정주령석사학위논문

Effect of Sodium Fluoroacetate (Compound 1080) and Bd on Survival, Metamorphosis and Behavior of Asiatic Toad (*Bufo gargarizans*) Tadpoles

포유류 살충제 1080와 Bd가 두꺼비 올챙이의 생존, 변태 그리고 행동에 미치는 영향

2015년 8월

서울대학교 대학원

생명과학부

정주령

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A Thesis presented by **Ju-Ryung Chung**

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이 논문을 이학석사 학위논문으로 제출함

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정주령의 석사학위논문을 인준함

2015 년 8 월

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Abstract

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Sodium fluoroacetate (compound 1080) is a mammalian pesticide widely used in Australia and New Zealand to control wild animals such as possums, wild dogs, rats and rabbits. Its mechanism on target species is to interrupt TCA cycle and kill them as they suffer from symptoms including cardiac attack and energy deprivation. Although the pesticide targets mammalian pests, its aerial bait campaigns have been a large issue as the baits may contaminate soil and water, and also may be consumed by non-target species. There have been many studies to identify whether or not there are non-target species, such as birds, invertebrates, and amphibians, under the risk of 1080. For amphibians, there are studies on the adults of various species but none on their eggs,

embryo, or larvae. Here I focused on the effects of 1080 on the Asiatic toad (*Bufo gargarizans*) considering the larvae stage as a critical period for further development. Acute or chronic pesticide exposure on the larvae could interfere in metamorphosis and other behavioral or developmental points. Amphibians at larval stages are easily exposed to various stimuli under the water. Not only environmental but also chemical stimuli may work independently or concurrently on those species in larval stages. In 2013, the effect of 1080 alone was observed in toad tadpoles as the data on their survival, development, metamorphosis and behavior were recorded. In 2014, I added *Batrachochytrium dendrobatidis* (Bd) to all individuals that were set up in the same condition as the previous year with 1080. Tadpoles in both 1080-alone and 1080+Bd treatments had higher mortality, slower development, and lower success to metamorphosis at higher 1080 concentration. None of the tapoles in the highest concentration (100ppm) survived to metamorphosis. Tadpoles in higher concentration displayed reduced swimming period and higher number of swirling. Those in lower concentration swam for a longer period, displayed few or no swirling, and displayed higher number of imbalances, which is considered as a long-term effect of 1080 as nerve system interruption. None of the tadpoles from 1080+Bd treatment were infected by Bd which indicate that Asiatic toad tadpole has resistance to the pathogen and that 1080 and Bd did not reduce or enhance properties of each other. These results suggest that 1080 can be toxic to amphibian larvae, as one of non-target species. Relating to this chronic exposure study, I predict that 1080 can impose an ample effect on amphibian larvae also with acute exposure leading

to developmental and behavior impairments through further development. An acute exposure experiment on various species would provide as a good resource for identifying more accurate amphibian susceptibility to 1080.

Keywords: ecotoxicology, amphibian population decline, synergistic effects.

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1. INTRODUCTION

Sodium fluoroacetate (1080) was first introduced as a mammalian rodenticide in the US in 1946 (Proudfoot et al., 2006). Its target species is mammals, as it is licensed in the US, in South Dakota, New Mexico, Montana, and Wyoming, for use as neck collars on sheep and goats against coyotes and in Australia and New Zealand to kill unwanted introduced species (Proudfoot et al., 2006). 1080 interrupts the tricarboxylic acid (TCA) cycle and causes accumulation of citrate in the blood which then leads to energy deprivation and, finally, death. In some localities, its widespread usage and possible effects on non-target species cause concerns among some members of the public. These concerns have not been abated by studies of residual effects of 1080 in stream water and soil (Eason, 2002; Twigg et al., 2003; Weaver, 2006; Giannitti et al., 2013). Among non-target species, many amphibians inhabit riparian zones that may be exposed to a wide variety of chemicals from water and soil treatment.

McIlroy (1986) studied the effects of 1080 on non-target animals in Australia. Among 171 species studied for time to appearance of poisoning, time to death, and time to recovery, amphibians and reptiles generally took the longest time to display poisoning, to die or to recover. They also had the highest LD_{50}s. This founding conforms to the previous study of McIlroy et al. (1985). Frogs were exposed to 1080 via intraperitoneal injection. They generally displayed prolonged period until they showed signs of poisoning or until they died. Signs of poisoning included lethargy and convulsion.

Amphibians and reptiles tended to be more tolerant to 1080 than most other species, but susceptibility differ among species and the actual risk they face depend on the degree of exposure to the baits or other sources of 1080. Similarly, Chenoweth (1949) stated that frogs are less sensitive to 1080 than birds and mammals as they had higher LD_{50} compared to any other species including mice, rabbits, pigeons, and dogs. This may be due to Amphibian's better capability to defluorinate fluoroacetate, which was tested *in vivo* and *in vitro* with liver acetone-powder that contribute to defluorination (Twigg & Mead, 1990). Fluoroacetate would be converted to fluorocitrate, leading to citrate accumulation and energy deprivation. Because amphibians have such mechanism, they turn out to be more tolerant than other species such as birds and mammals.

There have been studies done in order to investigate the effects of different pesticides on amphibians. These studies give awareness to the experts as well as general public on the human usage of pesticides to the field and wild nature. Pesticides generally affect animals more at higher concentrations, followed by results such as impaired or delayed growth and metamorphosis, impaired behavior and morph. Christin et al. (2004) found altered immune responses in frogs to a mixture of six pesticides including atrazine, metribuzine, endosulfan, lindane, aldricarb and dieldrin on *Xenopus laevis* and *Rana pipiens*. They suggested that these pesticides could increase amphibian vulnerability to other infections and thus lead to amphibian population decline. Another study by Gürkan & Hayretdag (2012) from Turkey examined copper sulfate, which is often used as a fungicide and

impedes growth of *Bufo viridis* tadpoles. It caused developmental deformities and growth impairment such as poor larval development and growth. Tadpoles displayed higher mortality with increasing concentrations of copper sulfate and displayed deformities such as reduced reaction to stimuli, loss of equilibrium, and shortening of swimming distance in higher concentrations. These results help support the impact of pesticides on the decline of amphibian population in those areas using pesticides.

Amphibians are known to be more tolerant (less susceptible) to 1080 than other species such as mammals (Chenoweth, 1949; Eisler, 1995; McIlroy et al., 1985; Twigg et al., 2009; Twigg & Mead, 1990). Also, the interaction between amphibians and 1080 is less known than other species since many studies on 1080 and its effect of wildlife have been done on mammal (Eason et al., 1993; Proudfoot et al., 2006), birds (Eason, 2002; Pierce & Montgomery, 1992), and invertebrates (Eason et al., 1993; Spurr & Drew, 1999; Spurr & Berben, 2004; Suren & Lambert, 2006) with few data available on other groups. Most amphibians have two distinct life stages in which they develop from an egg to aquatic larva and then to a frog as they metamorphose. Tadpoles may be more sensitive to the environmental stimuli especially when they metamorphose. Through metamorphosis, amphibians undergo radical transformation of their body systems to facilitate their transition from aquatic to terrestrial lifestyles.

While pesticides may themselves be dangerous to amphibians, these dangers increase when effects are compounded by disease or other stressors (Gaietto et al., 2014; Hanlon et al., 2015; Kleinhenz et al., 2012; McMahon et

al., 2013; Rohr et al., 2013; Rumschlag et al., 2014; Wise et al., 2014). *Batrachochytrium dendrobatidis* (Bd) is a major concern for amphibian worldwide, as it is known to be causing amphibian population decline (Gaietto et al., 2014; Kinney et al., 2011; Perfect & Bell, 2005; Vredenburg et al., 2010; Yang et al., 2009). Despite Bd causing declines in Central America and Australia (Rebollar et al., 2014), there is no evidence that Bd is causing amphibian declines in Korea (Bataille et al., 2013). However, Korea had a first report of Bd on the Asian mainland (Yang et al., 2009) and Bd prevalence is relatively high in most Korean amphibians (Bataille et al., 2013). In Korea, Bataille et al. (2013) demonstrated that there are both native and introduced Bd strains. Interactions between wildlife and factors such as human activity, climate, and diseases have been well documented (refs), we are interested in examining the combined effects of the pesticide 1080 and amphibian chytrid fungus (on amphibians).

In the present study, I investigated whether Asiatic toad (*Bufo gargarizans*) tadpoles are affected by 1080 in terms of survival to metamorphosis, time to metamorphosis, and behavior over two years, 2013 and 2014. Tadpoles were exposed to 1080 alone in the first year. In the second year, virulent strain of Bd was added to 1080 solution in order to examine whether tadpoles are more likely to be infected by Bd in presence of 1080. With either 1080 alone or 1080 and Bd together, we investigate how the pesticide can affect toad at larval stages.

2. METHODS

2.1. Animal Collection and Care

Fieldwork for animal collection was performed from February 25th to March 15th of 2013 and from February 17th until March 13th of 2014. Twenty pairs of amplexed toads were collected from each of two sites, Jeonju (35.784551°, 127.141503°) and Geumsan (36.137594°, 127.381514°), for a total of forty pairs. Amplexed pairs were captured with bare hands or a net and brought back to the laboratory in 7-L plastic containers. Four egg clutches from these pairs were randomly selected and reared for this experiment. Eggs were raised in 25-L plastic tubs (36 W x 47 L x 18 D cm) with air stones for oxygenation in a cold chamber at 15°C with a 12:12 light-dark cycle. UV-treated water was replaced every day for egg clutches and every four days for tadpoles. Tadpoles were fed with boiled and pureed Ah-wook, *Malva verticillata*.

2.2. Experiment Set-Up (Pesticide & Bd Preparation)

Twenty five individuals were randomly selected from each of the four clutches, when they are able to swim freely (Gosner stage 25) (Gosner, 1960), for a total of 100 individuals. They were kept in a separate room at 25±1°C with 12:12 light-dark cycle. Tadpoles were raised in individual 125 ml culture flasks (SPL Life Sciences T75 Cell Culture Flask, PS, Plug cap, from Dongin Bio, Seoul, Korea) until metamorphosis. Culture flasks were labeled accordingly and 100 mL of prepared 1080 solutions was added to each container before the addition of the tadpoles. Tadpoles were acclimated for 24 h prior to the start of the experiment. They were checked every day for any dead or metamorphosed individuals. When there were any deaths, they were noted and stored in 1.5 ml microtubes with 100% ethanol at -20°C. After metamorphosis, snout-vent lengths and mass were measured and those individuals were euthanized in a MS-222 solution, as approved by IACUC of Seoul National University (cert. # SNU-141120-5).

Analytical grade 1080 (MP Biomedicals, LLC, Solon, Ohio) was used was diluted in serial dilutions with UV-treated water to concentrations of 100, 10, 1, 0.1ppm. Control group consisted of UV-treated water without 1080.

