UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

ASSESSING THE AFFECTS OF IMIDACLOPRID AND BINARY MIXTURES OF THE NEONICOTINOIDS IMIDACLOPRID AND THIAMETHOXAM ON FATHEAD MINNOW LARVAE (*PIMEPHALES PROMELAS*)

A Chapter Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master's of Science, Biology

Anya Jeninga

College of Science and Health

August, 2022

ASSESSING THE AFFECTS OF IMIDACLOPRID AND BINARY MIXTURES OF THE NEONICOTINOIDS IMIDACLOPRID AND THIAMETHOXAM IN FATHEAD MINNOW LARVAE (*PIMEPHALES PROMELAS*)

By Anya Jeninga

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master's of Science, Biology

The candidate has completed the oral defense of the thesis.

Tisha King-Heiden, Ph.D. — 9/1/2022
Thesis Committee Chairperson — Date
On behalf of the committee members named below:

Elisabeth Harrahy, Ph.D.

David Schumann, Ph. D.

Cord Brundage, Ph. D

Thesis accepted

Meredith Thomsen, Ph.D. — 9/25/2022
Dean of Graduate Studies — Date

ABSTRACT

Jeninga, A.J. Assessing the effects of imidacloprid and binary mixtures of the neonicotinoids imidacloprid in fathead minnow larvae (*Pimephales promelas*). MS in Biology, August 2022, 77pp. (T. King-Heiden)

Neonicotinoid insecticides were designed to be selective for insect nicotinic acetylcholine receptors (nAChRs), however recent studies indicate they may cause subtle toxicity in fish. Imidacloprid (IM) and thiamethoxam (TM) are neonicotinoids found together in Wisconsin surface waters, yet no studies have evaluated their combined toxicities to fish. The aim of this study was to better understand the potential for IM and IM:TM mixtures to activate the nAChR and to affect the development (hatching, length, survival) or behavior (embryonic motor activity, predator escape response) of fathead minnows (*Pimephales promelas*). Embryos and larvae were exposed to IM or to mixtures of IM:TM. Only survival was significantly affected. Chronic exposure to 0.2 µg IM/L and 1:1 0.02 and 200 µg IM/L:µg TM/L resulted in reduced survival. Survival improved in those exposed to mixtures (1:1 2, 20 µg IM/L:µg TM/L, 1:4 0.05 µg IM/L: 0.20 µg TM/L, and 1:5 0.05 µg IM/L: 0.25 µg TM/L); however, high mortality was observed in controls. The effect on survival, but not behavior implies that IM and TM may have low affinities for the vertebrate nAChR and interact with a different system in the fish, which was supported by molecular modeling.

ACKNOWLEDGMENTS

First and foremost, I want to thank my advisor, Dr. Tisha King-Heiden, for her consistent support, willingness to teach at any moment, and encouragement to pursue opportunities I would not have considered otherwise. I am thankful for my committee members: Dr. Elisabeth Harrahy, Dr. David Schumann, and Dr. Cord Brundage for their insightful contributions to my research and education. I am so thankful for Shayla Victoria for her mentoring and molecular modeling skills which she has offered freely from the beginning of this project to the end. I am grateful to for the time and effort given by all the laboratory workers who helped with this project; Sara Duffy, Zion Wallace, Myah Fraundorf, Nicole Kooij, Kassidy Frame, and Jack Barette. I am incredibly grateful for the aid and embryos provided by the Wisconsin State Lab of Hygiene, this project would not have been possible without them. Additionally, I want to recognize UWL and the Wisconsin Department of Agriculture, Trade and Consumer Protection for their funding and resources which supported this project.

Finally, I greatly appreciate all my family, friends, and loved ones who have listened to me talk about fish and pesticides for the last 2 years; I know only some of you enjoyed it. I will always be amazed at how blessed I am to have had so many people who supported me through this experience.

