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Travis A. Moe
Northern Michigan University, tmoe@nmu.edu

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SUB-LETHAL AND LETHAL EFFECTS OF A NEONICOTINOID PESTICIDE ON THE DEVELOPMENT OF NORTHERN LEOPARD FROG TADPOLES.

By

Travis Moe

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Office of Graduate Education and Research

May 2017

SIGNATURE APPROVAL FORM

SUB-LETHAL AND LETHAL EFFECTS OF A NEONICOTINOID PESTICIDE ON THE DEVELOPMENT OF NORTHERN LEOPARD FROG TADPOLES.

This thesis by Travis Moe is recommended for approval by the student's Thesis Committee and Department Head in the Department of Biology and by the Assistant Provost of Graduate Education and Research.

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Committee Chair: Date

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First Reader: Date

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Second Reader (if required): Date

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Department Head: Date

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Dr. Lisa S. Eckert Date
Interim Director of Graduate Education

ABSTRACT

SUB-LETHAL AND LETHAL EFFECTS OF A NEONICOTINOID PESTICIDE ON THE DEVELOPMENT OF NORTHERN LEOPARD FROG TADPOLES.

By

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Well-known global declines in amphibian populations have sparked decades of studies into potential causes (Stuart et al. 2004). Pesticides are a suspected contributor to declining populations (Bruhl et al. 2013). Imidacloprid is the most widely used insecticide in the world, but few studies have considered its potential effects on anurans. I conducted a static-renewal experiment to monitor the lethal and sub-lethal, developmental effects in Northern leopard frog tadpoles exposed to three concentration levels (250 ng/L, 8.5 mg/L, and 85 mg/L) of imidacloprid in a laboratory setting. Survivorship was 0% by day 23 of exposure to imidacloprid at the previously lowest known LC50 value for frogs of 85 mg/L. This served as the high concentration level in this study. Tadpoles exposed to imidacloprid had reduced length at metamorphosis compared with the control group (one-way ANOVA, $p<0.001$). Imidacloprid exposure concentration was inversely related to the rate of development of tadpoles (Somers' d, $p=0.009$). Imidacloprid concentration level was positively associated with frequency of nuclear abnormalities. Exposure to imidacloprid may cause sub-lethal effects. Tadpoles exposed to 250 ng/L imidacloprid (a concentration found in a Canadian wetland area) exhibited sub-lethal effects (e.g. binucleated, blebbed, lobed and notched nuclei), suggesting that these effects may be observed in the environment with wild populations of frogs. More research is necessary to understand the lethal and sub-lethal effects of imidacloprid on Northern leopard frog tadpoles, but these results offer a basis for further research.

Copyright by

Travis Moe

May 2017

DEDICATION

To my advisor, Dr. Patrick Brown,
For the inspiring, and thought-provoking discussions and stories.

To my support system from the beginning to the end of this project,
Carissa Pischke, Cindy Eggen-Moe, and Greg Moe.

To everyone who recognizes the need for sound, scientific studies,
and protection for the environment and all its inhabitants.

"In nature nothing exists alone." – Rachel Carson, Silent Spring.

ACKNOWLEDGEMENTS

The author wishes to thank his thesis director, Dr. Patrick Brown, for his advice, support, and comments through the duration of this project. Particularly, with the direction offered in the beginning to narrow-in on a suitable topic within the realm of ecotoxicology, as well as consultation and direction throughout. I am extremely honored to be one of Dr. Brown's final graduate students prior to his retirement. The author also wishes to thank his thesis committee members, Dr. Leslie Putman and Dr. Alan Rebertus for their instruction and support through the duration of this project. Dr. Putman's advice was pivotal for the HPLC analysis, and the comments and questions on results were extremely helpful. Dr. Rebertus' inspiring work ethic and advice for statistical analysis were crucial in this project. The author also wishes to thank Dr. Jill B.K. Leonard for the use of the aquatics facility and advice in the early stages of this project, and Dr. Brent Graves for his early advice as my undergraduate advisor and encouraging my fondness for herpetofauna. Much of my interest in ecotoxicology and environmental chemistry stems from countless scientists and writers, to whom I owe so much for paving a path for this type of research. I also wish to acknowledge my colleagues for the support and mental breaks whenever they were needed most. I must express my gratitude to the sources of financial support, without which this project could not have been completed: Northern Michigan University's Excellence in Education Award, Northern Michigan University's Scholarly Activities Fund, and Northern Michigan University's Department of Biology, and contributions from Dr. Patrick Brown's Professional Development Fund.

This thesis is prepared using format from the Journal of Herpetology, which includes one of the journals that I will submit to for publication.

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ABSTRACT

Well-known global declines in amphibian populations have sparked decades of studies into potential causes (Stuart et al. 2004). Pesticides are a suspected contributor to declining populations (Bruhl et al. 2013). Imidacloprid is the most widely used insecticide in the world, but few studies have considered its potential effects on anurans. I conducted a static-renewal experiment to monitor the lethal and sub-lethal, developmental effects in Northern leopard frog tadpoles exposed to three concentration levels (250 ng/L, 8.5 mg/L, and 85 mg/L) of imidacloprid in a laboratory setting. Survivorship was 0% by day 23 of exposure to imidacloprid at the previously lowest known LC50 value for frogs of 85 mg/L. This served as the high concentration level in this study. Reduced length at metamorphosis was observed in the tadpoles exposed to imidacloprid compared with the control group (one-way ANOVA, $p<0.001$). An inverse relationship existed between imidacloprid exposure concentration and the rate of development of tadpoles (Somers' d, $p=0.009$). Imidacloprid concentration level was positively associated with frequency of nuclear abnormalities. Exposure to imidacloprid may cause sub-lethal effects. Tadpoles exposed to 250 ng/L imidacloprid (a concentration found in wetlands) exhibited sub-lethal effects (e.g. binucleated, blebbed, lobed and notched nuclei), suggesting that these effects may be observed in the environment with wild populations of frogs. More research is necessary to understand the lethal and sub-lethal effects of imidacloprid on Northern leopard frog tadpoles, but these results offer a basis for further research.

INTRODUCTION

Population declines, in some cases leading to extirpations, have been observed in amphibians around the world. Approximately 1/3rd of all amphibian species are now at risk for extinction, making this class of vertebrates the most threatened globally (Knapp et al. 2016, Sparling and Fellers 2009). For more than 25 years, many studies attempted to understand these global declines. The most likely causes of population declines include habitat destruction and fragmentation; emergent, widespread diseases such as chytridiomycosis; climate change; invasive species; and environmental pollutants, such as agrochemicals (Bruhl et al. 2013, Hof et al. 2011, Grant et al. 2016). Some or all of these factors may interact in ways that amplify or reduce their individual effects, complicating management and conservation efforts (Hof et al. 2011). For example, the use of newly developed agrochemicals (i.e. insecticides, herbicides, and fungicides) complicates possible interactions and may require multiple studies, across multiple taxa, to understand potential ecosystem effects. In addition, the impacts of these chemicals may go unnoticed due to their sub-lethal (e.g. immune suppression or endocrine disruption) or generational effects (Hayes et al. 2006, Mason et al. 2013). However, studies to understand the effects of individual causes remain vital, as many of them are poorly understood or the factors influencing them are constantly changing.

Approximately 40% of the global land surface is used for agricultural practices; consequently, the influence and exposure of agricultural zones to native populations is great and increasing (Foley et al. 2005). Much of this land was formerly grasslands or forests, and then converted to agricultural lands, which receive a disproportionate and increasing amount of pesticides (Figure 1, Bruhl et al. 2013). Chronic exposure to agrochemicals is common among non-target organisms within or near agricultural zones (Williams and Semlitsch 2009). Anurans

(i.e. frogs and toads), because of their life history characteristics, serve as excellent, non-target species-indicators of environmental pollution. These characteristics include thin, semi-permeable skin that absorbs moisture (and potential pollutants) from the environment, foraging on insects and small aquatic organisms where bioaccumulation may become a concern, overwintering and breeding in small to medium sized pools that may be exposed to chemicals via runoff or spray, and laying unshelled eggs in soil or water where they could absorb chemicals (Blaustein et al. 2003, Denton and Bernot 2011, Price et al. 2007). Despite these characteristics, amphibians are not part of the US Environmental Protection Agency's (EPA) ecological risk assessment for non-target species when testing (or re-registering) a new pesticide (US EPA). Few studies have investigated the effects of neonicotinoids (but see Feng et al. 2004, Ade et al. 2010, Puglis and Boone 2011, Ruiz de Arcaute et al. 2014).

Neonicotinoids are a group of relatively new, synthetic chemical insecticides (Hopwood et al. 2012). Currently, there are seven recognized neonicotinoids. Imidacloprid was the first one developed by Bayer Crop Science in 1985, and registered for use in the United States in the early 1990s (Tomizawa and Casida 2005). High levels and widespread use of neonicotinoids was evident in the mid- to late 90's (van der Sluijs et al. 2015). Neonicotinoids largely replaced the organophosphates and carbamates to become the most widely used group of insecticides in the world. Two primary reasons account for their widespread use, the first being their diverse mode of application (i.e., seed coatings, soil drenches, chemigation, or foliar sprays) (Hopwood et al. 2012). Secondly, their systemic mode of action makes them preferable to many other types of pesticides. Systemic insecticides often go against the use of integrated pest management practices because it treats a problem before one is present. In addition, studies have estimated that only around 5% of the active ingredient from seed coatings are actually taken up by the

target crop; the majority of neonicotinoid is left to accumulate in the soil or runoff into underground or surface bodies of water (Goulson 2013).