While the study in 2013 was done with 1080 only (1080-alone treatment), the same study was added with Bd in 2014 (1080+Bd treatment). The Australian Bd strain used in this experiment was AbercrombieR-Lbooroolongensis-09-LB1, which was obtained from Dr. Lee Berger (Centre for Public Health and Tropical Medicine, James Cook University, Townsville, Australia). Bd was cultured on TGhL media. Plates were prepared every one

to two weeks depending on the number of active zoospores remaining in the previous petri dish. To harvest Bd, petri dishes were flooded with sterile, filtered water and the number of zoospores in supernatant was counted with a disposable hemocytometer (inCYTO, C-CHIP DHC-N01-5) using inverted microscope (Axio Observer Z1, Zeiss). Each tadpole was inoculated with 10,000 zoospores, which is known to be a lethal dose in other species (Kinney et al., 2011; Vredenburg et al., 2010).

2.3. Confirmation of 1080 in Water

Concentrations of 1080 solutions were analyzed and confirmed with ion chromatography (Wang et al., 2004) by National Instrumentation Center for Environmental Management (NICEM) at Seoul National University.

2.4. Behavior Analysis

Forty days after the start of the experiment, 5 tadpoles were randomly selected from each treatment group for swimming behavior analysis. Selected individuals were transferred to petri dishes (14.5 cm diameter) filled with clean filtered water. Tadpoles were allowed to acclimate for 5 min and then behavior was recorded for 35 min using a digital camcorder (Sony DCR-SR82). Filming was conducted in a dark room with no lights except for an incandescent lamp which did not directly flash or reflect on the surface of the petri dish. The bulb was oriented towards the ceiling or on a wall to produce indirect light for filming. Care was taken to ensure even lighting with no shadows.

Recorded videos were manually observed with continuous sampling. Manual continuous sampling allowed observation of total time the tadpole spent swimming and abnormal behaviors such as swirling and losing balance (imbalance). Total time spent swimming was recorded manually in seconds and used in statistical analyses. Swirling can be described as a motion of fast twisting and rotating 360° sideways of the tadpoles. The number of swirls of each tadpole was also recorded for further analyses. We also detected that some tadpoles lost balance while or after swimming, as they lay on their ventral sides with “paralysis”, unable to move for few seconds. The number of times the tadpole lost balance throughout the 30-min period was also recorded for further analyses.

2.5. Bd Infection Determination

Bd infection was determined using a water filtration method (Shin et al., 2014) which is more accurate and less invasive than the commonly used swab method. The filter method was conducted after recording for swimming behavior. Tadpoles were transferred to 950 ml plastic containers with 150 ml filtered water. They were left in the water for 24 h to allow time for zoosporangia release into the water. After 24 h, approximately 60 ml of the water was filtered using a 25 mm syringe filter (Whatman Puradisc) with a 10 ml syringe. Filters were stored at -20°C until DNA extraction. Filters were opened with a pair of wire cutters to access the membranes. Membranes were transferred to 1.5 ml microtubes for DNA extraction and a highly sensitive nested PCR was done (Goka et al., 2009) to detect Bd. Final PCR products were loaded onto an agarose gel and examined under UV light for the presence of a 300 bp band.

2.6. Statistical Analysis

Survival of tadpoles in each treatment group was evaluated until metamorphosis (otherwise until all tadpoles died if not metamorphosed). Survival curves were produced using Kaplan-Meier product-limit method and were compared with log-rank test. In order to graph the survival plots, status of each tadpole needed to be determined. Those reached the endpoints, meaning death, were marked/coded 1 (“events”) and those survived were marked/coded 0 (“censored”). Survival curves were generated by the Kaplan-Meier estimator on SigmaPlot 8.0.

Two-way ANOVA was performed to test for the effects of different concentrations of 1080 and clutches on survival as well as development. Survival days were log transformed before running the test in order to normalize the data. Orthogonal contrast (of ANOVA) was run in order to compare the effects of 1080 among different concentrations, such as control vs. 1080 treatment groups, higher concentrations (10, 1ppm) vs. lower concentrations (0.1, 0.01ppm), and highest concentration (10ppm) vs. other concentrations (1, 0.1, 0.01ppm). Two-way ANOVA with orthogonal contrasts were generated using SAS Version 9.2. Boxplots were generated with SigmaPlot 8.0.

Development was measured as Gosner stages and two-way ANOVA was generated to test for the effects of different concentrations of 1080 and clutches. Orthogonal contrast was also performed.

Two-way ANOVA was also used for behavior analysis. Total swimming period (total time spent swimming), number of swirls and number

of imbalances were analyzed for different concentrations of 1080 and different clutches. Total swimming period data were log-transformed for normalization.

All tests were performed using a level of significance of $\alpha=0.05$.

3. Results

3.1. Tadpole survival

Seventeen and 36 out of 100 tadpoles survived to metamorphosis in 1080 alone and 1080+Bd treatments, respectively. Mortality was positively correlated ($P<0.0001$ in both treatments) with 1080 concentration while development was negatively correlated. Fastest metamorphosis rate was evident in the control groups, and no tadpoles metamorphosed in the highest concentration treatment in either treatment.

Kaplan-Meier survival plots (Figure 1a, b) show the survival rate at each day with survival curves and each dent on the curves represents a drop in survival rate as tadpoles reach their endpoints, which indicate death. In both treatments, tadpoles in highest concentration displayed a rapid decrease in survival compared to other groups. Those in control groups displayed slowest decrease and maintained highest survival rate at the end of the experiment. This survival analysis indicated that the higher concentration treatment groups die earlier, with faster rate.

In 1080-alone treatment, by the end of the experiment, survival rate dropped to 0, 0, 5, 30 and 50% for each treatment, 100, 10, 1, 0.1 ppm and control, respectively. Similarly, in 1080+Bd treatment, survival rate dropped to 0, 10, 65, 45 and 60% for each treatment. All individuals died in the highest concentration group at the end of the experiment.

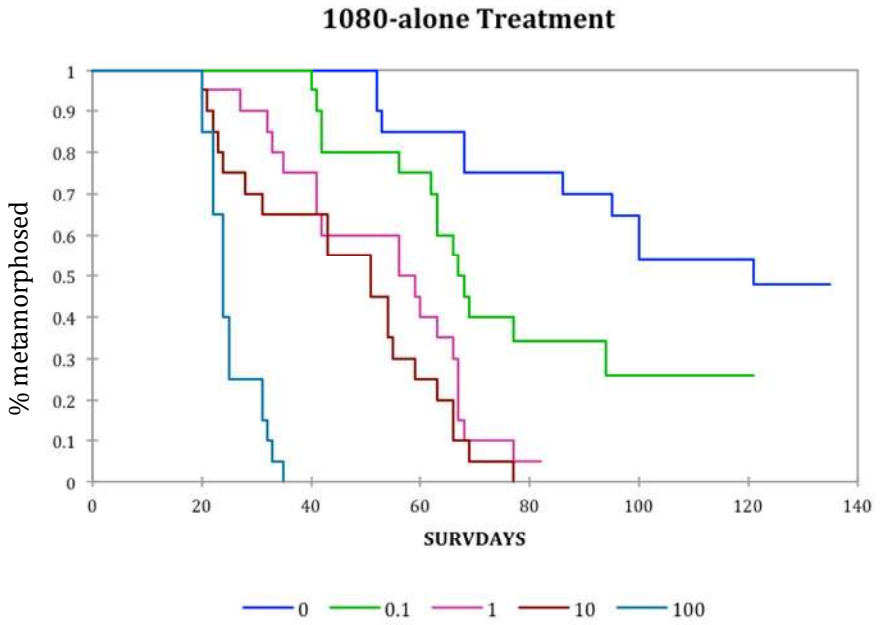

Figure 1a. Kaplan-Meier estimate curve for tadpoles in 5 experimental groups from 1080-alone treatment in 2013, representing % metamorphosed at the end of the experiment and the mortality rate with slope.

Table 1a. Mantel-Cox Log Rank test of tadpole survival from 1080-alone treatment.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	112.4554	4	P < 0.0001

Table 2a. Cases summary for Kaplan-Meier survival plot of 1080-alone treatment.

	Number of events [a]		Number censored [b]		
Factor	N	%	N	%	Total sample size
1	20	100.00	0	0.00	20
2	20	100.00	0	0.00	20
3	19	94.00	1	5.00	20
4	14	70.00	6	30.00	20
5	10	50.00	10	50.00	20
Overall	83	83.00	17	17.00	100

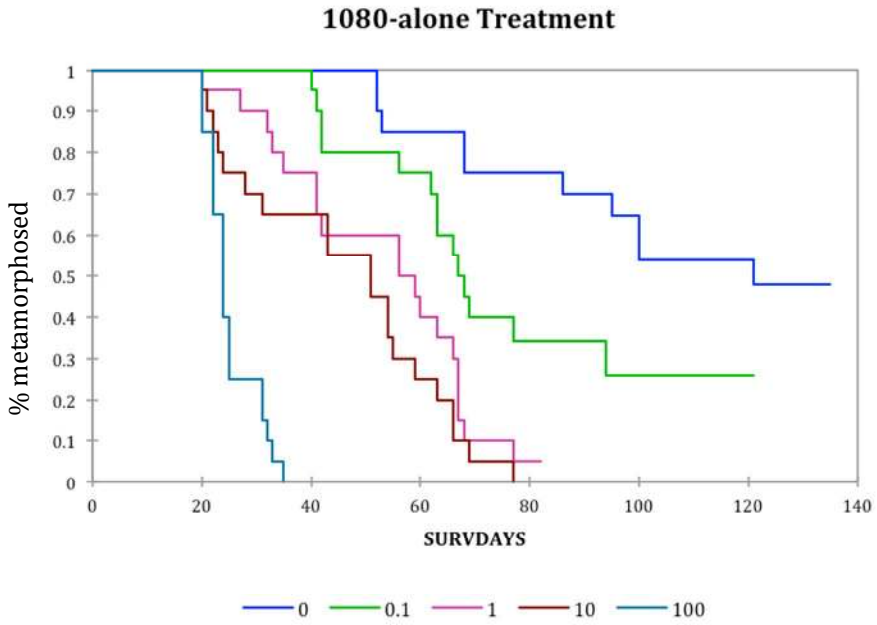

Figure 1b. Kaplan-Meier estimate curve for tadpoles in 5 experimental groups from 1080+Bd treatment in 2014, representing % metamorphosed at the end of the experiment and the mortality rate with slope.

Table 1b. Mantel-Cox Log Rank test of tadpole survival from 1080+Bd treatment.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	70.4570	4	P < 0.0001

Table 2b. Cases summary for Kaplan-Meier survival plot of 1080+Bd treatment.

	Number of events [a]		Number censored [b]		
Factor	N	%	N	%	Total sample size
1	20	100.00	0	0.00	20
2	18	90.00	2	10.00	20
3	7	35.00	13	65.00	20
4	11	55.00	9	45.00	20
5	8	40.00	12	60.00	20
Overall	64	64.00	36	36.00	100

3.2. Success to Metamorphosis

More tadpoles died in higher concentrations and thus, metamorphosed less. Two-way ANOVA on each tadpole's final Gosner stage at death or at the end of the experiment showed that tadpoles in lower concentration groups developed to higher Gosner stages in both treatments of 1080 alone and 1080+Bd (Table 3a, b). Clutches did not affect their development or metamorphosis ($F_{3,12} = 0.78$, $P = 0.5122$ in 1080 alone; $F_{3,10} = 0.18$, $P = 0.9091$ in 1080+Bd). More tadpoles reached metamorphosis in lower concentration groups, and the highest metamorphosis rate was found in control groups. (Figure 2a, b)

Table 3a. Two-way ANOVA of Gosner stages of tadpoles from 1080-alone treatment, log transformed.