Table of Contents

LIST OF FIGURES

FIGURE PAGE

LIST OF TABLES

CHAPTER I

ACUTE AND CHRONIC EXPOSURE TO IMIDACLOPRD DECREASES SURVIVAL IN FATHEAD MINNOWS BUT DOES NOT ALTER KEY MOTOR BEHAVIORS

Introduction

Neonicotinoids are a class of pesticides that after decades of widespread use are now a environmental contaminant in aquatic ecosystems (Borsuah et al., 2020; Goulson, 2013; Simon-Delso et al., 2015). In Wisconsin, there are currently eight neonicotinoids approved for use; thiamethoxam, imidacloprid, and clothianidin are the most frequently used (Senger, 2018). These pesticides are designed to specifically target the nicotinic acetylcholine receptors (nAChR) in the central nervous system (CNS) of invertebrates, similar to the natural pesticide nicotine, leading to paralysis and death (Goulson, 2013; Simon-Delso et al., 2015). These pesticides are highly specific for the nAChR of invertebrates due to their electron rich nitroethylene, nitroimine, or cyanoimine groups, and therefore are thought to be less toxic in vertebrates (Yamamoto et al., 1998).

The environmental risk of this class of pesticides has recently garnered public attention after discovering their impacts on non-target species such as pollinators. For example, exposure to imidacloprid, thiamethoxam, and clothianidin impairs the foraging

and homing behaviors of bees (*Bombus terrestris*) leading to their death and negatively effecting on honey-bee (*Apis mellifera*) populations (Gill et al., 2012; Henry et al., 2012; Rundlöf et al., 2015; Whitehorn et al., 2012). Negative effects have also been seen in other non-target invertebrates, such as midges (*Chironomus dilutus*), amphipods (*Gammarus pulex*), and wasps (*Nasonia vitripennis*), including decreased feeding rates, shifting sex ratios, delayed time-to-emergence, immobility, and changes in drift behavior (Beketov & Liess, 2008; Cavallaro, 2019; Hladik & Kolpin, 2016; Whitehorn et al., 2015). This evidence influenced the decision of the European Union to ban the use of neonicotinoids everywhere except for in greenhouses (Butler, 2018).

In the United States neonicotinoids are primarily used in agriculture, and are applied as a spray or a seed coating to crop seeds and can be found throughout the entire plant as it grows (Byrne & Toscano, 2006). This method of application reduces environmental exposure; however, since the pesticides are highly soluble in water, they can leach into agricultural runoff, rainwater, irrigation water, as well as the groundwater near their site of application (Anderson et al., 2015; Hladik & Kolpin, 2016; Huseth & Groves, 2014; Thompson, 2020). The U.S. Geological Survey reported that 53% of streams sampled across 24 states and Puerto Rico contained neonicotinoid pesticides, with imidacloprid being one of the most prevalent (Hladik & Kolpin, 2016). IM has been found in the groundwater of WI in the range of 0.052 to 1.59 µg/L and the surface water in the range 0.05 to 0.09 µg/L (Senger, 2018). Nationally, IM concentrations have been noted to be higher, and have been detected as high as 3.29 µg/L (Starner & Goh, 2012). These ranges are above the ecological thresholds, or safety limit, for invertebrates of 0.067 µg IM/L for long-term exposures (Morrissey et al., 2015). At these concentrations

it is unlikely that IM would cause mortality in fish, as IM was found to be non-toxic in fathead minnows exposure to concentrations below 5000 µg/L, prolonged exposure may lead to adverse long-term effects (Lanteigne et al., 2015). The 96 hour lethal concentration for 50% of organisms (LC_{50}) for rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) is 280 mg/L and 211 mg/L respectively (Tišler et al., 2009), concentrations that are well above what has been detected in the environment.

Neonicotinoids have been shown to impair development and alter behavior in fish such as impaired locomotion in fish larvae, decreased weight and length, and notochord degeneration (Beggel et al., 2010; DeCant and Barrett 2010; Gibbons et al., 2015). Zebrafish larvae exposed to the neonicotinoid acetamiprid at concentrations of 107, 537, 760 and 974 mg/L exhibited a time delay in their embryonic motor activity at low concentrations and restricted embryonic motor activity at the highest concentrations (Ma et al., 2019). Thiamethoxam, one of the most commonly used neonicotinoids in the U.S., has been shown to alter swimming activity in larval fish (Hladik & Kolpin, 2016; Liu et al., 2018). A decrease in survival of fathead minnows (*Pimephales promelas*) was also noted after embryonic exposure for 8 days to thiamethoxam $\geq$ 1.57 µg/L as well as embryonic motor activity (tail bends) at $\geq$ 1.57 µg/L, increased latency (time taken to respond to external stimuli) in their predator escape response behavior at $\geq$ 1.57 µg/L, and decreased burst speed at $\geq$ 155 µg/L (Victoria et al., 2022). Behavioral responses to environmental exposure, such as embryonic motor activity or avoidance behaviors like the predator escape response, can link the physiological and ecological consequences of chronic, low concentration exposure to pesticides (Shuman-Goodier & Propper, 2016).