In animals, imidacloprid's (IMI; 1-(6-chloro-3-pyridinylmethyl)-*N*-nitroimidazolidin-2-ylideneamine) mode of action is unclear, but it is thought to bind and affect the function of the nicotinic acetylcholine receptors (nAChRs). These receptor sites are much more abundant in insects than mammals, making them selectively more toxic to invertebrates (Kreutzweiser et al. 2007, Tomizawa and Casida 2005). Imidacloprid breaks down into several possible compounds (6-chloronicotinic acid, 6-hydroxynicotinic acid, chloronicotinic aldehyde, olefin-IMI, 5-OH-IMI, and 4-OH-IMI), each with differing levels of toxicity to target and non-target organisms. For example, one common metabolite of imidacloprid, 6-chloronicotinic acid (6-CNA), is also highly toxic to bees, thus increasing the exposure time to a potentially lethal compound or even causing a delay in lethality from imidacloprid application (or exposure) time (Simon-Delso et al. 2015). In addition to environmental exposure routes, food may also be a source for exposure because nicotine-derivative compounds, such as imidacloprid, can quickly and effectively cross the intestine barrier (Simon-Delso et al. 2015).

Imidacloprid is commonly used for controlling sucking insect pests (e.g. aphids, whiteflies, planthoppers, thrips, and some coleopteran pests) (Figure 2, Jeschke et al. 2010). A large portion (>80%) of the applied imidacloprid is not taken up by the plant, but released into the environment (Sur and Stork 2003, Tisler et al. 2009). Imidacloprid's long half-life in soil raises concern about its possible accumulation and transport to nearby water sources (Van Dijk et al. 2013). Laboratory and field studies estimate imidacloprid's 50% dissipation time ranges from 28 to 1250 days, depending on the soil type. Moreover, commonly reported concentrations in soil, water, and plants in field margins exceed LC50 levels for beneficial pollinators (Goulson

2013). Although imidacloprid use in agriculture is declining due to the more diverse application options of two closely related neonicotinoids, thiamethoxam and clothianidin, imidacloprid remains the most commonly detected neonicotinoid in urban/suburban settings (Main et al. 2016).

In non-target organisms exposed to pesticides, lethal effects (direct mortality) are relatively easy to detect, however, the sub-lethal effects are probably more common. Sub-lethal effects of pesticide exposure may include reduced growth, development, fecundity, impaired immune system, altered behavior, genotoxic effects, and cytotoxic effects (Boina et al. 2009, Perez-Iglesias et al. 2014). Although environmentally occurring concentrations of neonicotinoids are below reported lethal threshold levels for amphibians, sub-lethal effects are possible and are greatly understudied (van der Sluijs et al. 2015).

Biomonitoring markers provide a measurement for the level of cytogenetic damage produced from exposure to a toxic pollutant. One of the most reliable methods to measure cytogenetic damage is analysis of the frequency of micronuclei (MN) in circulating nucleated erythrocytes (Fenech 2000, Perez-Iglesias et al. 2014, Ruiz de Arcaute et al. 2014, Vera-Candioti et al. 2010). Micronuclei result from the loss of whole or partial chromosomes from daughter nuclei at mitosis and are relatively simple to detect and quantify, because they are separate from the main nucleus of the cell (Figure 3; Campana et al. 2003).

The goal of this study was to understand the lethal and sub-lethal effects of exposure to imidacloprid in Northern leopard frogs (*Lithobates pipiens*). Three exposure concentrations of imidacloprid were monitored for mortality, metamorphosis, morphological development from hatching to metamorphosis, growth (weight and length), cytotoxicity assay for micronuclei

analysis and other nuclear abnormalities, and an attempt to detect residues of imidacloprid in liver samples.

MATERIALS AND METHODS

Test organisms, lab conditions, and general experimental design

I obtained egg clusters of *L. pipiens* from Carolina Biological Supply (#146430) on March 15 2016, and nearly all eggs hatched by March 21. Fifteen individuals were transferred to each of eighteen, 10-gallon (37.85 L) aquaria in a laboratory setting. A 12/12 h light/dark cycle was maintained, and tank conditions included: 12 L of dechlorinated tap water with artificial aeration, a short PVC pipe with a plastic platform on top for metamorphs, and a dark sheet covering half of the tank to provide cover from foot-traffic in the lab (Figure 4). Average water conditions were as follows (mean ± S.E.): temperature, 17.66 ± 0.02 °C; pH, 7.94 ± 0.01; dissolved oxygen, 8.89 ± 0.01 mg/L. Tadpoles were fed tadpole pellets from Carolina Biological Supply (#146500) approximately every two days. An acclimation period of one week was allowed before the static-renewal dosing experiment began. I monitored the development, behavior, and morphology of tadpoles until the experimental endpoint (day 185). Tadpole development (weight and length over time) was measured every 10-15 days by randomly selecting five individuals from each tank. To randomly choose tadpoles, I selected five tadpoles to measure by choosing five numbers from 1 to 15 and measuring the tadpoles that were netted corresponding to those numbers. Morphological characteristics of developing tadpoles are described in detail and separated into stages of development (25-46) by Gosner (1960). I subdivided the number of tadpoles that remained at the experimental endpoint into three groups based on Gosner stages of development (Gosner 1960). These were stages 25-35, 36-41, and 42-44, which roughly corresponded to the physical characteristics “no legs,” “hind legs with tail,”

and “four legs with tail,” respectively. Frogs were euthanized by submersion in a buffered solution of MS-222 at 3 g/liter for 10 minutes or until movement ceased. Chemical euthanasia was followed by decapitation and pithing, in accordance to American Society of Ichthyologists and Herpetologists (ASIH) protocol. Frogs were euthanized near Gosner (1960) stage 46 (full resorption of tail and corner of mouth beyond eye). Immediately following euthanization, I dissected out the livers, wrapped them in foil, and stored in a freezer (~-20° C) for further analysis. At the same time, I created two slides per individual of peripheral blood smears via heart puncture.

Static renewal testing

Static-renewal testing began on 01 April 2016. Aquaria were cleaned and re-dosed every 10-15 days throughout the experiment. The static-renewal method was used in similar experiments with success (e.g., Brunelli et al. 2009, Mann and Bidwell 2001, Relyea 2004). Doses were administered blindly, with five aquaria each having a low, medium, and high concentration level of imidacloprid (250 ng/L, 8.5 mg/L, and 85 mg/L, respectively), and the three remaining aquaria served as the control group with no imidacloprid added. Only three control tanks were used due to space constraints (Figure 5).

Chemicals and reagents

I obtained imidacloprid from a commercial formula, Imidapro® 2SC (Agrisel™) (Figure 2). The Giemsa solution for staining cells was obtained from Sigma-Aldrich, all other chemicals and reagents were obtained from VWR International.

Measuring imidacloprid concentration

Methods for liver preparation were modified from Mohan et al. (2010). Livers were excised, weighed (Thermo Fisher Scientific balance accurate to 0.1 mg) and blended with the extraction solvent (100% acetone) by mortar-pestle. I transferred the mixture into a microcentrifuge tube and mixed further in an ultrasound bath before undergoing centrifugation at 7400 rpm for 5 minutes. The above methods for mixing livers into solution differed from Mohan et al. (2010) in that these researchers used an orbital shaker for two hours. The supernatant was transferred into a round-bottom flask and concentrated under vacuum using a rotary flash evaporator. I re-dissolved residues in as low a volume of acetonitrile as possible (approximately 100 to 600 µl) and loaded them onto SPE cartridges (Lichrolut RP-18; pre-washed with acetone), and eluted with acetonitrile. Samples were analyzed on HPLC (Varian 920-LC) equipped with ultraviolet (UV) detector. The column was Agilent Zorbax SB-C18 (3.5 µm, 3.0 x 150 mm) with an injection volume of 20 µL and acetonitrile:water (25:75) as mobile phase at a flow rate of 0.9 ml/min for 10 minutes per sample. The UV/Vis detector was set at 254 nm.

Recording micronuclei and other nuclear abnormalities.

Micronuclei and cytoxicity assays followed procedures used by Ruiz de Arcaute et al. (2014), Vera-Candioti et al. (2010), and Fenech (2000). I created peripheral blood smears following euthanasia of each animal onto clean, marked slides, air dried, and fixed with 100% cold methanol (4° C) for 20 min, and stained with 5% Giemsa solution for 15 min. I scored 1000 cells per frog under 1000x magnification on gridded slides. The MN criteria followed previous examination criteria from Vera-Candioti et al. (2010), that is, MN must possess (1) diameter smaller than 1/3 of the main nuclei, (2) non-refractability, (3) staining intensity similar to or

lighter than the main nuclei, (4) no overlapping, connection, or link, with the main nuclei, and (5) an MN boundary distinguishable from the main nuclei.