Dependent variable: Stages (log transformed)

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	19	0.022	0.001	2.02	0.0199
Error	63	0.036	0.001		
Corrected Total	82	0.057			

R-square= 0.378203, Coeff Var= 1.549552, Root MSE= 0.023818, Stage Mean= 1.537098

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Conc	4	0.017	0.0041	7.30	<.0001
Clutch	3	0.0013	0.0004	0.78	0.5122
Conc*Clutch	12	0.0036	0.0003	0.54	0.8836

Table 3b. Two-way ANOVA of Gosner stages of tadpoles from 1080+Bd treatment, log transformed.

Dependent variable: Stages (log transformed)

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	17	0.170	0.010	8.04	<.0001
Error	46	0.057	0.001		
Corrected Total	63	0.228			

R-square= 0.748193, Coeff Var= 2.352498, Root MSE= 0.035290, Stage Mean= 1.500121

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Conc	4	0.1253	0.0313	25.16	<.0001
Clutch	3	0.0007	0.0002	0.18	0.9091
Conc*Clutch	10	0.0510	0.0051	4.10	0.0005

Table 4a. Quantitative description of development in Gosner stages in 1080-alone treatment.

Variable	CONC (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
STAGE	100	33.0500	0.3939	1.7614	28.0000	33.0000	33.5000	34.0000	35.0000
	10	34.1000	0.3154	1.4105	30.0000	33.2500	34.0000	35.0000	36.0000
	1	35.2000	0.6867	3.0711	30.0000	34.0000	34.5000	36.0000	46.0000
	0.1	38.0500	0.9907	4.4305	33.0000	35.0000	36.0000	43.0000	46.0000
	Control	41.150	1.106	4.945	34.000	36.000	42.500	46.000	46.000

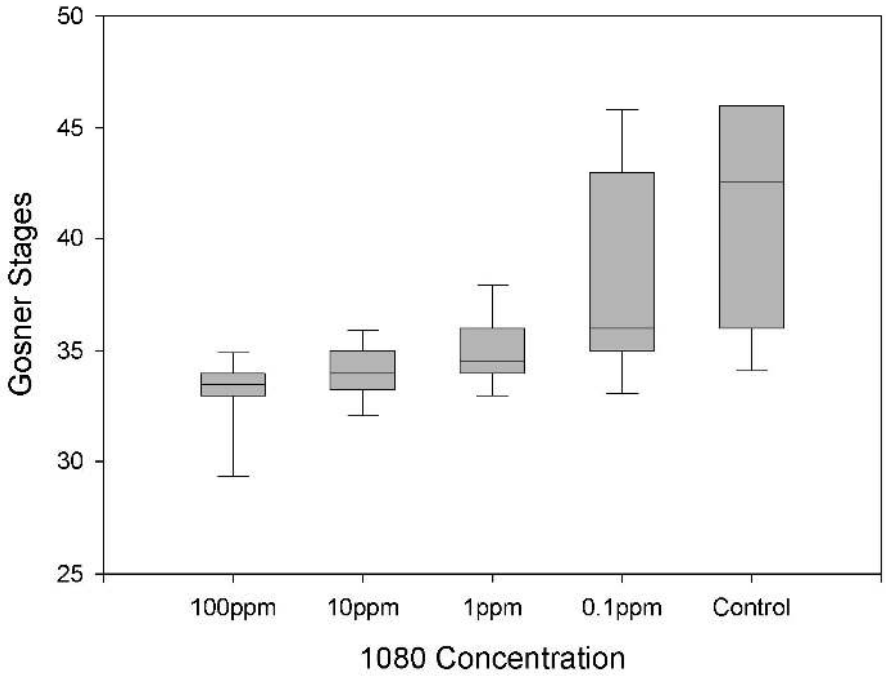

Figure 2a.The effect of different 1080 concentrations on tadpole Gosner stages from 1080-alone treatment. Medians, quartiles and ranges are displayed.

Table 4b. Quantitative description of development in Gosner stages in 1080+Bd treatment.

Variable	CONC (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
STAGE	100	29.0000	0.6113	2.7338	27.0000	27.0000	27.5000	31.7500	35.0000
	10	32.1000	1.2010	5.3700	27.0000	28.0000	31.5000	33.7500	46.0000
	1	39.6500	1.6010	7.1620	27.0000	33.2500	41.5000	46.0000	46.0000
	0.1	39.1000	1.2710	5.6840	30.0000	33.2500	39.5000	45.5000	46.0000
	Control	42.0000	0.8367	3.7417	35.0000	40.0000	41.5000	46.0000	46.0000

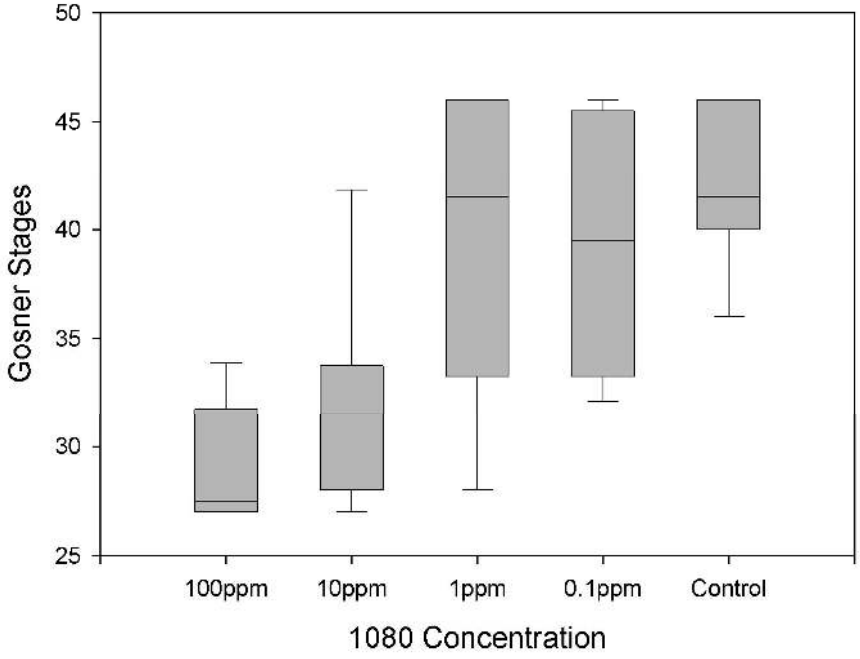

Figure 2b. The effect of different 1080 concentrations on tadpole Gosner stages from 1080+Bd treatment. Medians, quartiles and ranges are displayed.

3.3. Survival Measurement

The results from two-way ANOVA showed that survival of tadpoles in both treatments was significantly affected by 1080 exposure and by the different concentrations of 1080 ($P < 0.0001$) while clutches did not affect their survival ($P = 0.4006$ in 1080 alone, $P = 0.2041$ in 1080+Bd) (Table 6a, b). Tadpoles in higher concentrations lived for a shorter period of time compared to those in lower concentrations (highest concentration survival days mean = 25.25 in 1080-alone, 14.75 in 1080+Bd) (Table 5a, b). Orthogonal contrasts were used to compare different concentration groups and find relationships between survival days and 1080 concentrations. Contrast results showed that survival was strongly associated with 1080 concentrations as survival rate increased with lower concentrations (Table 6a, b) (Figure 3a, b). In 1080-alone treatment, survival of control groups differed significantly from 1080-exposed groups ($P < 0.0001$). Survival of control groups was significantly different from both higher concentration groups (100ppm & 10ppm) and lower concentration groups (1ppm & 0.1ppm). Survival of higher concentration groups differed significantly from lower concentration groups ($P < 0.0001$). In 1080+Bd treatment, survival of control groups differed significantly from 1080-exposed groups as well as higher concentration groups ($P < 0.0001$) but not from lower concentration groups ($P = 0.0221$). Survival of higher concentration groups and lower concentration groups showed difference as well ($P < 0.0001$).

Table 5a. Quantitative description of survival days of tadpoles from 1080-alone treatment.

Variable	CONC (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Survival	100	25.250	1.026	4.587	20.000	22.000	24.000	29.500	35.000
(Days)	10	46.000	4.124	18.445	20.000	25.000	51.000	62.000	77.000
	1	52.950	3.954	17.683	20.000	36.500	57.500	67.000	82.000
	0.1	72.000	5.476	24.488	40.000	57.500	67.500	87.250	121.000
	Control	101.400	6.683	29.888	52.000	72.500	100.500	131.500	135.000

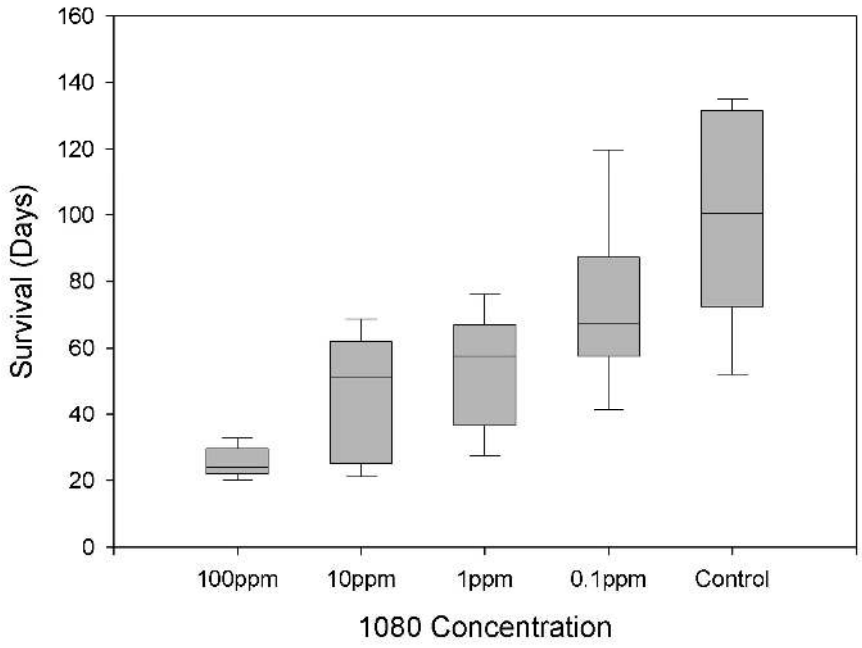

Figure 3a. The effect of different 1080 concentrations on tadpole survival (days) from 1080-alone treatment. Medians, quartiles and ranges are displayed.

Table 5b. Quantitative description of survival days of tadpoles from 1080+Bd treatment.

