: C1 vs C2 ($F_{1,18}$=4.836, P=0.041) and D1 vs D2 ($F_{1,19}$=6.148, P=0.023). Body masses did not show significant differences between A1 vs A2 ($F_{1,20}$=0.001, P=0.982) and B1 vs B2 ($F_{1,22}$=2.220, P=0.150). Body lengths showed significant differences between D1 vs D2 ($F_{1,19}$=11.693, P=0.003) but not between A1 vs A2 ($F_{1,20}$=0.174, P=0.681), B1 vs B2 ($F_{1,22}$=3.373, P=0.100), and C1 vs C2 ($F_{1,18}$=0.236, P=0.633).

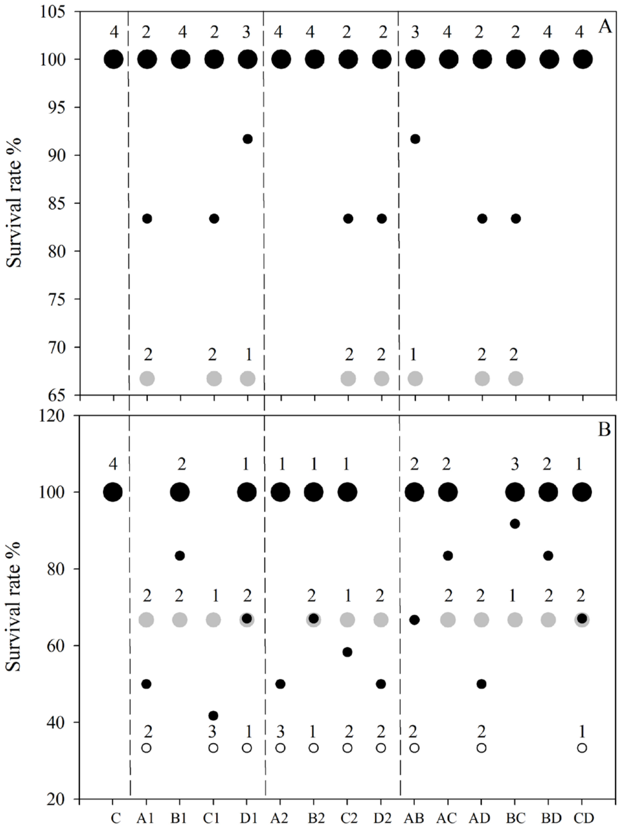

Fig. 1. The survival of *F. limnocharis* (A) and *M. fissipes* (B) tadpoles when exposed to different concentrations of separate and combined pesticides. Treatments are abbreviated as follows: C, Control; A, Chlorantraniliprole; B, Penoxsulam; C, Pymetrozine; D, Haloxyfop-P. Large black circle, grey circle, white circle and small black circle represent 100%, 66.7%, 33.3% and mean survival, respectively. The numbers above on the circles represent the treatment numbers.

The body masses and body lengths of *M. fissipes* tadpoles under the control treatment were higher than those under the 1 mg/L (body mass: $F_{4,36}$=11.721, P<0.001; body length: $F_{4,36}$=8.313, P<0.001), 2 mg/L (body mass: $F_{4,34}$=9.628, P<0.001; body length: $F_{4,34}$=18.922, P<0.001), and combined (body mass: $F_{6,58}$=11.112, P<0.001; body length: $F_{6,58}$=11.043, P<0.001) treatments (Fig. 3). Body mass and body length did not show significant differences under individual treatment with all four pesticides at low and high concentrations (all P>0.05).