Other nuclear abnormalities were scored in the same manner and followed criteria previously reported (Ruiz de Arcaute et al. 2014, Strunjak-Perovic et al. 2009, and Cavas and Ergene-Gozukara 2003). Specifically, binucleated cells contained two nuclei, blebbed nuclei were cells with one nucleus presenting a relatively small evagination of the nuclear membrane, lobed nuclei were larger evaginations than blebbed, which could have several lobes, and notched nuclei possessed vacuoles and substantial depth into a nucleus, without containing nuclear material (Figure 3). The cytotoxicity assay was accomplished using the same slides as above, and determined by counting the total number of erythrocytes and erythroblasts out of 1000 cells and expressed as a frequency.

Statistical Analysis

I used a one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test to test for differences in the number of frogs that reached metamorphosis among treatments, and the weights and lengths of frogs that reached metamorphosis across all treatments. The number of mortalities and the number of tadpoles remaining at the experimental endpoint were analyzed using Kruskal-Wallis test, because the data did not meet the equal variances test (Levene's F-test) for one-way ANOVA. A directional measures (ordinal by ordinal) Somers' d test was used to analyze the developmental stages of tadpoles still alive at the experimental endpoint. The independent and dependent variables were concentration level and development stage, respectively. Both weights and lengths over time were analyzed by linear mixed effects model (LMM) with 'treatment' and 'days' set as fixed effects with 'days' as a covariate, and 'tanks' assigned as random effects to check for any block effects. Weights were log-transformed to

meet the assumptions of the analysis and the high-level treatment groups were removed from developmental analysis for LMM because all tadpoles were deceased by day 23. All physical measures of aquaria conditions including temperature (^{o}C), pH, and dissolved oxygen (mg/L) were analyzed using a one-way ANOVA with Tukey's HSD post-hoc test.

The weights of livers extracted from the frogs that reached metamorphosis were analyzed using one-way analysis of covariance (ANCOVA) with Bonferroni adjustment for multiple comparisons. The covariate for this test was days until metamorphosis, to adjust for any size differences as a result of additional development time.

I tested the micronuclei assay and other nuclear abnormality data using one-way ANOVA with Tukey's HSD post-hoc or Kruskal-Wallis test. The number of notched nuclei and the category of combined 'other abnormalities' (blebbed + notched + lobed), were log transformed to meet the assumptions for ANOVA. All parametric and non-parametric ANOVAs/ANCOVAs were carried out in SPSS Statistical Program for the Social Sciences, version 24. Linear mixed models were carried out in the program RStudio (version 3.3.3) for the developmental data of tadpole weights and lengths.

RESULTS

Lethal Effects

The low, medium, and high concentration groups each started with 75 tadpoles and exhibited 33.3, 28.0 and 100.0% mortalities, respectively. The control group started with 45 tadpoles and exhibited 42.2% mortality (Table 1; Figure 6). Significantly more mortalities occurred in the high concentration group (Kruskal-Wallis, df=3, p=0.008) than all other groups. The mean proportion of tadpoles that reached metamorphosis differed among treatments (Figure

7; one-way ANOVA, $F_{3,14}$=18.207, p<0.001). The high treatment group had significantly fewer tadpoles reach metamorphosis compared with all other groups. The proportion of tadpoles alive at the experimental endpoint (day 185), was significantly lower in the high-level treatment group (Kruskal-Wallis, df=3, p=0.010) than in other groups (Table 2).

Sub-lethal Effects

For all sub-lethal, developmental effects, the high treatment group was absent from analysis because all tadpoles were dead by day 23. As the imidacloprid treatment concentration increased, the development rate of tadpoles was slowed (Somers' d, dev. dependent statistic = -0.299, p=0.009). No significant differences in weights at metamorphosis were observed (Figure 8; $F_{2,75}$=2.849, p=0.064). However, significant differences in lengths at metamorphosis were observed (Figure 8; $F_{2,75}$=10.304, p<0.001). Shorter lengths were observed in the low and medium treatment groups compared with the control group. Developmental weights and lengths were analyzed with a linear mixed effects model (Figure 9; Figure 10). No significant differences existed between treatments in weights over sampling days ($F_{2,204}$=2.16, p=0.117), but length was significantly different between the control and medium treatment level groups ($F_{2,10}$=5.33, p=0.026). The mean tank conditions (pH, temperature, and dissolved oxygen) were not significantly different between treatment and control groups (Table 3).

There were no significant differences in the liver weights between treatment groups when development days were accounted for as a covariate (Figure 11; $F_{2,72}$=2.906, p=0.061). The covariate 'days' did not appear to interact with the group. The HPLC methods used in this study resulted in an imidacloprid retention time of approximately 3.07 min. using a 99.9% pure imidacloprid standard (Figure 12). None of the chromatograms revealed any indication that

imidacloprid was present at detectable levels in the frogs' livers, as evidenced by lack of a peak at the retention time of imidacloprid (3.07 min) (Figure 12).

Blood smears from a total of 70 individuals analyzed for micronuclei and other nuclear abnormalities revealed no significant differences in the number of MN produced by this pesticide across all groups (Table 4; Kruskal-Wallis, p=0.071). However, the number of binucleated cells in the control, low, and medium level treatment groups were significantly different from each other, with frequency increasing with concentration level (Table 4; Kruskal-Wallis, p=0.002). I observed significantly more notched nuclei in the medium level treatment group compared with the low and control groups (Table 4; $F_{2,125}$=8.366, p<0.001). The number of blebbed or lobed nuclei were not significantly different between all groups (Table 4; blebbed: $F_{2,125}$=2.525, p=0.084; lobed: p=0.139). When the 'notched,' 'blebbed,' and 'lobed' categories were combined into a single 'other nuclear abnormalities' category, the numbers produced were significantly higher in the medium-level treatment group compared with the low and control groups (Figure 13; $F_{2,125}$=5.155, p=0.007). The number of erythrocytes in 1000 cells was not significantly different between any group (Figure 14; Kruskal-Wallis, p=0.315). These genotoxicity assays did not include the high treatment group because all tadpoles were dead by day 23 of the experiment.

Tadpole behavior and morphology was monitored daily throughout the experiment. Any oddities were noted and summarized at the end of the experiment (Table 5). The most commonly observed behaviors included slow-to-no response to a disturbance (e.g. shifting the tank or skimming out old food) and appearing disoriented in the tank. The latter was most often observed in the tadpoles exposed to 85 mg/L imidacloprid and was characterized by tadpoles swimming or floating on their sides.

DISCUSSION

Exposure to 85 mg/L imidacloprid caused 100% mortality by day 23 of the experiment (Figure 6). Although this concentration is higher than what is likely to occur in the environment, it is the lowest LC_{50} value reported for frogs exposed to imidacloprid. The previous lowest LC_{50} value comes from a study in Argentina on the Montevideo tree frog (*Hypsiboas pulchellus*) (Ruiz de Arcaute et al. 2014). My results suggest that the LC_{50} value for *L. pipiens* exposed to imidacloprid is lower than that for *H. pulchellus*. Although the design of my study was inadequate to calculate a true LC_{50} value, I was able to estimate a value ranging from ~27 to 40 mg/L (Figure 15). Future research should follow an EPA protocol design for calculating an accurate LC_{50} value for this species. My results offer evidence for a lower LC_{50} value for imidacloprid exposed *L. pipiens*. This range may be more informative for future studies using North American ranid frogs than values such as the one for *H. pulchellus*.

Mortalities occurred in all experimental groups, primarily in the early stages of development. This is typical in wild populations of *L. pipiens* (Calef 1973). However, lab-reared tadpoles were provided all the food needed for survival with no threat of predation, contrary to wild populations. Thus, a similar level of mortality is slightly unexpected. Tadpoles that survived to the experimental endpoint, but did not reach metamorphosis, were placed into three categories based on their level of development (Gosner stages 25-35, 36-41, 42-44). On average, tadpoles in the control group developed faster than those exposed to 250 ng/L imidacloprid at the experimental endpoint. The low treatment group was also more developed than the tadpoles exposed to 8.5 mg/L imidacloprid. Exposure to higher concentrations of imidacloprid resulted in slower developmental rates, up to a point where primarily mortalities occurred. Slower development could affect the survival and fitness of wild populations of *L. pipiens*. Tadpoles

found in ephemeral ponds may only have a narrow window to develop into frogs before standing water is no longer available and a longer development time may expose them to a longer period of predation risk (Alvarez and Nicieza 2002). In such a scenario, delayed development could result in higher than normal mortality rates. Delayed development could also affect the mating success of individuals that breed early in the season, especially when accounting for reduced size at metamorphosis. In addition to slower development, the size of frogs that reached metamorphosis was reduced with increasing exposure concentrations (Figure 8). Smaller frogs might suffer from lower fitness and reduced ability to escape predation.

One concern of my experiment involved the rearing temperature. Although temperatures between experimental groups were not significantly different from each other, they were below the preferred range for *L. pipiens*. In addition to the imidacloprid treatments, this could influence the rate of development. Hatching to metamorphosis in *L. pipiens* typically ranges from 70 to 110 days, with the longer periods occurring in the colder, northern areas of the species range. Previous studies demonstrated, as expected, that populations of *L. pipiens* located in southern Canada tolerated colder temperatures better than populations from northern Mexico (Goldstein 2007). Goldstein (2007) performed an experiment in which relict leopard frog tadpoles (*L. onca*) were acclimated to temperatures ranging from 15 to 35 ^{o}C and allowed to develop at acclimation temperatures. The 15 ^{o}C group took longer to develop than any other; however, no significant differences existed in survival. Because the temperatures in my study were around 17.7 ^{o}C on average, and *L. pipiens* is very closely related to *L. onca*, longer development may have benn influenced in part by temperature, but mortality was not temperature dependent. It is worth noting that Goldstein (2007) used a species native to Southwestern United States, and these results may be irrelevant for a population local to Michigan's Upper Peninsula, which deals with

much colder water temperatures during development. Further studies are needed to ensure that temperature effects were not an important influence in tadpole survival.