Variable	CONC (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Survival	100	14.750	1.930	8.632	4.000	9.000	12.000	20.750	33.000
(Days)	10	24.800	4.136	18.498	5.000	13.250	19.000	33.000	66.000
	1	40.650	4.430	19.813	11.000	24.750	42.000	52.000	85.000
	0.1	45.700	4.608	20.609	17.000	29.500	45.000	56.250	87.000
	Control	52.350	2.831	12.659	32.000	40.750	52.000	59.750	81.000

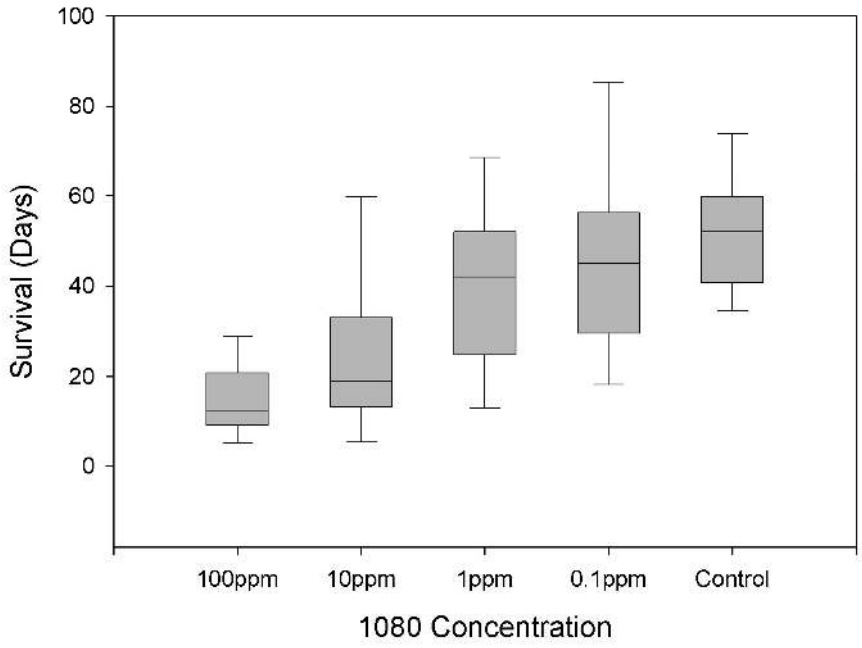

Figure 3b. The effect of different 1080 concentrations on tadpole survival (days) from 1080+Bd treatment. Medians, quartiles and ranges are displayed.

Table 6a. Two-way ANOVA and orthogonal contrasts with survival days of tadpoles from 1080-alone treatment, log transformed.

Dependent variable: Survival days (log transformed) (1080-alone)

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	19	4.247	0.224	9.42	<.0001
Error	80	1.897	0.024		
Corrected Total	99	6.144			

R-square=0.691173, Coeff Var=9.023031, Root MSE=0.154007, Surv. Mean=1.706824

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Conc	4	3.942	0.986	41.55	<.0001
Clutch	3	0.071	0.024	0.99	0.4006
Conc*Clutch	12	0.234	0.019	0.82	0.6275

Dependent variable: Survival days (log transformed)

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Compare Control vs 1080	1	1.930	1.930	81.38	<.0001
Compare Higher vs Lower Conc	1	1.305	1.305	55.01	<.0001
Compare Control vs Lower Conc	1	0.643	0.643	27.12	<.0001
Compare Control vs Higher Conc	1	3.009	3.009	126.86	<.0001

Table 6b. Two-way ANOVA and orthogonal contrasts with survival days of tadpoles from 1080+Bd treatment, log transformed.

Dependent variable: Survival days (log transformed) (1080+Bd treatment)

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	19	6.692	0.352	6.23	<.0001
Error	80	4.523	0.057		
Corrected Total	99	11.215			

R-square= 0.596702, Coeff Var= 16.52245, Root MSE= 0.237776, Surv. Mean= 1.439110

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Conc	4	4.949	1.237	21.88	<.0001
Clutch	3	0.266	0.089	1.57	0.2041
Conc*Clutch	12	1.477	0.123	2.18	0.0205

Dependent variable: Survival days (log transformed)

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Compare Control vs 1080	1	1.791	1.791	31.67	<.0001
Compare Higher vs Lower Conc	1	2.665	2.665	47.13	<.0001
Compare Control vs Lower Conc	1	0.308	0.308	5.45	0.0221
Compare Control vs Higher Conc	1	3.565	3.565	63.05	<.0001

3.4. Swimming Behavior

1080 exposure had a significant effect on tadpole swimming behavior. Total swimming time ($F_{4,12} = 15.13$, $p = 0.0053$ in 1080-alone; $F_{4,12} = 4.31$, $p = 0.026$ in 1080+Bd) and total number of swirls ($F_{4,95} = 8.95$, $p = 0.0168$ in 1080-alone; $F_{4,12} = 7.35$, $p = 0.025$ in 1080+Bd) were negatively related to 1080 concentration (Table 7a, b & 9a, b). Both variables were not affected among different clutches ($F_{4,12} = 1.66$, $p = 0.3003$ for swimming; $F_{4,12} = 4.65$, $p = 0.0506$ for swirls in 1080-alone; $F_{4,12} = 1.39$, $p = 0.349$ for swimming; $F_{4,12} = 1.54$, $p = 0.313$ for swirls in 1080+Bd). Number of times the tadpoles lost balance was not significantly affected by either 1080 or clutches (Table 7c, 9c).

In both treatments, total swimming time increased when exposed to less or 1080 while the number of swirls decreased with less 1080 exposures. No tadpoles displayed swirling motion or imbalance in control groups. Although balance was not statistically significant from two-way ANOVA results, a trend existed in which the average number of the behavior displayed was higher in lower concentration groups (1 & 0.1ppm). From video analysis, tadpoles in higher concentration groups tended to display unstable swimming (random speed, swirls, strong and random tail motion), as they tilted their heads more when swimming and waivered side-to-side stronger than those in lower concentrations.

Table 7a. Two-way ANOVA with total time spent swimming of tadpoles from 1080-alone treatment, log transformed.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Conc	4	2.005	0.501	15.13	0.005
Clutch	3	0.084	0.028	0.85	0.525
Conc*Clutch	12	0.660	0.055	1.66	0.300
Error	5	0.166	0.033		
Total	24	2.793			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.181972673	94.07%	71.55%	*

Table 7b. Two-way ANOVA with total time of swirl of tadpoles from 1080-alone treatment.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Conc	4	182.486	45.621	8.95	0.017
Clutch	3	141.490	47.163	9.25	0.018
Conc*Clutch	12	284.597	23.716	4.65	0.051
Error	5	25.500	5.100		
Total	24	607.760			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
2.25831796	95.80%	79.86%	*

Table 7c. Two-way ANOVA with total time of imbalance in tadpoles from 1080-alone treatment.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Conc	4	313.657	78.414	1.16	0.428
Clutch	3	137.173	45.724	0.68	0.603
Conc*Clutch	12	441.508	36.792	0.54	0.821
Error	5	338.500	67.700		
Total	24	1285.040			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
8.22800097	73.66%	0.00%	*

Table 8a. Quantitative description of total time spent swimming of tadpoles from 1080-alone treatment.

Variable	Conc (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Swim	100	217.4	57.3	128.1	89.0	109.0	162.0	353.5	378.0
	10	563.2	122.6	274.1	222.0	357.5	533.0	784.0	985.0
	1	564.2	179.2	400.6	249.0	268.0	428.0	928.5	1230.0
	0.1	850.8	140.3	313.7	439.0	548.0	884.0	1137.0	1241.0
	Control	1221.2	39.1	87.4	1090.0	1135.5	1243.0	1296.0	1302.0

Table 8b. Quantitative description of total number of swirl of tadpoles from 1080-alone treatment.

Variable	Conc (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Swirl	100	6.6	2.0	4.4	1.0	3.0	6.0	10.5	13.0
	10	6.0	4.0	8.9	0.0	0.0	2.0	14.0	21.0
	1	3.4	1.5	3.4	0.0	0.5	2.0	7.0	8.0
	0.1	2.2	1.0	2.3	0.0	0.5	2.0	4.0	6.0
	Control	0	0	0	0	0	0	0	0

Table 8c. Quantitative description of total time of imbalance in tadpoles from 1080-alone treatment.

Variable	Conc (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Imbalance	100	5.6	2.9	6.6	0.0	0.5	2.0	12.5	15.0
	10	4.4	4.4	9.8	0.0	0.0	0.0	11.0	22.0
	1	11.8	3.5	7.9	3.0	4.5	12.0	19.0	23.0
	0.1	6.8	2.4	5.4	1.0	1.0	10.0	11.0	12.0
	Control	0	0	0	0	0	0	0	0

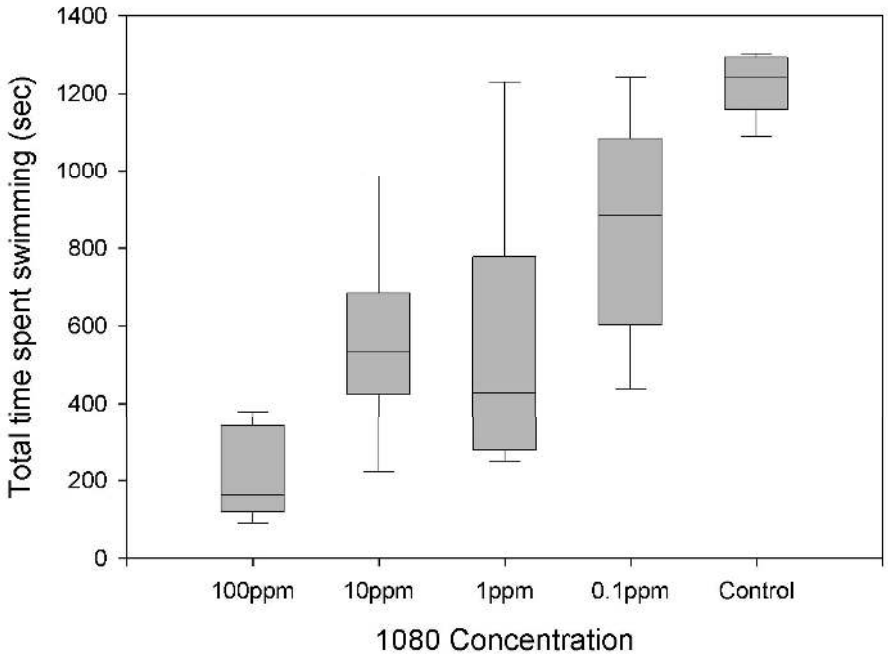

Figure 4a. The effect of different 1080 concentrations on tadpoles' total time spent on swimming (sec) during the 30-min recording from 1080-alone treatment. Medians, quartiles and ranges are displayed.