Few studies have been done to explore potential effects of chronic exposure to low concentrations of IM due to its low toxicity in vertebrates (Ihara & Matsuda, 2018; Lanteigne et al., 2015). Chronic exposure to relatively high concentrations of IM was linked with a decrease in acetylcholinesterase enzyme activity, and increased oxidative stress at $\geq$ 10 mg IM/L after 15 days in rainbow trout embryos (Topal et al., 2017). IM exposure for 14 days resulted in a thickening of muscle fibers in zebrafish (*Danio rerio*) embryos and larvae exposed to >2 mg IM/L as well as decreased total length and physical deformities in Japanese medaka embryos and larvae (*Oryzias latipes*) at $\geq$ 0.2 µg IM/L (Vignet et al., 2019). This limited information makes it difficult to predict how this pesticide may behave in when exposed to fish in the environment.

Since neonicotinoid pesticides are designed to cause toxicity through overactivation of the nAChR, and the nAChR plays an important role in the development and coordination of behavioral movements, it is possible for chronic exposure to neonicotinoid pesticides to disrupt neuromuscular activity. Elucidating the potential effects of chronic exposure to sublethal concentrations of IM on fish health and behavior is necessary to better understand the potential the risk that IM poses to wild fish populations. The goals of this project were to determine the potential for IM to activate the nAChR and affect motor behaviors during early and later stages of development, as well as the hatching success, growth, and survival of fathead minnow larvae. Fathead minnows were used to test the hypothesis that the nAChR will be activated by exposure to sublethal concentrations of IM increasing embryonic motor activity and decreasing the predator escape response behavior, growth, hatching success and survival.

Materials and Methods

Chemicals

Imidacloprid (>99% purity) was purchased from Sigma-Aldrich, Inc. Dosing solutions were prepared through serial dilutions of 20,000 µg/L stock solutions with moderately hard water (80-100 mg/L $CaCO_3$, pH 7.4-7.8, US EPA 2006). Concentrations were selected to show the range of responses to exposure to IM, from below (0.02), at (0.2 and 2) and above environmentally documented concentrations (20, 200 µg/L each). Concentrations in this study were not confirmed and are nominal.

Test Species and Animal Protocols

All experiments were approved by the University of Wisconsin-La Crosse Institutional Animal Care and Use Committee (Animal Use Protocol 3-19) and all tests followed standard toxicity protocols outlined by the US EPA and Organization for Economic Co-operation and Development (OECD) (OECD, 2012; U. S. EPA, 2002). Fathead minnow eggs were supplied from the Wisconsin State Lab of Hygiene (Madison, WI). Embryos were removed from spawn tiles, sorted by age under a dissecting microscope and raised at 25º C with a 16-hour light cycle.

Potential for IM to Activate the nAChR on Motor Activity in Fathead minnow Embryos

Spontaneous contractions of the tail of fish embryos are reflexive motor responses associated with activation of the nAChR (Thomas et al., 2009; Victoria et al., 2022). Eggs were collected from 3 different spawn groups to incorporate biological replicates. Eggs from each spawn group were sorted and allocated so that 10 embryos were placed

in each well of a 24 well plate. When the embryos displayed the tail bend behavior (1 day post fertilization) embryos were placed in 1 mL of standard culture water under a dissecting microscope attached to a Nikon 80i camera (Nikon Instruments, Inc., Mellville, NY, USA) set at 43 frames per second (fps, maximum possible speed) with the NIS Elements software (Nikon Instruments, Inc., Mellville, NY, USA). Fathead minnow embryos were placed under the dissecting microscope and allowed to acclimate for one minute and then were dosed with the appropriate concentrations of IM to produce the target dosing solutions (0, 0.02, 0.2, 2, 20, 200 µg/L). A 1-minute recording was taken for evaluation. Video recordings were taken in a cycle pattern (1 well/dose of each treatment group) to eliminate time as a source of variability. Video analysis was performed blind and embryonic motor activity evaluated by counting movements (tail bend and release) per minute (n = 4 wells/dose, 3 repeat experiments, total n = 12 per dose). The number of tail bends/min for all fish within a dosing solution group were averaged before statistical analysis (n = 4 beakers per dose from 3 spawn groups for a total of n = 12 per dose).