Liver weights did not appear to be influenced by imidacloprid exposure, while controlling for body weight (Figure 11). In tadpoles, the liver is involved with the hormonal cascades that drive metamorphosis as well as creating blood cells, immune function, and metabolic processes (Hartigan et al. 2012). No tadpoles exposed to 85 mg/L imidacloprid reached metamorphosis and all were too small at time of death for liver extraction, thus were excluded from HPLC analysis. In all tadpole treatments, imidacloprid was not detected or occurred below detectable levels (< 2 ng/mL; Figure 12). The nature of the static-renewal experiment may have allowed time for imidacloprid to be metabolized or broken down within the tadpoles before I measured residues within the liver.

Micronuclei presence in peripheral blood erythrocytes is a reliable measure for genotoxicity in amphibians produced by pesticides (Vera Candioti et al. 2009). Micronuclei formation occurs when dividing cells do not distribute genetic material equally between the two daughter cells, because of chemical or radiation damage (National Toxicology Program, 2017). Increasing concentrations of imidacloprid resulted in increasing genotoxic effects, at varying significances, per 1000 cells (Table 4). Every category showed a trend of increasing nuclear abnormalities quantified (MN, binucleated, blebbed, lobed, notched) with increasing imidacloprid concentrations. Imidacloprid seems capable of inducing chromosomal damage. Chemicals capable of inducing chromosomal damage in somatic cells are potentially carcinogenic, or cancer causing. Further, chromosomal damage in germ cells could result in reduced reproductive output or even birth defects in tadpoles (National Toxicology Program, 2017).

Abnormal tadpole behavior, such as odd swimming behaviors could impair predator avoidance and increase energy use from constantly restoring their up-right position. Two individuals (one exposed to 250 ng/L and one exposed to 8.5 mg/L) displayed an odd deformity in the tail as a 'notch' near the base (Figure 17). Both of these individuals swam in an awkward manner and the tail interfered with swimming once hind limbs developed. The tails appeared to reabsorb normally near the end stages of development and both reached metamorphosis successfully. Only two individuals in the experiment exhibited this morphological deformity, and there is no evidence to suggest exposure to imidacloprid was the cause. In the wild, individuals with this deformity might not reach adulthood, as they would make an easy prey item.

The imidacloprid-based formulation used in this study contained 21.4% imidacloprid and the remainder was listed as 'other ingredients'. These ingredients are not typically made available to the public, and often contain surfactants, solvents, and adjuvants, some of which may have their own toxic effects on organisms (Vera Candioti et al. 2010). Experiments using commercially prepared formulations are relevant because these are also the formulations used in agricultural settings. Ideally, imidacloprid should also be tested in a pure form to understand its true effects to an organism. There is likely some variety of inactive ingredients used across multiple manufacturers, so every formulation could potentially have different effects in organisms.

Pesticides, by definition, have inherent toxicity (Ecobichon 1993), which is noticeable in target species but can elude detection in non-target species. Due to their mode of action, neonicotinoids should be less toxic to non-target organisms, such as mammals, birds, amphibians, and fish. However, increasing numbers of studies, focusing on long-term sub-lethal

effects, reveal that neonicotinoids may pose just as much of a threat to some populations as broad-spectrum pesticides (Goulson 2013; Kohler and Triebskorn 2013). Single-pesticide exposure in the environment is rare-- it is more common that multiple pesticides exist and interact in the environment at a time. The toxicity of neonicotinoids and their metabolites increase when used in combination with other pesticides (Simon-Delso et al. 2015). As a result, the sub-lethal effects caused by laboratory exposure to environmentally realistic concentrations of a single pesticide may become amplified in the environment. Although studies investigating the effects of multiple pesticide exposure scenarios at environmentally realistic concentrations are not common, they should be considered for management and conservation decisions. Long-term studies are necessary to reveal some sub-lethal effects and generational effects; however, such studies cannot keep up with the rate of newly introduced pesticides into the environment. In addition to potential interactions with surfactants, solvents, metabolite compounds, and other pesticides present in the environment, numerous new neonicotinoids are in development or approved for use in China. All of these new compounds are *cis*-neonicotinoids at the nitro or cyano group, which are known to cause very different toxic effects compared to the *trans*-neonicotinoids (Simon-Delso et al. 2015).

Detrimental effects to developing *L. pipiens* tadpoles at moderate concentrations of imidacloprid exposure are evident from this study. Imidacloprid concentrations of 85 and 8.5 mg/L are not likely found in the environment, but the effects at these levels reveal the potential effects caused by imidacloprid exposure. More importantly, sub-lethal effects appear to occur at the cellular level when exposed to imidacloprid concentrations of only 250 ng/L. This concentration level was measured in surface water wetland areas of southern Canada, near agricultural zones. More recently, surface water samples from agricultural regions of California

have exceeded imidacloprid concentrations of 1.05 µg/L, more than four-fold the lowest concentration used in this study (Sadaria et al. 2016). The US EPA reported the Aquatic Life Benchmark for imidacloprid at 1.05 ug/L, based upon the most sensitive aquatic toxicity data for multiple taxa (https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/aquatic-life-benchmarks-pesticide-registration). Further research should investigate if similar sub-lethal effects occur in wild populations of anurans during development in these areas where imidacloprid exposure is common. Well-informed decisions regarding the proper timing, use, and amounts of imidacloprid, and other pesticides, should incorporate studies such as this one, that focus on overlooked, but susceptible, non-target organisms.

TABLES AND FIGURES

Table 1: Survivorship summary including the starting number of *L. pipiens* tadpoles raised under different concentrations of imidacloprid, those that that reached metamorphosis, those that died, and those that were still alive at the experimental endpoint (day 185). *Significant differences in metamorphs, mortalities, and tadpoles alive at experimental endpoint were observed between the high-level concentration group and all others.

Concentration Level	No. of tadpoles	No. of metamorphs	Metamorphs (%)	No. of mortalities	Mortalities (%)	No. of tadpoles alive at day 185	Tadpoles alive at day 185 (%)
Control	45	16	35.60	19	42.20	10	22.20
Low (250 ng/L)	75	32	42.70	25	33.30	18	24.00
Medium (8.5 mg/L)	75	30	40.00	21	28.00	24	32.00
High (85 mg/L)	75	0*	0.00*	0*	100.00*	0*	0.00*

Table 2: Stage of development of all *L. pipiens* tadpoles still alive at the experimental endpoint (day 185). Stages were determined based on physical development features, described in Gosner (1960). As treatment concentration increased, the number of tadpoles at higher stages of development decreased.

Concentration Level	Tadpoles alive at experimental endpoint (%)	No. of tadpoles at particular stages of development.		
		Stage 25-35 days (no legs)	Stage 36-41 days (hind legs)	Stage 42-44 days (four legs)
Control	22.2	0	7	3
Low (250 ng/L)	24.0	5	9	4
Medium (8.5 mg/L)	32.0	13	6	5

Table 3: Physical conditions (temperature, pH, dissolved oxygen) of experimental tanks that housed L. pipiens, under three different levels of imidacloprid shown as mean ± SE. Note: Measurements for high concentration-level tanks were only recorded until all tadpoles were dead (within 23 days).

Concentration Level	Avg. Temp (°C)	Avg. pH	Avg. D.O. (mg/L)
Control	17.76 ± 0.05	7.92 ± 0.03	8.93 ± 0.03
Low (250 ng/L)	17.71 ± 0.04	7.97 ± 0.02	8.89 ± 0.02
Medium (8.5 mg/L)	17.69 ± 0.05	7.91 ± 0.02	8.86 ± 0.02
High (85 mg/L)	16.39 ± 0.15	7.93 ± 0.02	8.93 ± 0.03

Table 4: Micronuclei assay and other nuclear abnormalities from blood smears obtained from metamorphosed *L. pipiens* tadpoles raised in tanks with different levels of imidacloprid. . Two blood smears were obtained from each frog and 1000 cells per individual were counted. Abnormalities are reported as mean ± S.E. *Significant differences in binucleated cells and 'notched' abnormalities.

Concentration	No. of Frogs Sampled	No. of Cells Analyzed	Avg. no. of MN Cells	Avg. no. of BN Cells	Avg. no. of other nuclear abnormalities		
					Blebbed	Notched	Lobed
Control	15	25,000	0.48 ± 0.16	0.20 ± 0.10*	1.36 ± 0.32	1.44 ± 0.39*	0.52 ± 0.15
Low (250 ng/L)	27	51,000	1.16 ± 0.25	0.59 ± 0.14*	1.86 ± 0.31	2.24 ± 0.30*	0.63 ± 0.12
Medium (8.5 mg/L)	28	52,000	1.92 ± 0.44	1.23 ± 0.22*	2.51 ± 0.37	3.73 ± 0.43	1.15 ± 0.20

Table 5: Anecdotal observations of behavior and physical abnormalities of *L. pipiens* for each of the imidacloprid treatment groups. Disturbances were implemented by shifting the tanks. Disorientation was observed when a tadpole turned sideways while floating or swimming; most individuals would orient themselves upright after a short period of time. Only two physical deformities were observed in the low and medium treatment groups.