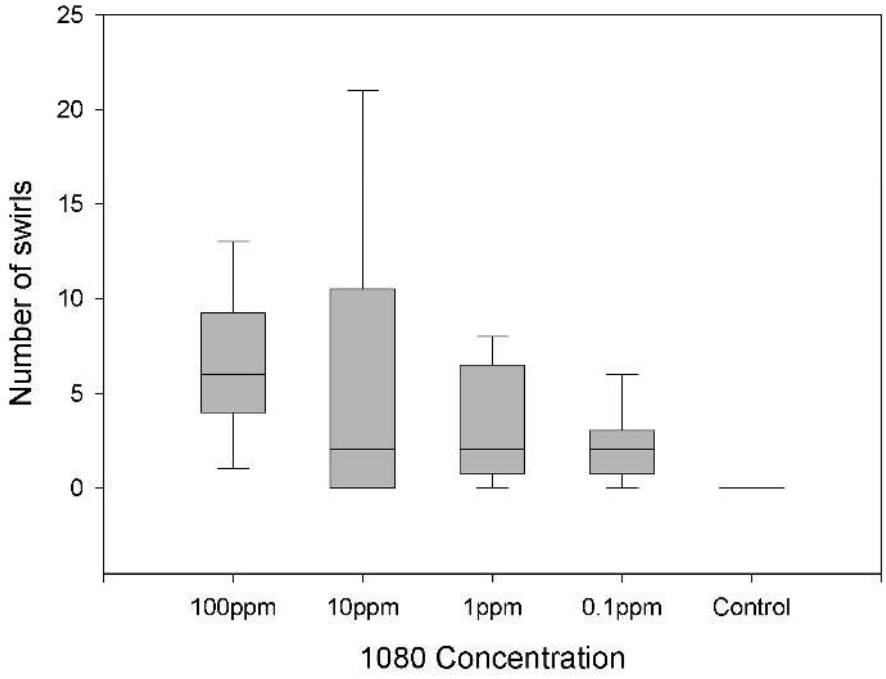

Figure 4b. The effect of different 1080 concentrations on the number of swirls by tadpoles during the 30-min recording from 1080-alone treatment. Medians, quartiles and ranges are displayed.

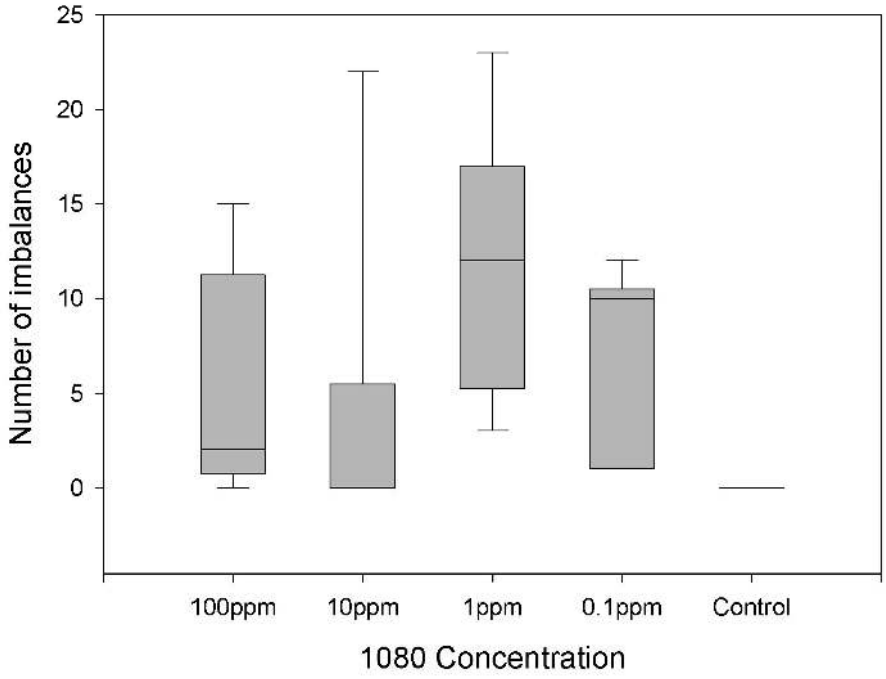

Figure 4c. The effect of different 1080 concentrations on the number of imbalances by tadpoles during the 30-min recording from 1080-alone treatment. Medians, quartiles and ranges are displayed.

Table 9a. Two-way ANOVA with total time spent swimming of tadpoles from 1080+Bd treatment, log transformed.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Conc	4	0.895	0.224	7.31	0.026
Clutch	3	0.127	0.042	1.39	0.349
Conc*Clutch	12	0.202	0.017	0.55	0.817
Error	5	0.153	0.031		
Total	24	1.424			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.174938	89.25%	48.40%	*

Table 9b. Two-way ANOVA with total time of swirl of tadpoles from 1080+Bd treatment.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Conc	4	1696.1	424.01	7.35	0.025
Clutch	3	150.7	50.23	0.87	0.515
Conc*Clutch	12	478.0	39.83	0.69	0.723
Error	5	288.5	57.70		
Total	24	2852.0			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
7.59605	89.88%	51.44%	*

Table 9c. Two-way ANOVA with total number of imbalance in tadpoles from 1080+Bd treatment.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Conc	4	489.5	122.38	1.80	0.266
Clutch	3	166.3	55.43	0.82	0.538
Conc*Clutch	12	665.0	55.42	0.82	0.644
Error	5	339.5	67.90		
Total	24	1778.0			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
8.24015	80.91%	8.35%	*

Table 10a. Quantitative description of total time spent swimming of tadpoles from 1080+Bd treatment.

Variable	Conc (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Swim	100	259.40	26.18	58.55	198.00	205.50	254.00	316.00	342.00
	10	515.4	108.9	243.6	278.0	327.5	399.0	761.5	878.0
	1	680.60	98.14	219.46	363.00	465.50	743.00	864.50	926.00
	0.1	797.80	65.86	147.26	649.00	662.50	765.00	949.50	986.00
	Control	948.2	145.4	325.2	399.0	657.0	1090.0	1168.5	1208.0

Table 10b. Quantitative description of total number of swirl of tadpoles from 1080+Bd treatment.

Variable	Conc (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Swirl	100	23.00	11.13	24.88	0.00	0.00	22.00	46.50	59.00
	10	15.000	5.030	11.247	8.000	9.000	11.000	23.000	35.000
	1	6.400	2.293	5.128	1.000	2.500	4.000	11.500	14.000
	0.1	2.200	1.356	3.033	0.000	0.000	0.000	5.500	6.000
	Control	0	0	0	0	0	0	0	0

Table 10c. Quantitative description of total number of imbalance in tadpoles from 1080+Bd treatment.

Variable	Conc (ppm)	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Imbalance	100	2.400	1.435	3.209	0.000	0.500	1.000	5.000	8.000
	10	8.800	2.764	6.181	0.000	3.500	9.000	14.000	17.000
	1	11.800	3.455	7.727	4.000	5.000	9.000	20.000	21.000
	0.1	12.000	6.075	13.583	0.000	0.000	7.000	26.500	28.000
	Control	0	0	0	0	0	0	0	0

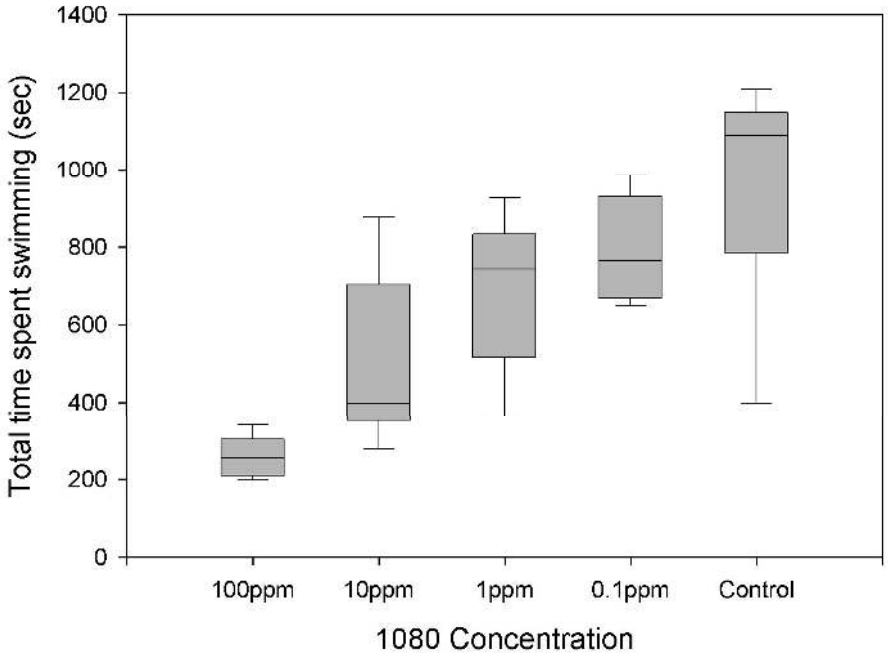

Figure 5a. The effect of different 1080 concentrations on tadpoles' total time spent on swimming (sec) during the 30-min recording from 1080-alone treatment. Medians, quartiles and ranges are displayed.

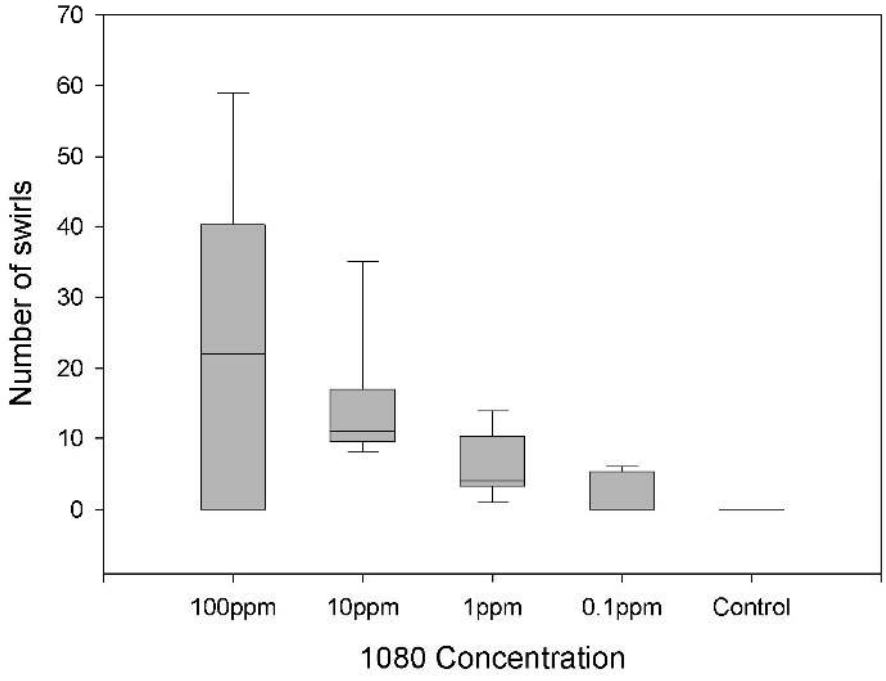

Figure 5b. The effect of different 1080 concentrations on the number of swirls by tadpoles during the 30-min recording from 1080+Bd treatment. Medians, quartiles and ranges are displayed.