Effects of Chronic Exposure to Imidacloprid in Fathead Minow Embryos and Larvae

Eggs from different spawn groups were sorted by age and allocated so that 10 embryos were placed in each well of a 24 well plate. Each well had 2 mL of dosing solutions and a 100% complete renewal of the solutions was performed every day for 8 days. The fathead minnow larvae were transferred to corresponding 50 mL beakers post hatch (6 dpf) to accommodate their larger size with 30 mL of the same dosing solution which was also 100% renewed each day (n = 4 well/beaker per dose from 3 spawn groups

for a total of n = 12 per dosing solution). Effects on growth, development, and behavior were assessed as described below.

Impacts on Survival, Hatching and Growth After Exposure to IM for 8 days

Mortality and hatching were recorded daily throughout the exposure period to calculate survival and the proportion of fish that hatched at 6dpf. At the end of the exposure, total length (TL) of two fish from each beaker were measured and used as an indicator of growth. ImageJ software (National Institute of Health and Laboratory for Optical and Computational Instrumentation, Madison, Wisconsin, USA) was used to measure TL from images isolated from videos recorded for the predator escape response (outlined below). TL was determined by measuring the distance between the tip of the nose to the end of the tail (n = 12).

Behavioral indicators of neurotoxicity

Behavioral indicators of neurotoxicity were analyzed with two assays, the embryonic motor activity assay performed on day 1 and the predator escape response assay performed on day 8. The embryonic motor activity assay was set up and analyzed in the same method as described above (n = 12). Four wells were removed from the control exposure groups due to mortality(n = 8).

The predator escape response, made up of the latency, burst speed, length and total escape response, was assessed for subsampled larvae (2 from each beaker; n = 4 beakers per dose with 3 experiments, total n = 24 per dosing solution), according to the methods described in Painter et al., (2009) and McGee et al., (2009). A dissecting microscope was set up with a 1 by 1 mm grid attached to a stimulus plate with a vibrating

buzzer in place of the glass base piece. A Phantom MiroC210 camera (Vision Research, Wayne, NJ, USA) was attached to the dissecting microscope and set at 1000 fps. A ring light and two-headed external light source were arranged on the microscope to increase visibility. Videos were recorded using the Phantom Camera Control Application software (Vision Research, Wayne, NJ, USA). The camera and the buzzer on the vibrational stimulus plate were activated simultaneously by a trigger. Fish were placed in a plastic dish with 2 mL of standard culture water. This dish was placed on the vibrational stimulus plate and the larva was allowed 1 minute to acclimate prior to the trigger for the camera and buzzer being activated and the predator escape response being recorded. If the larva failed to produce a predator escape response after 2 stimuli attempts, a new larva from the same beaker was selected and tested in the original larva's place. Videos were recorded in cycles for all experiments (1 larva/beaker in each treatment group) to remove time a source of variability. Video analysis was performed blind using the ImageJ software (National Institute of Health and Laboratory for Optical and Computational Instrumentation, Madison, Wisconsin, USA) following methods described by Diamond et al., (2016). Videos were used to measure latency (amount of time between stimulus and initiation of response), burst speed (speed of initial 40 ms of response), and total escape response (combination of latency, length of larva, and burst speed).

Data Analysis

Data analyses were conducted using SigmaStat (Ver 4 integrated with SigmaPlot11, Spss Inc, Chicago, IL, USA). All experimental data except survival data were analyzed using the one-way analysis of variance (ANOVA) with Tukey's post-hoc tests at 95% confidence comparing each dosage group (0.02, 0.2, 2, 20, 200 µg/L) to the

control (0 µg/L), and assumptions of normality and homoscedasticity were checked using Shapiro-Wilk and Levene's test respectively. If data failed to pass normality, then ranked ANOVA was used to complete analysis. Survival data were analyzed using the Kaplan-Meier survival analysis with the Gehan-Breslow significance test.