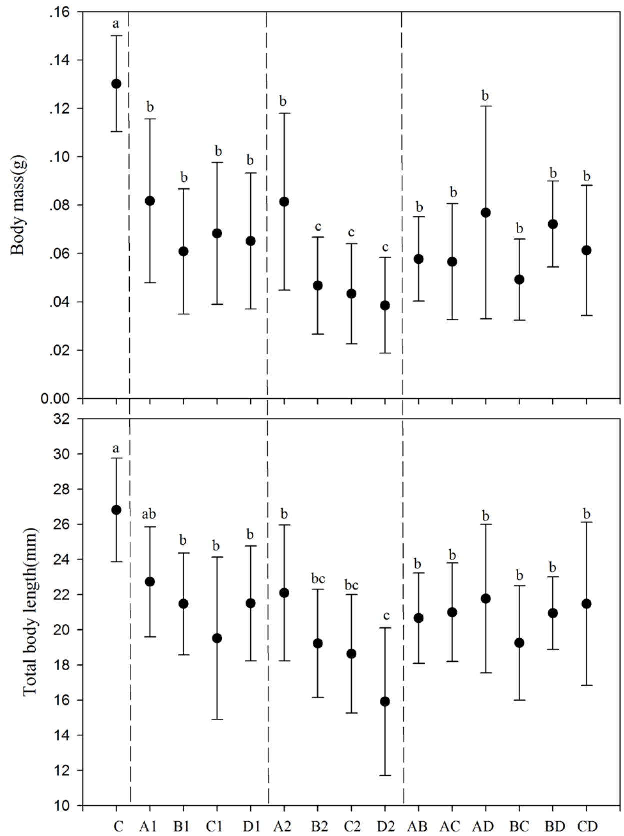

Fig. 2. The growth of *F. limnocharis* tadpoles exposed to different concentrations of separate and combined pesticides compared with control group. Treatments are abbreviated as Figure 1. Types with different superscripts differ significantly (Tukey's test, α = 0.05, a>b>c).

Comparisons of survival and growth between the two species

MANOVA results for interspecies comparisons showed that survival and growth traits were drastically affected by individual pesticides, concentrations, and experimental species (Table I). Survival rates were affected by pesticide types and species (Table I). Body mass was affected by pesticide types, species, pesticide concentrations, interactions between pesticides and concentrations, and interactions between species and pesticide concentration (Table I). Total body length was affected by pesticide types, species, and pesticide concentrations (Table I). MANOVA results showed that pesticides and species drastically affected the growth of tadpoles under treatment with the six pairwise combinations of pesticides (Table II). The survival rates, body masses, and body lengths of *F. limnocharis* were all significantly higher than those of *M. fissipes* (Table II).

Table I. Effects of individual pesticides, pesticide concentration, and their interactions on growth traits (survival, body mass, and total body length) of experimental tadpoles.

	df	F	P
MANOVA (Wilks λ)			
Pesticide	9.136	3.306	0.001*
Species	3.56	105.556	<0.001*
Concentration	3.56	4.475	0.007*
ANOVA			
Survival			
Pesticide	3. 48	3.109	0.035*; A^{ab}, B^{a}, C^{b}, D^{ab}
Species	1. 48	39.500	<0.001*; FL^{a}, MF^{b}
Concentration	1. 48	0.042	0.839
Pesticide × Species	3. 48	0.481	0.697
Pesticide×Concentration	3. 48	1.136	0.344
Species × Concentration	1. 48	0.370	0.546
Pesticide × Species × Concentration	3. 48	0.589	0.625
Body mass			
Pesticide	3. 48	7.581	0.001*; A^{a}, B^{b}, C^{b}, D^{b}
Species	1. 48	53.717	<0.001*; FL^{a}, MF^{b}
Concentration	1. 48	4.589	0.037*; I^{a},II^{b}
Pesticide × Species	3. 48	2.369	0.082
Pesticide×Concentration	3. 48	3.713	0.018*
Species × Concentration	1. 48	8.715	0.005*
Pesticide × Species × Concentration	3. 48	0.656	0.583
Total Body Length			
Pesticide	3. 48	5.115	0.004*; A^{a}, B^{ab}, C^{b}, D^{b}
Species	1. 48	231.114	<0.001*; FL^{a}, MF^{b}
Concentration	1. 48	13.869	0.001*; I^{a}, II^{b}
Pesticide × Species	3. 48	1.411	0.251
Pesticide×Concentration	3. 48	3.020	0.487
Species×Concentration	1. 48	3.261	0.077
Pesticide × Species × Concentration	3. 48	2.672	0.06

Note: *indicates significant differences at P<0.05. A, B, C, and D indicate chlorantraniliprole, penoxsulam, pymetrozine and haloxyfop-P, respectively. FL, *F. limnocharis*, MF, *M. fissipes*. I and IInrepresent 1 and 2 mg/L of pesticide concentration, respectively. Types with different superscripts show statistically significant differences (Tukey's test, α=0.05, a>b).

Table II. Effects of pesticide pairwise combinations on the growth traits of experimental tadpoles.

	df	F	P
MANOVA (Wilks λ)			
Pesticide	15.108	2. 818	0.001*
Species	3.39	81.641	<0.001*
ANOVA			
Survival			
Pesticide	5. 36	1.873	0.124
Species	1. 36	11.763	0.002*; FL^{a}, MF^{b}
Pesticide × Species	5. 36	1.248	0.307
Body Mass			
Pesticide	5. 36	6.161	0.001*; AB^{b}, AC^{b}, AD^{a}, BC^{b}, BD^{ab}, CD^{b}
Species	1. 36	25.942	<0.001*; FL^{a}, MF^{b}
Pesticide × Species	5. 36	1.647	0.173
Total Body Length			
Pesticide	5. 36	3.925	0.006*; AB^{ab}, AC^{ab}, AD^{ab}, BC^{b}, BD^{ab}, CD^{a}
Species	1. 36	210.730	<0.001*; FL^{a}, MF^{b}
Pesticide × Species	5. 36	1.395	0.249

Note: *indicates significant differences at P<0.05. A, B, C, and D indicate chlorantraniliprole, penoxsulam, pymetrozine and haloxyfop-P, respectively. FL, *F. limnocharis*, MF, *M. fissipes*. Types with different superscripts show statistically significant differences (Tukey's test, α=0.05, a>b).