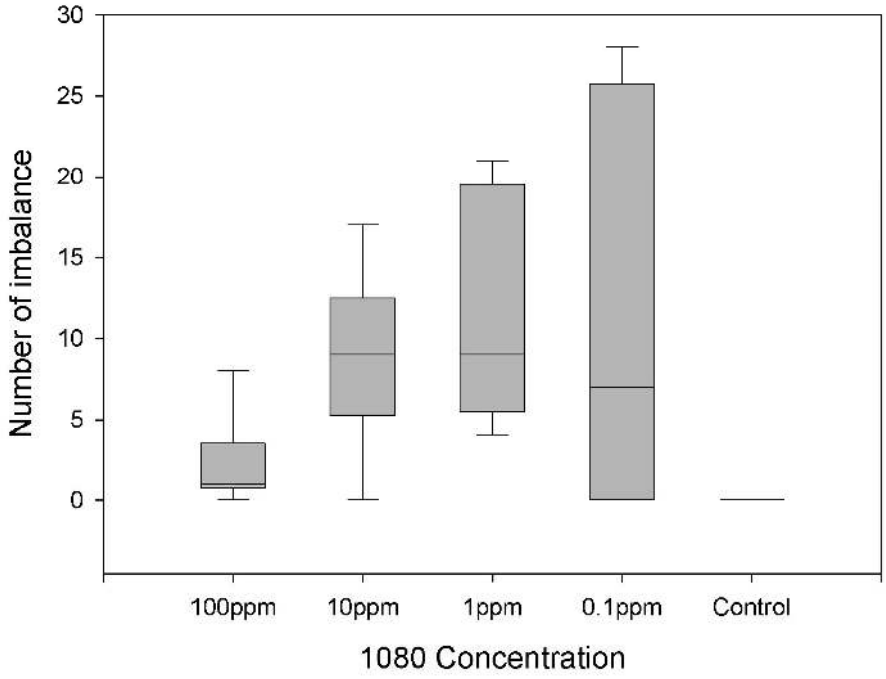

Figure 5c. The effect of different 1080 concentrations on the number of imbalances by tadpoles during the 30-min recording from 1080+Bd treatment. Medians, quartiles and ranges are displayed.

4. DISCUSSION

4.1. Summary and Interpretation of the Results

In this study, I focused on the effects of 1080 on tadpoles considering the larval stage as a critical period for further development. Acute or chronic pesticide exposure on the larvae could interfere in metamorphosis and other behavioral or developmental points. Amphibians at larval stages are easily exposed to various cues under the water. Not only environmental cues but also chemical cues may work independently or concurrently with other stressors on those species at larval stages. Different pesticides can affect them, whether acute or chronic, which impair their survival, development or behavior. (Bridges, 2000; Brühl et al., 2011; Brunelli et al., 2009; Christin et al., 2004; Denoël et al., 2010; Denoël et al., 2012; Denoël et al., 2013; Feng et al., 2004; Gurushankara et al., 2007; Kleinhenz et al., 2012; Lavorato et al., 2013; Marco & Blaustein, 1999; McMahon et al., 2013; Rohr et al., 2013). Rumschlag et al. (2014) exposed tadpoles to different temperatures, 2 different insecticides (carbaryl, malathion), and Bd, where they observed longer time to tail absorption when exposed to both Bd and insecticides and reduced survival at high and fluctuating temperatures. Their study suggests abiotic stressors contributing the interactions between host and pathogen but their findings showed that insecticides alone were not lethally affecting tapdoles. Unlike lab conditions that can be controlled, natural, wild conditions have multiple interactions and cofactors that can negatively or positively affect tadpoles. Rohr et al. (2013) confirmed enduring atrazine effects when

exposed at an early larval stage. This study serves a good example for interactions in the environment. Although atrazine itself did not have a significant effect on tadpole survival, their acute exposure allowed tadpoles to be more susceptible to pathogens, such as Bd, resulting in a mortality twice as higher than those exposed to Bd without atrazine. By taking different life stages of amphibian into consideration, Bridges (2000) examined different concentrations of carbaryl, acute and chronic, on different life stages of Southern Leopard frog such as egg stage, embryo stage, and tadpole stages. Carbaryl caused significant mortality as well as deformities, such as missing hind limb, three front limbs, and bent tails, in larvae when exposed chronically. Deformities were observed in low concentrations as well, which claim our understanding on the persistency of insecticides and pesticides in the environment. Even at low concentration, insecticide or pesticide distributed and left at a single area can remain and affect inhibiting tadpoles and other species. We need to understand these consequences and their potential risks.

From our chronic exposure experiment with sodium fluoroacetate and Bd in *B. gargarizans* tadpoles, significant effects of 1080 were observed in terms of mortality, success to metamorphosis, development and behavior. Especially high mortality was observed in the highest concentration that is above the range of concentration found in the wild or natural streams after 1080 baiting campaign (Eason et al., 1993). It is known that amphibians are not affected by 1080 (McIlroy et al., 1985; Perfect & Bell, 2005; Spurr & Powlesland, 1997). All of these studies were done on adult frogs without

taking their different life stages into account. Although 1080 baits may not affect adults, their acute or chronic exposure to developing larvae may increase their risks to infection and cause deformities in further development. Currently there is no mortality or development data available for tadpoles, which then make this study an important step towards investigating the impact of 1080 and its operation in the environment.

Some deaths were still observed in 0.1 ppm and control groups with or without Bd present. This may be due to limitations to their laboratory incubating conditions. Tadpoles were incubated individually in a culture flask to provide more accurate and measured exposure of 1080 to each individual tadpole. Although such deaths were observed, all tadpoles were exposed to the same conditions and I was able to obtain a correlation between 1080 and mortality from it.

This experiment showed that the exposure to 1080 pesticide alters survival at various concentrations. All tadpoles from the highest concentration died before the experiment ended in both treatments. Also, none of them reached metamorphosis. Although the experiment did not entirely mimic natural environments including the presence of plants or microorganisms, interaction with each other or with other species, stream water itself, I was able to produce a risk assessment on the non-target species of 1080 at a wide range of concentrations with or without amphibian pathogen.

4.1.1. Compound 1080 in Nature

Compared to mammals, reptiles and amphibians are considered to possess an innate tolerance to 1080, as they were able to detoxify fluoroacetate by defluorination at a faster rate than mammals (Twigg et al., 1986). This implication is based on the responses from adults, and further investigation on tadpoles would be necessary as they can be affected by pesticides in early life stages. For adult amphibians, the actual risk they face during a poisoning campaign depends on the amount and concentration of 1080 bait in soil or water they are exposed to (McIlroy et al., 1985). As 1080 baits are most likely to end up in soil or in water such as stream or pond, amphibians are exposed to the pesticide in many possible ways, as they are both terrestrial and aquatic species, living in riparian zones.

Many toxicity data on adult amphibians are obtained from studies using either intraperitoneal or oral injection, but it is questionable whether this is comparable to field situations, because of the differences in processes such as absorption, distribution in the body, and transport to organs may differ depending upon delivery method. Amphibians have water permeable skin that protects amphibians from external stimuli but also consumes and releases water. This skin can provide a great route to transport pesticides within water into the body system (Stebbins & Cohen, 1997).

In nature, 1080 degradation has been shown to differ depending on the temperature, surrounding materials, and humidity (Northcott et al., 2014; Saunders et al., 2000; Twigg & Socha, 2001; Twigg et al., 2000). Degradation rate was slowest on oat grains and soil samples, presumably because 1080

does not bind with these materials as they would with meat which is often used as bait material (Wong et al., 1995). They also differ among various soils at different sites depending on the presence of microorganisms that are able to degrade fluoroacetate as well as the number of such microorganisms present at the site (Twigg & Socha, 2001). 1080 is generally known to have a short-term persistence in water (Booth et al., 1999; Eason et al., 1993; Eason et al., 1992; Ogilvie et al., 1996), thus lowering the possibility of long-term exposure of 1080 to aquatic species (Bridges, 2000). However, Twigg & Socha (2011) showed that 10-50% of 1080 in soil was degraded by 12 days at 27°C. and its half-life in soil of many arid regions may be 40 days or less, which is a longer persistency than in water (Booth et al., 1999). This persistency also highly depends on temperature and humidity; 1080 was degraded faster after heavy rainfall (Twigg & Socha, 2001). Northcott et al. (2014) suggested that the temperature is an important factor affecting the degradation of 1080, as 1080 degraded faster with higher temperature, possibly due to microbial activities at increasing temperature. Several studies showed that amphibians could be affected by pesticides at field-relevant concentration (Davidson, 2004; Hayes et al., 2002; Johansson et al., 2006; Relyea, 2005; Sparling et al., 2001). Pesticides often exist with other pesticides or insecticides as well as pathogens. Amphibians are mostly exposed to water and their skin, which is an important organ to protect themselves and to exchange gas and water, is prone to such external stimuli. Thus, pesticides or insecticides at field-relevant concentrations along with costressors may affect amphibians sublethally and even lethally. As suggested,

there are lots of variables and factors affecting amphibian infection and 1080 degradation, which inhibit us and many other researchers from achieving the exact results of the effects of the pesticide. Presence of 1080 in soil increases the potential risk to non-target species as it stays in soil and as it leaches into groundwater or stream water. Considering such possibility of constant leaching from soil and 1080 poisoning campaign followed by no rainfall, it is necessary to examine responses and symptoms of aquatic species from chronic exposure. I exposed the tadpoles to chronic exposure conditions as I observed various responses ranging from prolonged metamorphosis, impaired behavior, and to high mortality with outbreak of death, with several concentrations. This will be able to provide information on the relationship between tadpole and 1080, acknowledging their susceptibility to the pesticide resulting in various disease signs.

4.1.2 Relationship of Pesticide and Bd with Tadpole Survival and Metamorphosis

There are only few toxicity data of 1080 on amphibians, which are done on adults for their sensitivity or susceptibility to the chemical (McIlroy et al., 1985; Perfect & Bell, 2005) or on the frog population in nature (Greene et al., 1995; McNaughton & Greene, 1994; Spurr & Powlesland, 1997). Amphibians tend to be more susceptible to external contamination at lower larval stages (Prabhaker et al., 1989). Here I investigated the effect of exposure to 1080 and Bd during larval stage instead of adults. The experimental treatments were designed to test the effect of 1080 at a various range of concentrations that reflect environmental to lethal concentrations with or without Bd. Tadpoles displayed rapid mortality and lowest metamorphosis rate in highest concentration, 100 ppm. Since it was known that 1080 does not have lethal impact on amphibians (Perfect & Bell, 2005), I actually did not expect such mortality from tadpoles. However, the results confirmed a strong, positive correlation between 1080 concentrations and mortality – higher concentration of 1080 resulted in higher mortality rate. Concentration of 1080 exposure was also related to tadpole development as more tadpoles reached metamorphosis at low concentration, and highest metamorphosis was observed when there was no 1080 exposure at all. These results were equivalent when tadpoles were exposed to 1080 alone and to 1080+Bd. I expected 1080 to reduce the impact of Bd, which would result in higher mortality in controls due to Bd infection. However, none of the tadpoles were infected by Bd, including control groups, thus implying that

Bufo gargarizans tadpoles were not susceptible to Bd infection. However, the reduction in survival days and higher development rate in 1080+Bd treatment suggest various possibilities. There may have been an interaction between low concentration of 1080 and the presence of Bd. Since Bd was added along with flooded water from the TGhL media, there may have been a positive or negative effect of it as well. All tadpoles were tested negative and they had a same pattern of survival and development as those in 1080-alone treatment. This similar pattern is due to the pesticide effect and Bd seemed to have altered their survival and development as it shortened the larval period, accelerated metamorphosis but reduced the survival rate. Although pesticides or insecticides may or may not alter the susceptibility to pathogens, which is also affected by different species, *Bufo gargarizans* tadpoles were observed to be tolerant and resistant to Bd pathogens with or without the presence of pesticide 1080. Bd zoospores require a host with keratin but tadpoles, especially in earlier stages, lack tissues with keratin besides mouthparts, which makes them less susceptible than adult amphibians. As observed from the study results, Bd could have an impact on tadpole growth in presence of other pesticides (Wise et al., 2014). Wise et al. (2014) observed a significantly decreased survival of American toad tadpoles with Bd and lower mass in metamorphs that were exposed to malathion. Although not significant, when Bd was added to malathion, it lowered the mass of tadpoles even more compared to tadpoles that were exposed to malathion only. The effect of Bd cannot be determined with results from one species. Due to its inconsistency and various effects on different species, amphibians require thorough and

broad study in order to make a precise conclusion on its susceptibility to amphibians.