Treatment	Observations
Control	Three individuals out of 45 exhibited slow reaction or no reaction to netting or disturbance. No apparent physical deformities were observed in this group.
Low (250 ng/L)	Eight individuals exhibited slow or no reaction to netting or disturbance. Eleven individuals exhibited disorientation while swimming or floating. These individuals turned onto their sides and would right themselves. One individual developed a deformity with its tail (see Figure 16).
Medium (8.5 mg/L)	Five individuals exhibited slow or no reaction to netting or disturbance. Six individuals exhibited disorientation while swimming or floating. One individual developed a tail deformity in the same way that another did from the low treatment group (see Figure 16).
High (85 mg/L)	At least half of the individuals in these treatment tanks exhibited disorientation and turning on their sides. Many also exhibited slow or no reaction to netting or disturbance. No apparent physical deformities were observed, however, all tadpoles were dead by day 23 of the experiment and were very small.

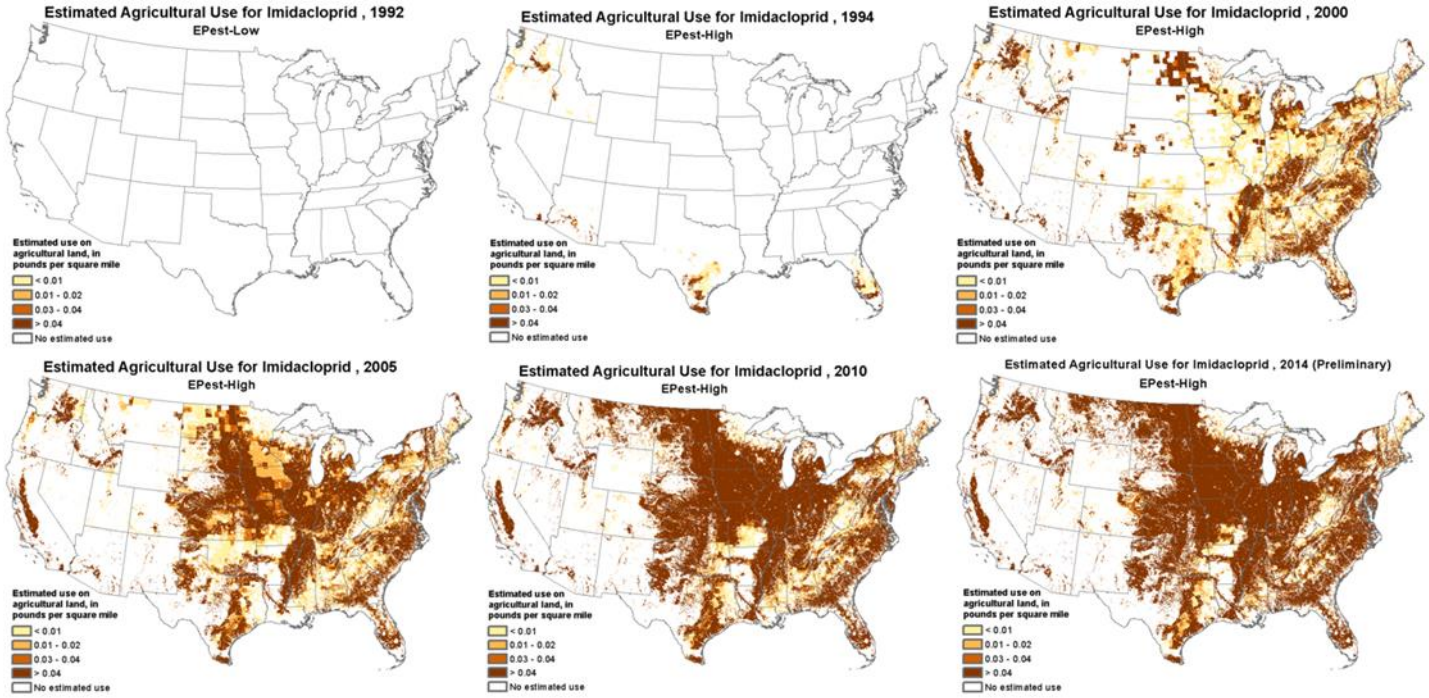

Figure 1: Map of United States depicting the estimated use of imidacloprid (in pounds per square mile) on agricultural lands beginning in 1992 (top left) to 2014 (bottom right). These maps were obtained from the United States Geological Survey as part of the National Water-Quality Assessment Program. (https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=1992&map=IMIDACLOPRID&hilo=L).

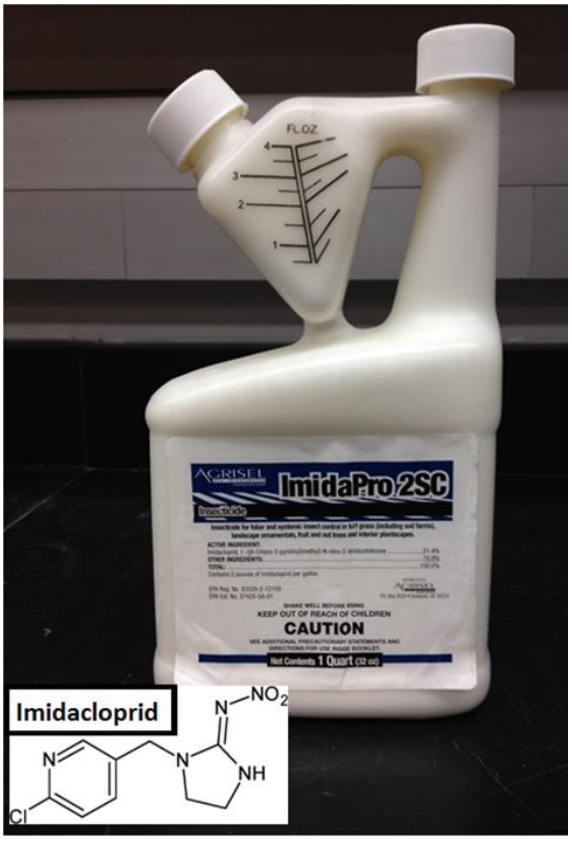

Figure 2: Agrisel ImidaPro 2SC® served as the imidacloprid-based pesticide (active ingredient 21.4%) used in this study. Chemical structure of imidacloprid displayed in lower-left of image.

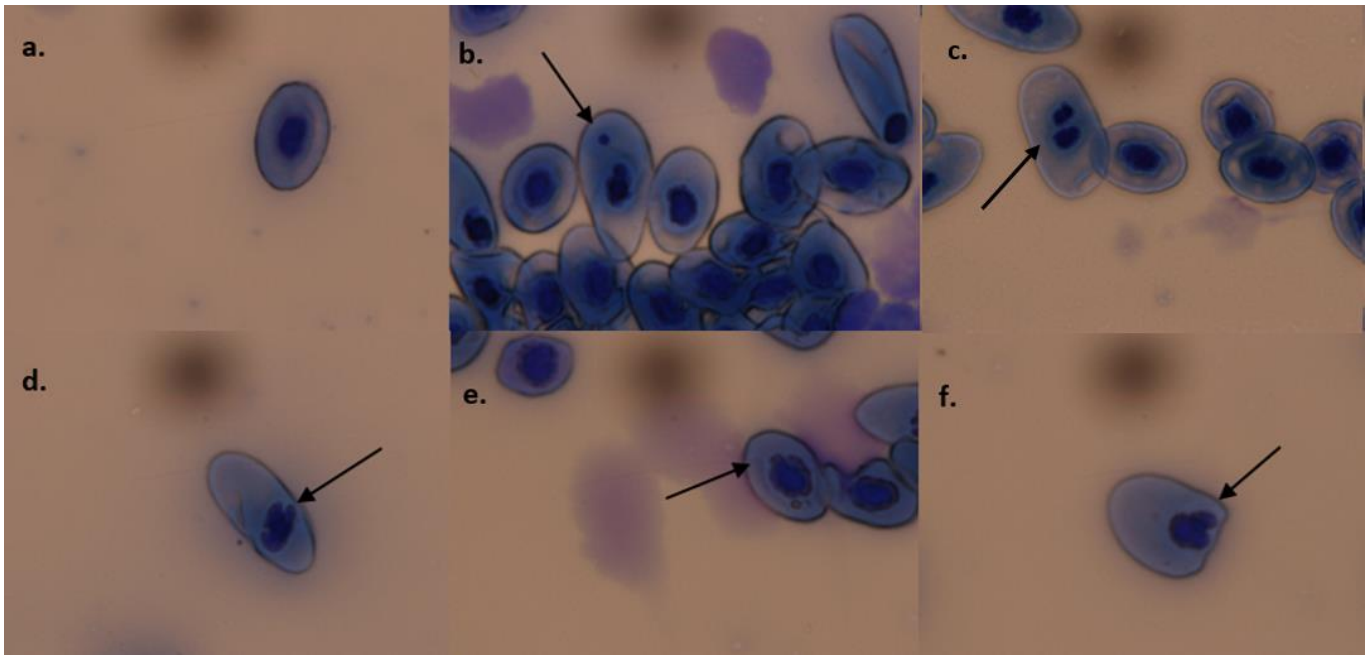

Figure 3: Examples of *L. pipiens* erythrocytes from tadpoles exposed to three levels of imidacloprid observed under 1000x magnification. a) Normal erythrocyte; b) Micronucleus; c) Binucleated cell; d) Notched nucleus; e) Blebbed nucleus; f) Lobed nucleus.