DISCUSSION

The results of this study indicate that individual pesticides and their pairwise combinations drastically affected the survival and growth traits of the tadpoles of *F. limnocharis* and *M. fissipes*. Tadpole survival was severely affected under treatment with all four individual pesticides at concentrations of 1 and 2 mg/L. Treatment with 2 mg/L penoxsulam and chlorantraniliprole did not cause mortality among *F. limnocharis* tadpoles. By contrast, all four pesticides caused mortality among *M. fissipes* tadpoles. The different effects of the pesticides on the mortality rates of the two species may be correlated to the physiological structures of the two frog species: *M. fissipes* tadpoles have completely transparent bodies, whereas *F. limnocharis* tadpoles have opaque bodies (Distel and Boone, 2015). Thus, environmental contaminants may more easily permeate the bodies of *M. fissipes* tadpoles than those of *F. limnocharis* tadpoles. *F. limnocharis* tadpoles showed the most severe toxicity symptoms under treatment with 1 mg/L chlorantraniliprole, 1 mg/L pymetrozine, 2 mg/L pymetrozine, and 2 mg/L haloxyfop-P-methyl. By contrast, *M. fissipes* tadpoles presented the most severe toxicity

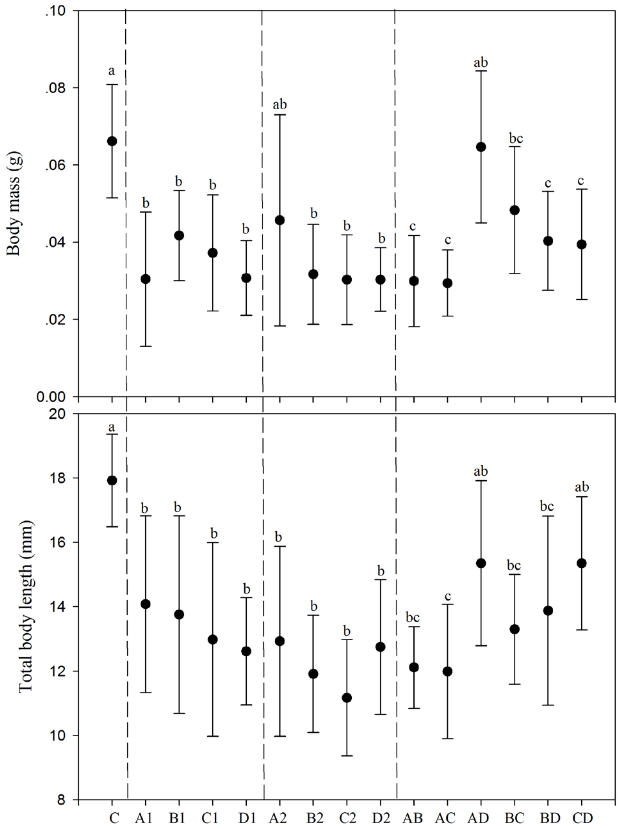

Fig. 3. The growth of M. fissipes tadpoles exposed to different concentrations of separate and combined pesticides compared with control group. Treatments are abbreviated as Figure 1. Types with different superscripts differ significantly (Tukey's test, α = 0.05, a>b>c).