There may be differences in susceptibility to pesticides depending on the species phylogeny (Junges et al., 2012). *B. gargarizans* may have strong or weak susceptibility to the pesticide but it would need more studies done on other species as well to observe the broad effect of the pesticide as well as susceptibility of other species at larval stages.

Although pesticides are produced into baits under regulations, debate on chemical use in wildlife is still ongoing. The chemical effects may be subtle (Boone et al., 2009) but the effects on amphibians need further studies as the sensitivity to pesticides differ greatly among species (Boone et al., 2009). It may be more difficult to investigate the effects on amphibians as they undergo various life stages from egg, larva, froglet and to full-grown adult, and usually live in riparian zones with diverse temporal and spatial conditions.

4.1.3. Swimming behavior

1080 not only affects survival but also opens tadpoles to behavior impairment. I found that the pesticide could affect swimming behaviors of the tadpoles such as total time of swimming, number of swirls and number of times the tadpole lost balance throughout the recording session. Behaviors of swirling motion and losing balance were not observed in control groups but were displayed only by those exposed to 1080. Swimming time decreased and swirling motion increased with higher 1080 concentration. Imbalance did not have strong relationships with 1080 concentration in 1080-alone treatment but had a negative relationship trend with concentration in 1080+Bd treatment as the behavior increased with decreased concentrations. In both treatments, this behavior was found in the tadpoles in lower concentration, which are the ones who swirl less but swim more. Tadpoles in lower concentration groups swim more at more stable motion and swirl less, but they seemed to lose balance instead of swirling. This may indicate a nerve system interruption by 1080 from high to low concentrations. These behaviors may result from lack of energy due to 1080 interrupting TCA cycle and also from 1080 acting on the nervous system (Chenoweth & Gilman, 1946). Energy deprivation will not allow tadpoles to swim for a long period of time as normal tadpoles would. Swirling motion was also observed in other studies as well. Lavorato et al. (2013) reported abnormal swimming patterns such as shorter distance of swimming, swirling, resting and unusual use of space from *Rana dalmatina* tadpoles with endosulfan exposure. Similar impaired swimming behaviors were also observed in other pesticides or insecticides as well (Brunelli et al.,

2009; Denoël et al., 2010; Denoël et al., 2012; Denoël et al., 2013; Gürkan & Hayretdağ, 2012; Lavorato et al., 2013). Denoël et al. (2013) also observed shorter distance of swimming, less swimming activity, and abnormal use of space from *Rana tempora* tadpoles at higher endosulfan concentration. Similar results were found from the effect of copper sulfate as tadpoles swam shorter distance and lost equilibrium (Gürkan & Hayretdağ, 2012). Gürkan & Hayretdağ (2012) stated that impaired development of skeletal muscles, general deformities of tadpole morph, and impaired liver cells and pronepheric tubules due to copper sufate contribute to muscle development. Muscles are important for tadpoles for swimming and such deformities may contribute to swimming behaviors which result in shortened swimming distances and immobility. Many factors contribute to swimming behavior itself such as skeletal muscles, energy from TCA cycle, and nervous system. It may be difficult to attribute one factor to abnormal behaviors that are observed from not only 1080 exposure but also other pesticides and insecticides. One or all factors could affect and result in these behaviors but will need further study.

Reference

Bataille, A., Fong, J. J., Cha, M., Wogan, G. O., Baek, H. J., Lee, H., Min, M. S., & Waldman, B. (2013). Genetic Evidence for a High Diversity and Wide Distribution of Endemic Strains of the Pathogenic Chytrid Fungus Batrachochytrium Dendrobatidis in Wild Asian Amphibians. *Molecular ecology, 22*(16), 4196-4209.

Boone, M. D., Davidson, C., & Bridges-Britton, C. (2009). Evaluating the Impact of Pesticides in Amphibian Declines. *Amphibian Decline: Diseases, Parasites, Maladies and Pollution. Surrey Beatty and Sons, Chipping Norton, Inglaterra*, 3183-3207.

Booth, L., Ogilvie, S., Wright, G., & Eason, C. (1999). Degradation of Sodium Monofluoroacetate (1080) and Fluorocitrate in Water. *Bulletin of environmental contamination and toxicology, 62*(1), 34-39.

Bridges, C. (2000). Long-Term Effects of Pesticide Exposure at Various Life Stages of the Southern Leopard Frog (Rana Sphenocephala). *Archives of environmental contamination and toxicology, 39*(1), 91-96.

Brühl, C. A., Pieper, S., & Weber, B. (2011). Amphibians at Risk? Susceptibility of Terrestrial Amphibian Life Stages to Pesticides. *Environmental Toxicology and Chemistry, 30*(11), 2465-2472.

Brunelli, E., Bernabò, I., Berg, C., Lundstedt-Enkel, K., Bonacci, A., & Tripepi, S. (2009). Environmentally Relevant Concentrations of Endosulfan Impair Development, Metamorphosis and Behaviour in Bufo Bufo Tadpoles. *Aquatic Toxicology, 91*(2), 135-142.

Chenoweth, M. B. (1949). Monofluoroacetic Acid and Related Compounds.

Pharmacological reviews, 1(3), 383-424.

Chenoweth, M. B., & Gilman, A. (1946). Studies on the Pharmacology of Fluoroacetate I. Species Response to Fluoroacetate. *Journal of Pharmacology and Experimental Therapeutics, 87*(2), 90-103.

Christin, M., Menard, L., Gendron, A., Ruby, S., Cyr, D., Marcogliese, D., Rollins-Smith, L., & Fournier, M. (2004). Effects of Agricultural Pesticides on the Immune System of Xenopus Laevis and Rana Pipiens. *Aquatic toxicology, 67*(1), 33-43.

Davidson, C. (2004). Declining Downwind: Amphibian Population Declines in California and Historical Pesticide Use. *Ecological Applications, 14*(6), 1892-1902.

Denoël, M., Bichot, M., Ficetola, G. F., Delcourt, J., Ylieff, M., Kestemont, P., & Poncin, P. (2010). Cumulative Effects of Road De-Icing Salt on Amphibian Behavior. *Aquatic Toxicology, 99*(2), 275-280.

Denoël, M., D'Hooghe, B., Ficetola, G. F., Brasseur, C., De Pauw, E., Thomé, J.-P., & Kestemont, P. (2012). Using Sets of Behavioral Biomarkers to Assess Short-Term Effects of Pesticide: A Study Case with Endosulfan on Frog Tadpoles. *Ecotoxicology, 21*(4), 1240-1250.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.-F., & De Pauw, E. (2013). Effects of a Sublethal Pesticide Exposure on Locomotor Behavior: A Video-Tracking Analysis in Larval Amphibians. *Chemosphere, 90*(3), 945-951.

Eason, C. (2002). Sodium Monofluoroacetate (1080) Risk Assessment and Risk Communication. *Toxicology, 181*, 523-530.

Eason, C., Gooneratne, R., Wright, G., Pierce, R., & Frampton, C. (1993). *The Fate of Sodium Monofluoroacetate (1080) in Water, Mammals and Invertebrates.* Paper presented at the Proceedings of the New Zealand plant protection conference.

Eason, C., Wright, G., & Fitzgerald, H. (1992). Sodium Monofluoroacetate (1080) Water-Residue Analysis after Large-Scale Possum Control. *New Zealand journal of ecology*, 47-49.

Eisler, R. (1995). *Sodium Monofluoroacetate (1080) Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review*: US Department of the Interior, National Biological Service.

Feng, S., Kong, Z., Wang, X., Zhao, L., & Peng, P. (2004). Acute Toxicity and Genotoxicity of Two Novel Pesticides on Amphibian, Rana N. Hallowell. *Chemosphere, 56*(5), 457-463.

Gaietto, K. M., Rumschlag, S. L., & Boone, M. D. (2014). Effects of Pesticide Exposure and the Amphibian Chytrid Fungus on Gray Treefrog (Hyla Chrysoscelis) Metamorphosis. *Environmental Toxicology and Chemistry, 33*(10), 2358-2362.

Goka, K., Yokoyama, J., Une, Y., Kuroki, T., Suzuki, K., Nakahara, M., Kobayashi, A., Inaba, S., Mizutani, T., & Hyatt, A. D. (2009). Amphibian Chytridiomycosis in Japan: Distribution, Haplotypes and Possible Route of Entry into Japan. *Molecular Ecology, 18*(23), 4757-4774.

Gosner, K. L. (1960). A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification. *Herpetologica*, 183-190.

Greene, B. S., McNaughton, A., & Singh, A. (1995). *Hochstetter's Frog (Leiopelma Hochstetteri) Survey in the Hunua Ranges 1995*: Auckland Regional Council Parks Service.

Gürkan, M., & Hayretdağ, S. (2012). Morphological and Histological Effects of Copper Sulfate on the Larval Development of Green Toad, Bufo Viridis. *Turk J Zool, 36*(2), 231-240.

Gurushankara, H., Krishnamurthy, S., & Vasudev, V. (2007). Effect of Malathion on Survival, Growth, and Food Consumption of Indian Cricket Frog (Limnonectus Limnocharis) Tadpoles. *Archives of environmental contamination and toxicology, 52*(2), 251-256.

Hanlon, S. M., Lynch, K. J., Kerby, J. L., & Parris, M. J. (2015). The Effects of a Fungicide and Chytrid Fungus on Anuran Larvae in Aquatic Mesocosms. *Environmental Science and Pollution Research*, 1-12.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., & Vonk, A. (2002). Hermaphroditic, Demasculinized Frogs after Exposure to the Herbicide Atrazine at Low Ecologically Relevant Doses. *Proceedings of the National Academy of Sciences, 99*(8), 5476-5480.

Johansson, M., Piha, H., Kylin, H., & Merilä, J. (2006). Toxicity of Six Pesticides to Common Frog (Rana Temporaria) Tadpoles. *Environmental Toxicology and Chemistry, 25*(12), 3164-3170.

Kinney, V. C., Heemeyer, J. L., Pessier, A. P., & Lannoo, M. J. (2011). Seasonal Pattern of Batrachochytrium Dendrobatidis Infection and Mortality in Lithobates Areolatus: Affirmation of Vredenburg's

"10,000 Zoospore Rule". *PLoS One, 6*(3), e16708.

Kleinhenz, P., Boone, M. D., & Fellers, G. (2012). Effects of the Amphibian Chytrid Fungus and Four Insecticides on Pacific Treefrogs (Pseudacris Regilla). *Journal of Herpetology, 46*(4), 625-631.