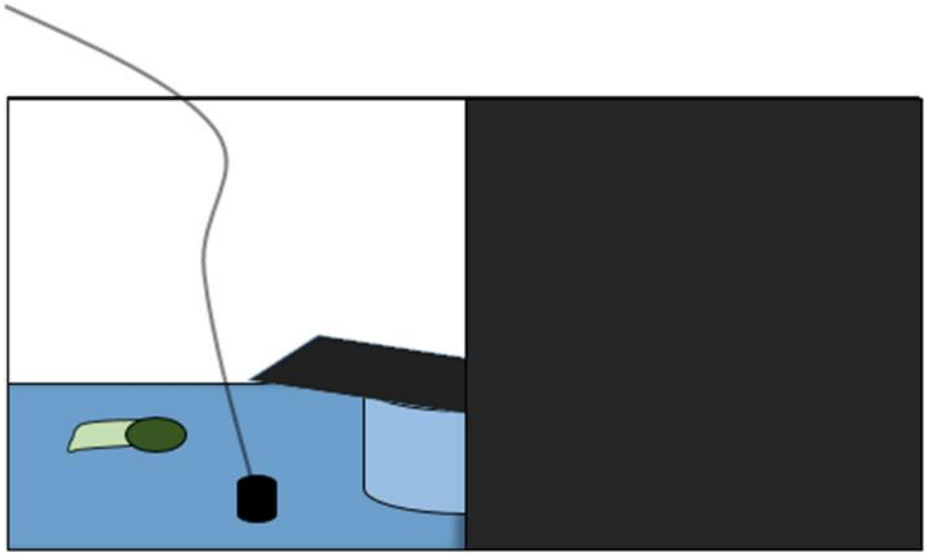

Figure 4: Side-view of experimental tank design used to test the effect of imidacloprid on tadpoles. Every tank had a covered and uncovered half, pvc pipe with a platform resting at water level near the center for leopard frog metamorphs to climb onto, air tube with an air stone attached, and 12 L of dechlorinated water.

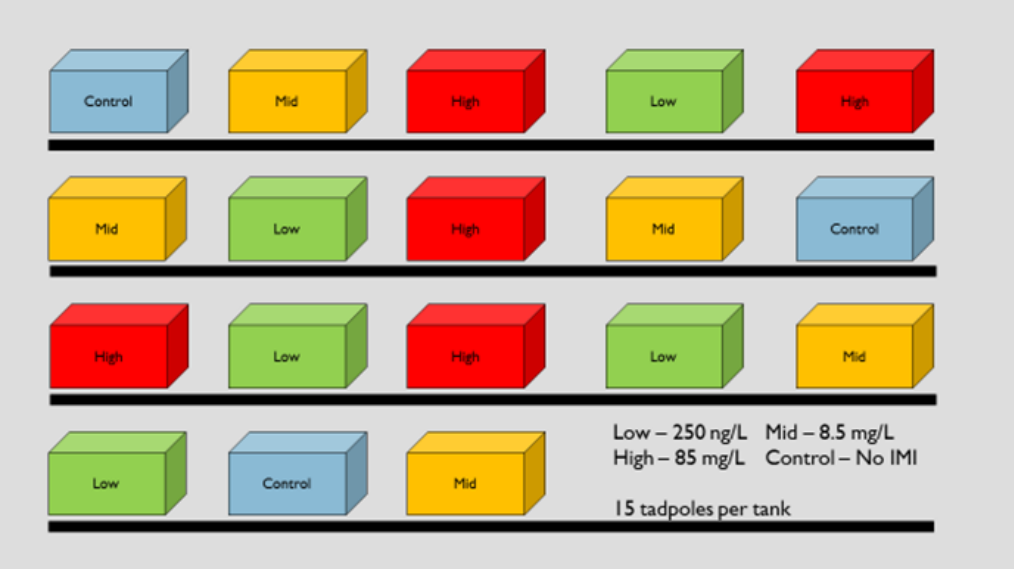

Figure 5: Experimental design displaying all experimental tanks with their imidacloprid treatment concentrations (lower right) and locations on shelves in the aquatics facility. Treatments were randomly assigned and applied blindly so that the experimenter did not know which tanks received treatments.

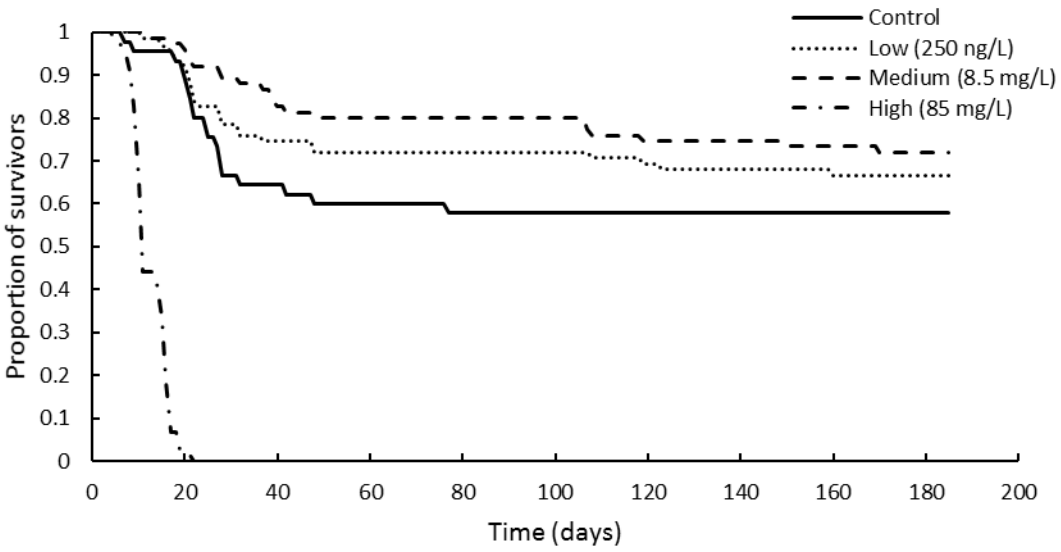

Figure 6: Survivorship of *L. pipiens* tadpoles through duration of experiment in which tadpoles were exposed to three concentration levels of imidacloprid. Treatment groups started with 75 individuals and the control group started with 45 individuals.

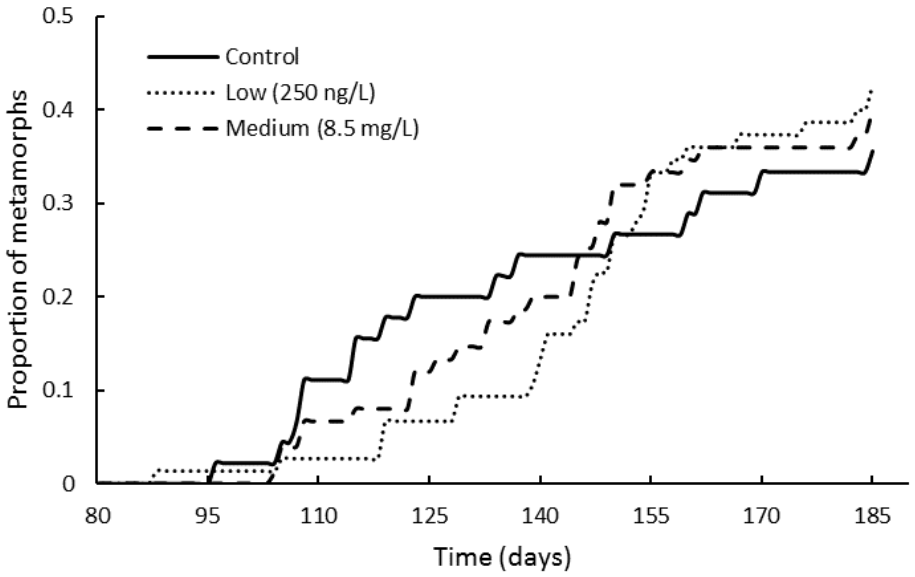

Figure 7: Cumulative metamorph curve of *L. pipiens* tadpoles through duration experiment in which tadpoles were exposed to three concentration levels of imidacloprid. Note: the high treatment-level group (85 mg/L) had no metamorphs.