symptoms under treatment with 1 mg/L pymetrozine and 2 mg/L haloxyfop-P-methyl. These results support our first prediction that different agrochemicals exert different toxicity effects on the two experimental frog species. The survival rates of *F. limnocharis* tadpoles under treatment with individual pesticides at low (1 mg/L) and high (2 mg/L) concentrations did not show significant differences, whereas those of *M. fissipes* showed significant differences under treatment with three of the four pesticides, except for pymetrozine. These results do not completely support our second hypothesis and, in agreement with the results of previous toxicity studies on other species (Relyea, 2004; Johansson *et al.*, 2006; Choung *et al.*, 2011; Wei *et al.*, 2014; Aronzon *et al.*, 2016), illustrate that the mortality patterns of the two frog species are pesticide- and species-specific. We also found that mortality rates under treatment with pairwise combinations of pesticides were rarely higher than those under treatment with individual pesticides at concentrations of 1 and 2 mg/L (Fig. 1). This result implies that tadpoles are exposed to multiple pesticides in the field (LeNoir *et al.*, 1999; Relyea, 2004; Melvin *et al.*, 2014). Here, we also found some mixed results in support of the third prediction. For example, the combinations of chlorantraniliprole/pymetrozine, penoxsulam/haloxyfop-P-methyl, and pymetrozine/haloxyfop-P-methyl did not cause mortality among *F. limnocharis* tadpoles. By contrast, all six pairwise combinations of pesticides caused mortality among *M. fissipes* tadpoles. These results could be attributed to the additive, synergistic, and antagonistic interactions among various agrochemicals (Loureiro *et al.*, 2009; Zhang *et al.*, 2011; Svartz *et al.*, 2016). Our results reveal that the interactive effect of chlorantraniliprole and penoxsulam resulted in higher mortality rates among the two frog species than chlorantraniliprole alone and lower mortality rates than penoxsulam. Our present results are in agreement with our previous study on Chinese tiger frog (*Hoplobatrachus chinensis*) tadpoles (Wei *et al.*, 2014). Even if chlorantraniliprole, penoxsulam, and flubendiamide–abamectin individually show high toxicity toward experimental tadpoles, their pairwise combinations showed antagonistic and synergistic effects at different exposure times (McConnell *et al.*, 1998; Relyea, 2004).

Growth and development are important factors of amphibian fitness that drastically affect amphibian life history (Boone and Semlitsch, 2002; Briggs, 2013; Melvin *et al.*, 2014). In this study, we found that the effects of pesticides on tadpole growth were more subtle than those on tadpole survival. Growth indexes under pesticide treatments were always lower than those under control treatments. This result further suggests that agrochemical contaminants are one of the main causes of the worldwide decline in amphibian populations (Davidson, 2004; Alford, 2010; Mesléard *et al.*, 2016). The growth indexes of the two species under treatments with individual pesticides at concentrations of 1 or 2 mg/L and with combinations of pesticides at concentrations of 1 mg/L did not show statistically significant differences (Figs. 2 and 3). Growth indexes under treatment with 1 mg/L pesticide were almost higher than or equal to those under treatment with 2 mg/L pesticide. These results indicate that tadpole growth and survival may be dependent on pesticide dose and concentration (Ezemonye and Tongo, 2009). In addition, similar to survival rate, growth rates under treatment with pairwise combinations of pesticides were higher than those under treatment with individual pesticides at concentrations of 1 and 2 mg/L. Our results are in agreement with those of previous studies on the effects of pesticides on amphibians, such as *Rana sphenocephala* (Bridges, 1999), *R. pipiens*, *Hyla versicolor* (Relyea, 2004), and *Pelophylax perezi* (Mesléard *et al.*, 2016). Therefore, we predict that although amphibian tadpoles often survive exposure to various pesticides in nature, they exhibit altered growth and behavior to adapt to environmental changes (Alford, 2010). Tadpoles may regulate their growth in the presence of pesticides via density (survival) and joint toxicity effects (Johansson *et al*., 2006). In this study, we found that although the growth rate of *F. limnocharis* was higher than that of *M. fissipes*, the growth rates of the two species under treatment with pairwise combinations of pesticides were not lower than those under treatment with individual pesticides. These observations indicate that tadpoles exhibit plastic growth in response to environmental constraints on larval growth and development and adaptive plastic responses to environmental variations (Tarvin *et al*., 2015). Thus, high survival rates, fast growth, large size at metamorphosis, and maturity may favor the long-term population dynamics of amphibians (Cabrera-Guzmán *et al*., 2013).

In China, approximately 100 registered pesticides are used in agricultural production each year. Understanding how each of these pesticides and their combinations affect a wide variety of organisms is highly challenging. The results of this study suggest that various pesticide treatments can reduce the survival and growth of the two species of frogs. However, individual pesticides and their pairwise combinations exerted species-specific toxicity effects (Distel and Boone, 2015; Afza *et al*., 2019). Additional pesticide types and their combination patterns, such as triple or quadruple combinations of pesticides, should be tested with other amphibian species.

CONCLUSION

In conclusion, the individual pesticides and their pairwise combinations could result in different toxic effects including survival, growth and development on anuran species. Thus, the impact of agricultural pesticides on aquatic animal and human health should be concerned. We should take some steps to manage the application of pesticide reasonably.

ACKOWLEDGEMENTS

This study was supported by the Zhejiang Provincial Natural Science Foundation of China (LY19C040001), the Key Research and Development Project of Zhejiang Province(2018C02031) and the Key Research Projects of Lishui City (20151206, SH2017001). We thank Fang LIU for her assistance with laboratory work.

Statement of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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