Lavorato, M., Bernabo, I., Crescente, A., Denoël, M., Tripepi, S., & Brunelli, E. (2013). Endosulfan Effects on Rana Dalmatina Tadpoles: Quantitative Developmental and Behavioural Analysis. *Archives of environmental contamination and toxicology, 64*(2), 253-262.

Marco, A., & Blaustein, A. R. (1999). The Effects of Nitrite on Behavior and Metamorphosis in Cascades Frogs (Rana Cascadae). *Environmental Toxicology and Chemistry, 18*(5), 946-949.

McIlroy, J., King, D., & Oliver, A. (1985). The Sensitivity of Australian Animals to 1080 Poison Viii.* Amphibians and Reptiles. *Wildlife Research, 12*(1), 113-118.

McMahon, T. A., Romansic, J. M., & Rohr, J. R. (2013). Nonmonotonic and Monotonic Effects of Pesticides on the Pathogenic Fungus Batrachochytrium Dendrobatidis in Culture and on Tadpoles. *Environmental science & technology, 47*(14), 7958-7964.

McNaughton, A., & Greene, B. S. (1994). *The Effect of 1080 on the Hochstetter's Frog (Leiopelma Hochstetteri) Population in the Hunua Ranges 1994*: Auckland Regional Council Parks Service.

Northcott, G., Jensen, D., Ying, L., & Fisher, P. (2014). Degradation Rate of Sodium Fluoroacetate in Three New Zealand Soils. *Environmental toxicology and chemistry, 33*(5), 1048-1058.

Ogilvie, S., Hetzel, F., & Eason, C. (1996). Effect of Temperature on the Biodegradation of Sodium Monofluoroacetate (1080) in Water and in Elodea Canadensis. *Bulletin of environmental contamination and toxicology, 56*(6), 942-947.

Perfect, A. J., & Bell, B. D. (2005). *Assessment of the Impact of 1080 on the Native Frogs Leiopelma Archeyi and L. Hochstetteri*: Department of Conservation Wellington.

Pierce, R. J., & Montgomery, P. (1992). *The Fate of Birds and Selected Invertebrates During a 1080 Operation*: Head Office, Department of Conservation.

Prabhaker, N., Toscano, N., & Coudriet, D. (1989). Susceptibility of the Immature and Adult Stages of the Sweetpotato Whitefly (Homoptera: Aleyrodidae) to Selected Insecticides. *Journal of Economic Entomology, 82*(4), 953-958.

Proudfoot, A. T., Bradberry, S. M., & Vale, J. A. (2006). Sodium Fluoroacetate Poisoning. *Toxicological reviews, 25*(4), 213-219.

Rebollar, E. A., Hughey, M. C., Harris, R. N., Domangue, R. J., Medina, D., Ibáñez, R., & Belden, L. K. (2014). The Lethal Fungus Batrachochytrium Dendrobatidis Is Present in Lowland Tropical Forests of Far Eastern Panamá. *PloS one, 9*(4), e95484.

Relyea, R. A. (2005). The Impact of Insecticides and Herbicides on the Biodiversity and Productivity of Aquatic Communities. *Ecological applications, 15*(2), 618-627.

Rohr, J. R., Raffel, T. R., Halstead, N. T., McMahon, T. A., Johnson, S. A.,

Boughton, R. K., & Martin, L. B. (2013). Early-Life Exposure to a Herbicide Has Enduring Effects on Pathogen-Induced Mortality. *Proceedings of the Royal Society B: Biological Sciences, 280*(1772), 20131502.

Rumschlag, S. L., Boone, M. D., & Fellers, G. (2014). The Effects of the Amphibian Chytrid Fungus, Insecticide Exposure, and Temperature on Larval Anuran Development and Survival. *Environmental Toxicology and Chemistry, 33*(11), 2545-2550.

Saunders, G., McLeod, S., & Kay, B. (2000). Degradation of Sodium Monofluoroacetate (1080) in Buried Fox Baits. *Wildlife Research, 27*(2), 129-135.

Shin, J., Bataille, A., Kosch, T. A., & Waldman, B. (2014). Swabbing Often Fails to Detect Amphibian Chytridiomycosis under Conditions of Low Infection Load. *PloS one, 9*(10), e111091.

Sparling, D. W., Fellers, G. M., & McConnell, L. L. (2001). Pesticides and Amphibian Population Declines in California, USA. *Environmental Toxicology and Chemistry, 20*(7), 1591-1595.

Spurr, E., & Drew, K. (1999). Invertebrates Feeding on Baits Used for Vertebrate Pest Control in New Zealand. *New Zealand Journal of Ecology*, 167-173.

Spurr, E., & Powlesland, R. (1997). Impacts of Aerial Application of 1080 on Non-Target Native Fauna. *Science for conservation (Wellington, NZ), 62*(632.9510993), 20.

Spurr, E. B., & Berben, P. H. (2004). Assessment of Non-Target Impact of

1080-Poisoning for Vertebrate Pest Control on Weta (Orthoptera: Anostostomatidae and Rhaphidophoridae) and Other Invertebrates in Artificial Refuges. *New Zealand Journal of Ecology, 28*(1), 63-72.

Stebbins, R. C., & Cohen, N. W. (1997). *A Natural History of Amphibians*: Princeton University Press.

Suren, A. M., & Lambert, P. (2006). Do Toxic Baits Containing Sodium Fluroacetate (1080) Affect Fish and Invertebrate Communities When They Fall into Streams? *New Zealand journal of marine and freshwater research, 40*(4), 531-546.

Twigg, L., Lowe, T., & Martin, G. (2009). Characteristics and Use.

Twigg, L., & Mead, R. (1990). Comparative Metabolism of, and Sensitivity to, Fluoroacetate in Geographically Separated Populations of Tiliqua-Rugosa (Gray)(Scincidae). *Australian journal of zoology, 37*(6), 617-626.

Twigg, L., Mead, R., & King, D. (1986). Metabolism of Fluoroacetate in the Skink (Tiliqua Rugosa) and the Rat (Rattus Norvegicus). *Australian journal of biological sciences, 39*(1), 1-16.

Twigg, L., & Socha, L. V. (2001). Defluorination of Sodium Monofluoroacetate by Soil Microorganisms from Central Australia. *Soil Biology and Biochemistry, 33*(2), 227-234.

Twigg, L. E., Eldridge, S. R., Edwards, G. P., Shakeshaft, B. J., & Adams, N. (2000). The Longevity and Efficacy of 1080 Meat Baits Used for Dingo Control in Central Australia. *Wildlife Research, 27*(5), 473-481.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., & Briggs, C. J. (2010).

Dynamics of an Emerging Disease Drive Large-Scale Amphibian Population Extinctions. *Proceedings of the National Academy of Sciences, 107*(21), 9689-9694.

Wang, F., Dicinoski, G. W., Zhu, Y., & Haddad, P. R. (2004). Simultaneous Determination of Monofluoroacetate, Difluoroacetate and Trifluoroacetate in Environmental Samples by Ion Chromatography. *Journal of Chromatography A, 1032*(1), 31-35.

Wise, R. S., Rumschlag, S. L., & Boone, M. D. (2014). Effects of Amphibian Chytrid Fungus Exposure on American Toads in the Presence of an Insecticide. *Environmental Toxicology and Chemistry, 33*(11), 2541-2544.

Wong, D., Kinnear, J., & Runham, C. (1995). A Simple Rapid Bioassay for Compound 1080 (Sodium Fluoroacetate) in Bait Materials and Soil-Its Technique and Applications. *Wildlife Research, 22*(5), 561-568.

Yang, H., Baek, H., Speare, R., Webb, R., Park, S., Kim, T., Lasater, K. C., Shin, S., Son, S., & Park, J. (2009). First Detection of the Amphibian Chytrid Fungus Batrachochytrium Dendrobatidis in Free-Ranging Populations of Amphibians on Mainland Asia: Survey in South Korea. *Diseases of aquatic organisms, 86*(1), 9-13.

국문초록

Sodium fluoroacetate(1080)는 다양한 야생동물을 관리하기 위해 호주와 뉴질랜드에서 흔히 쓰이는 포유류 살충제이다. 이것은 목표 동물의 캘빈회로를 망가뜨려서 죽게 만들며 흔히 관찰되는 증상에는 심정지와 에너지 손실이 있다. 이 살충제는 포유류의 야생동물을 목표로 하지만, 공중에서 다양한 지역으로 살포되는 방식은 토양과 수질 오염의 위험이 우려되며 그 외에 목표가 아니었던 동물까지도 살충제에 노출이 될 수 있어 논란이 되고 있다. 이러한 목표 동물 외에 새, 무척추동물, 양서류 등의 다른 동물들도 1080의 위험에 노출되어 있는지 확인하기 위한 연구들이 많았다. 양서류의 경우, 성체형에 대한 연구는 많았지만 그들이 알, 배아 또는 유충에 대한 연구는 없었다. 살충제의 급성 또는 만성적인 노출은 양서류의 변태와 그 외 행동이나 성장에 영향을 미칠 수 있다. 양서류는 유충 단계에서 물 속에서 지내며 다양한 자극에 노출될 수 있다. 환경적 요인 뿐만 아니라 화학적 자극 또한 유충에게 독립적으로 또는 다른 요인과 함께 영향을 줄 수 있다. 이 논문에서는 유충 단계가 앞으로의 성장에 있어서 중요한 시기임을 고려하여 1080가 두꺼비 유충(올챙이)에 미치는 영향을 연구하였다. 2013년에는 두꺼비 올챙이의 생존, 변태와 행동에 미치는 1080의 독립적인 영향(1080-alone)을 알아보았으며, 2014년에는 Bd라는 항아리곰팡이균을 추가하여 1080와 함께 미치는 영향(1080+ Bd)을 알아보았다. 올챙이는 1080-alone 그리고 1080+ Bd 관찰 그룹에서 높은 농도의 살

충제에 노출되어 있을수록 높은 치사율, 더딘 생장 그리고 낮은 변태율을 보였다. 제일 높은 농도(100ppm)에 노출되었던 올챙이는 전부 변태를 하지 못했다. 높은 농도에 노출되어 있을수록 올챙이는 낮은 수영주기와 잦은 스월링(빙빙 도는 모습) 움직임을 보였다. 낮은 농도에 있던 올챙이에서는 더 오랜 수영주기가 관찰되었고 스월링 움직임은 거의 관찰되지 않았지만, 균형을 잃는 모습은 더 자주 관찰되었다. 이는 살충제의 만성적인 노출이 신경계를 방해한 결과라고 볼 수 있다.

1080+ Bd 관찰 그룹의 올챙이는 Bd에 감염되지는 않았으며 이는 두꺼비(*Bufo gargarizans*) 올챙이가 Bd에 대한 면역성이 있음을 보여주며 살충제가 Bd의 효과를 저해하거나 높이지는 않음을 나타낸다. 이러한 결과는 살충제 1080가 목표 포유류 동물이 아닌 양서류의 유충에 유독할 수 있음을 제시한다. 이 연구결과에 따라 살충제 1080가 양서류 유충의 추후 생장과 생존에 충분한 영향을 미칠 수 있다고 판단되며, 다양한 종에 살충제의 급성 또는 만성 노출 실험을 한다면 보다 정확한 양서류의 1080에 대한 민감성을 판단할 수 있을 것이다.

키워드: 환경독성학, 양서류 개체군 감소, 상승효과.

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