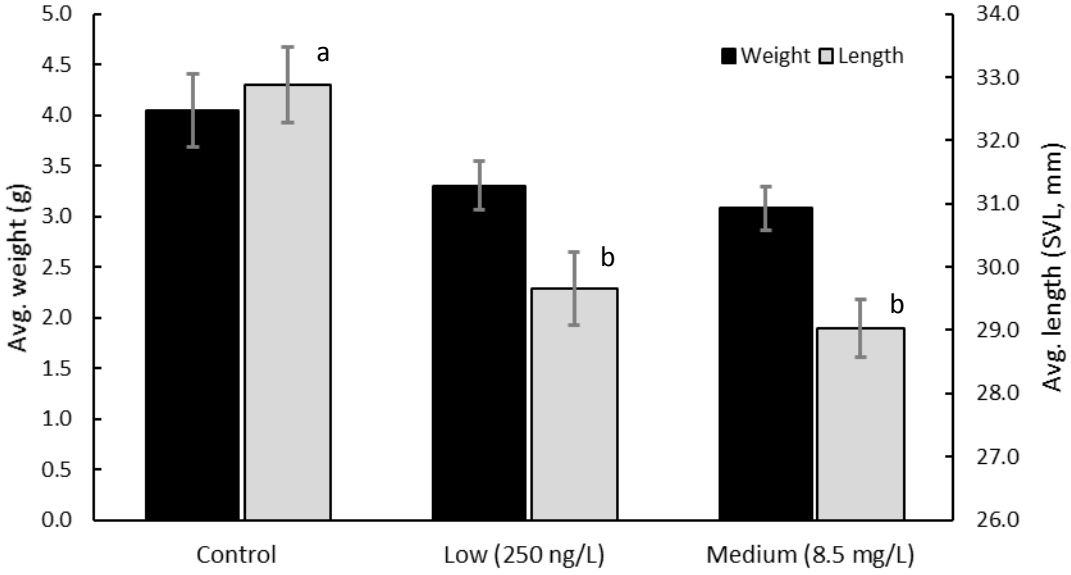

Figure 8: Average weights and lengths of *L. pipiens* at metamorphosis in tadpoles exposed to different levels of imidacloprid. Weights of treatment and control groups were not significantly different ($p=0.064$). [a,b] On average, imidacloprid-exposed frogs were significantly shorter than the control group at metamorphosis ($p<0.001$). Bars surrounding averages are standard errors.

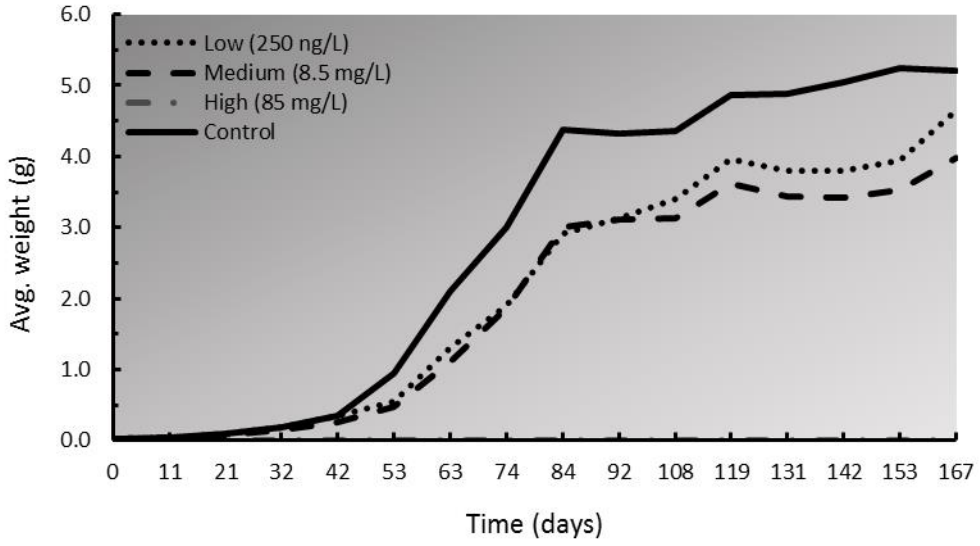

Figure 9: Average weights of *L. pipiens* tadpoles exposed to different levels of imidacloprid throughout the experiment. Weights of five randomly selected tadpoles per tank were recorded on static-renewal days. No significant differences existed in developmental weights over time between treatment groups (p=0.117). Note: the high-level treatment group was ignored for statistical analysis because of high mortality..

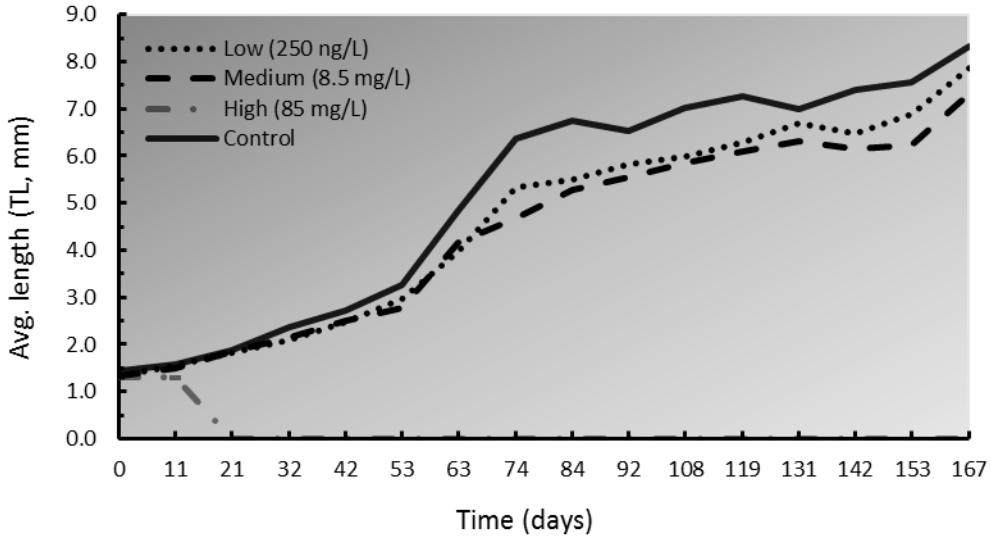

Figure 10: Average length of *L. pipiens* tadpoles over time, measured as total length (TL) in relation to level of imidacloprid. Lengths of five randomly selected tadpoles per tank were measured on static-renewal days. Significantly different developmental lengths existed between control and medium group tadpoles (p=0.026). The high-level treatment group was ignored for significance.

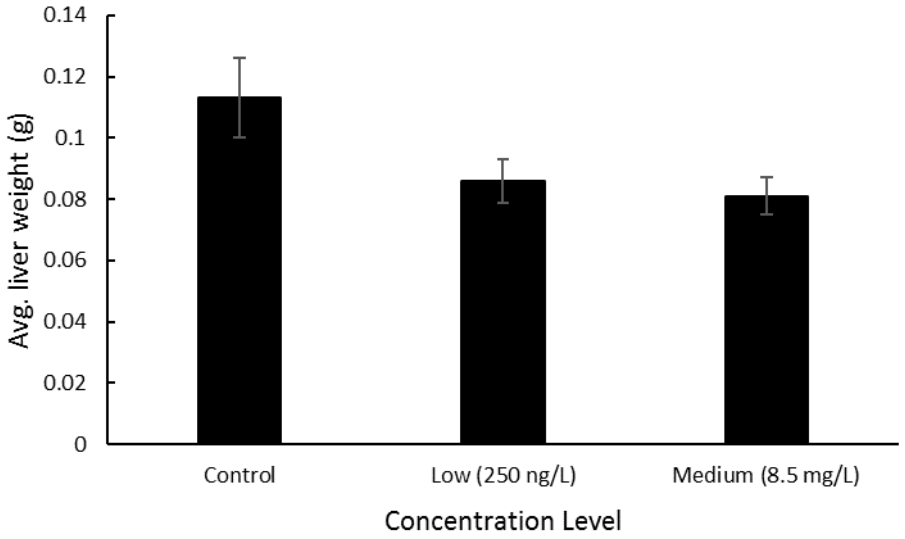

Figure 11: Average weight of livers extracted from metamorphosed *L. pipiens* tadpoles raised in tanks with different levels of imidacloprid. There were no significant difference across the groups (p=0.061). Bars surrounding averages are standard errors.

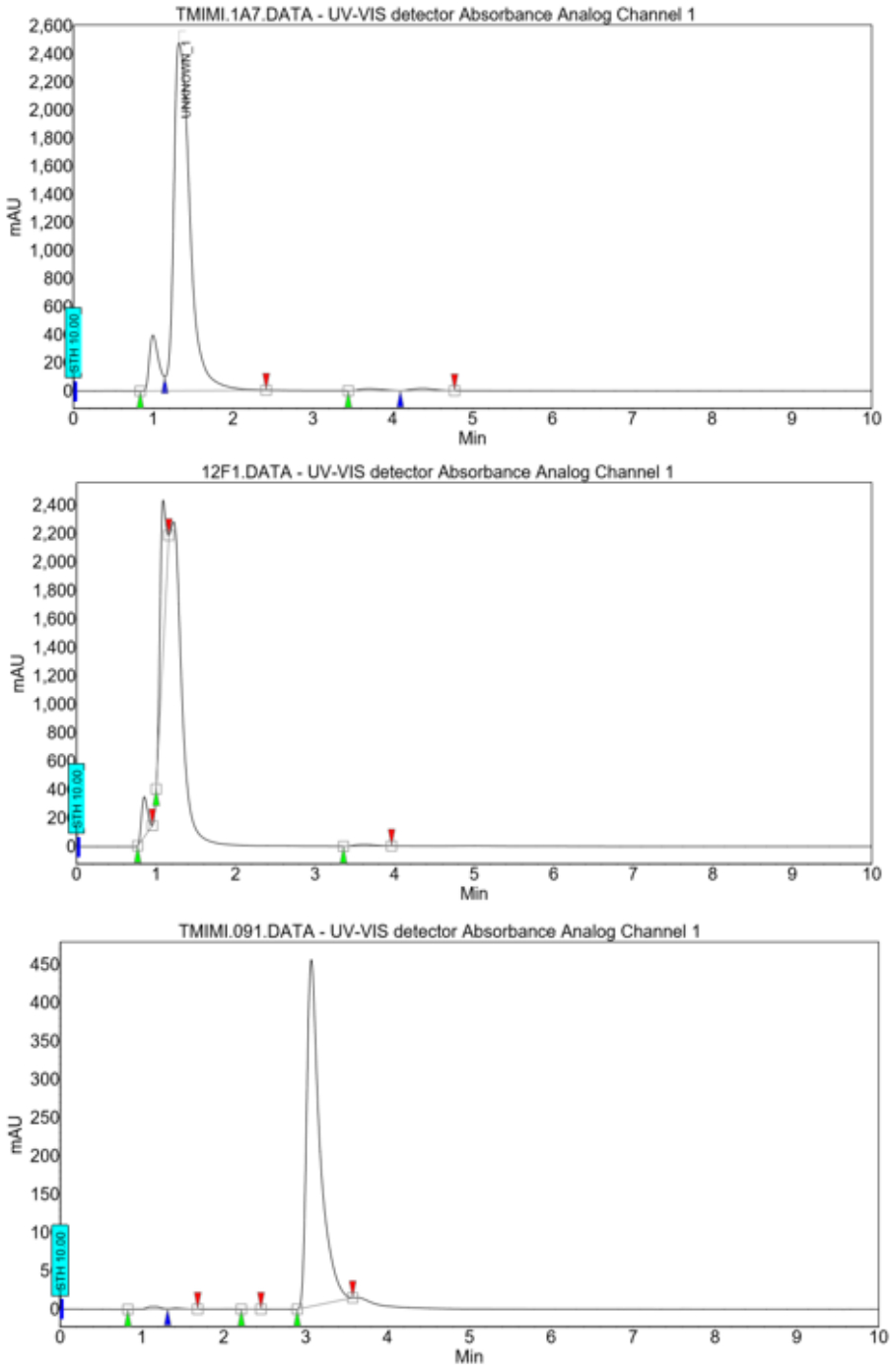

Figure 12: Chromatograms of (a) *L. pipiens* liver solution from control group; (b) *L. pipiens* liver solution from low-treatment group; (c) imidacloprid (PESTANAL®) 99.9% pure standard prepared at a concentration of 0.09 mg/L. Retention time of imidacloprid peak is 3.07 min. Absorbance was measured at 254 nm using Varian-LC UV-VIS detector. Visible peaks in (a) and (b) are dead time (t_M) and no imidacloprid peak was present in any treatment solutions.

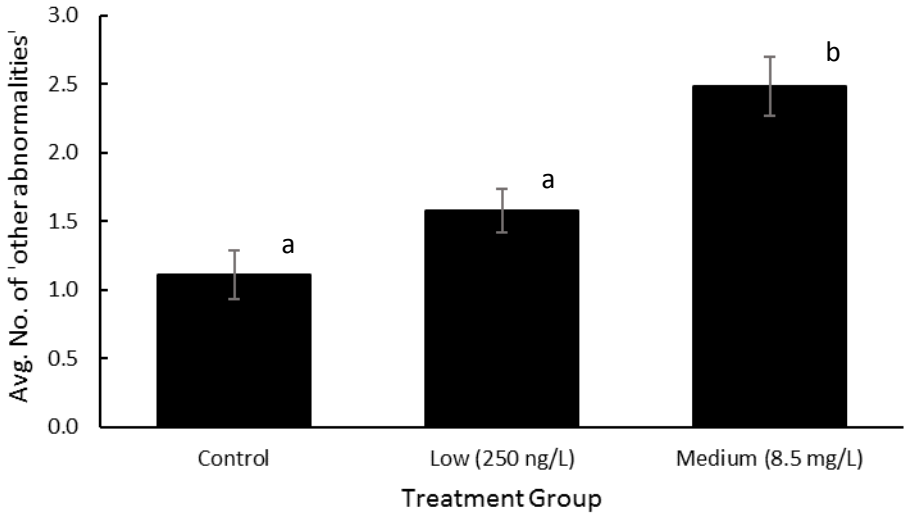

Figure 13: Average number of blebbed, notched, and lobed nuclear abnormalities (combined) from *L. pipiens* tadpoles raised in different levels of imidacloprid. These abnormalities were combined and analyzed as one category labeled 'other nuclear abnormalities.' Control and low treatment groups had significantly fewer abnormalities than the medium treatment group (p=0.007). Bars surrounding averages are standard error.

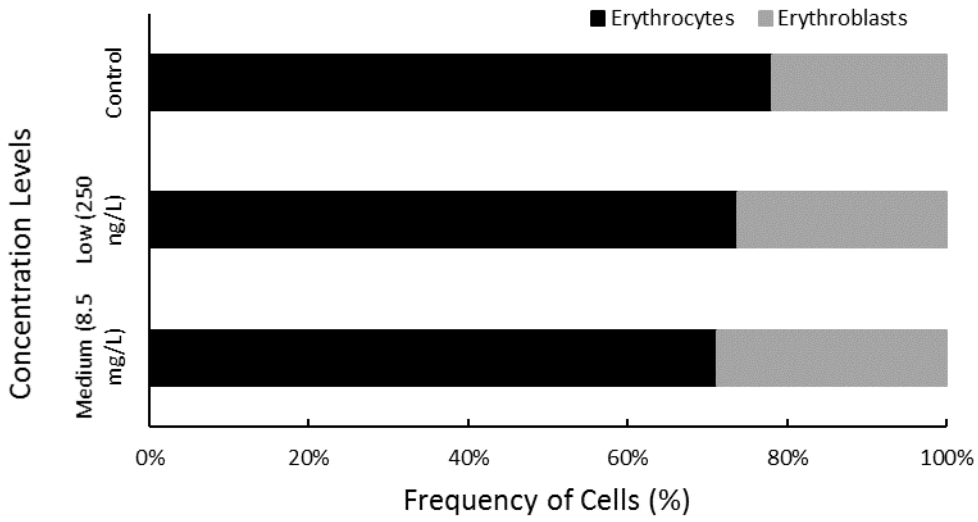

Figure 14: The number of erythrocytes and erythroblasts per 1000 cells were counted from MN assays from *L. pipiens* that were raised at different levels of imidacloprid. They are represented as a percentage (1000 cells = 100%), and no significant differences were observed between the groups.

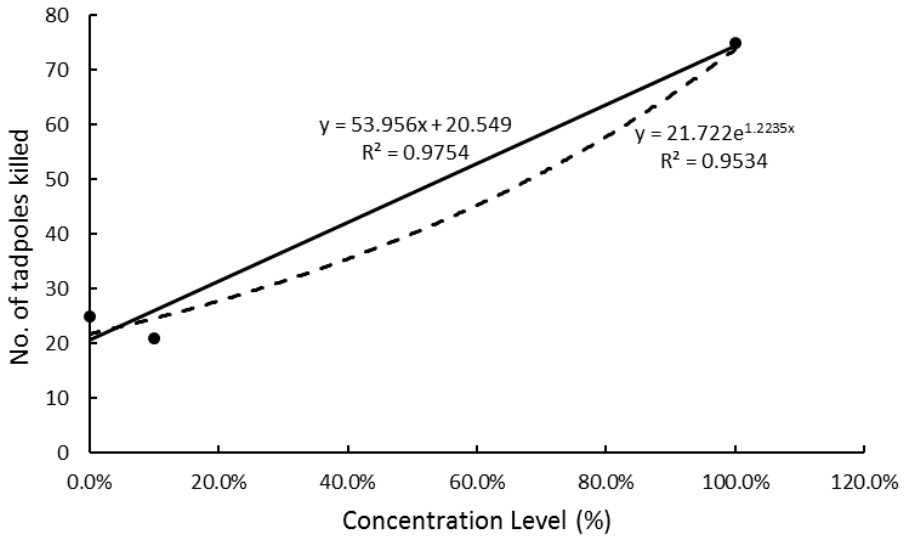

Figure 15: Number of *L. pipiens* tadpoles killed at each concentration level. Data were plotted with the high treatment level (85 mg/L) designated as 100% concentration level because all tadpoles died from this treatment. Both linear (solid) and exponential (dashed) equations, R^2 values, and trendlines are displayed and LC-50 values were calculated for both relationships. Calculated LC-50 values, linear = 27.26 mg/L and exponential = 39.56 mg/L, are estimates using the three data points available.

Figure 16: Images of an individual *L. pipiens* tadpole during development with a tail deformity. The above individual is from a low concentration-level treatment tank (250 ng/L). Another individual with the same deformity was monitored from a medium concentration-level treatment tank (8.5 mg/L).

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