Results

Impact of Acute Activation of nAChR on Embryonic Motor Activity

Acute exposure of fathead minnow embryos to IM at concentrations as high as 200 µg/L did not exhibit changes to embryonic motor activity or paralysis when compared with the control (Figure 1, Kruskal-Wallis One Way ANOVA on Ranks, H = 0.495, df = 5, p = 0.992).

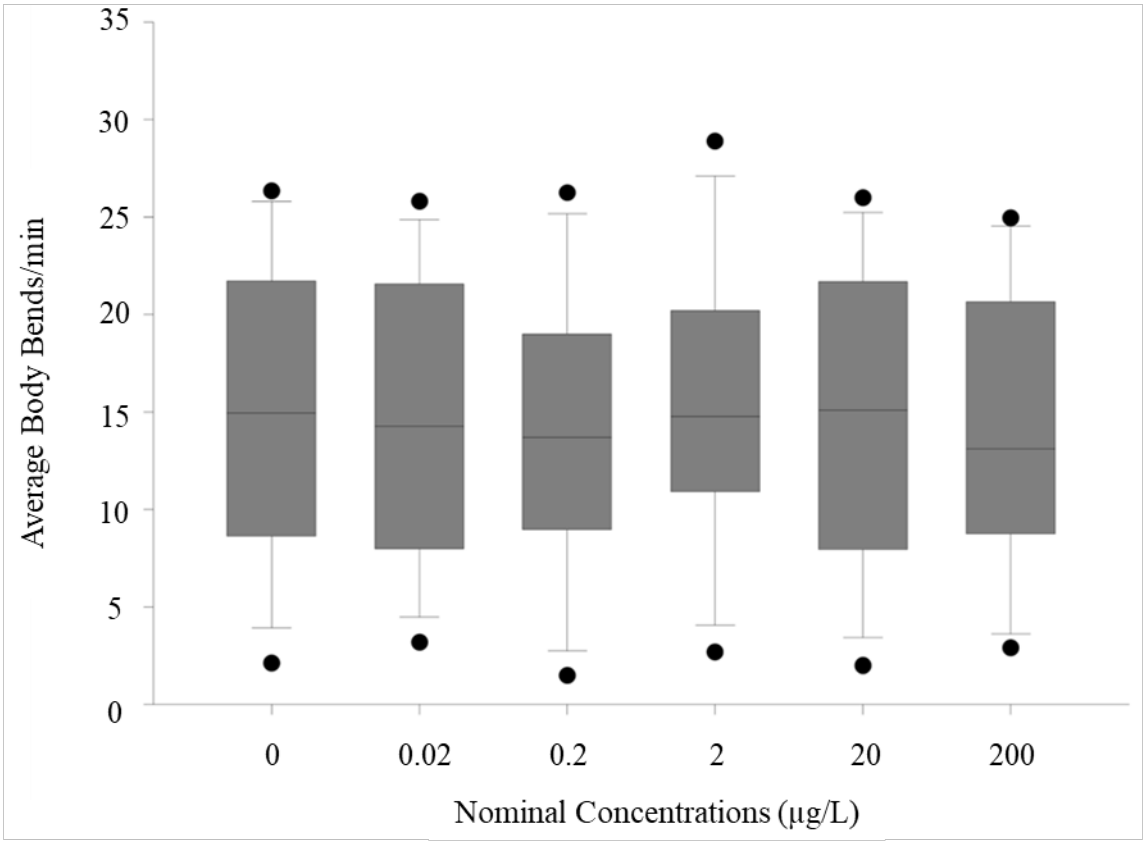


Figure 1. Effects of acute activation of nAChR of fathead minnow embryos spiked with increasing concentrations of IM.

Impact of IM Chronic Exposure on Hatching

Chronic exposure to IM at 6 days post fertilization did not impair hatching success of fathead minnow embryos (Figure 2, One Way ANOVA, F = 1.888, df = (5,56), P = 0.111).

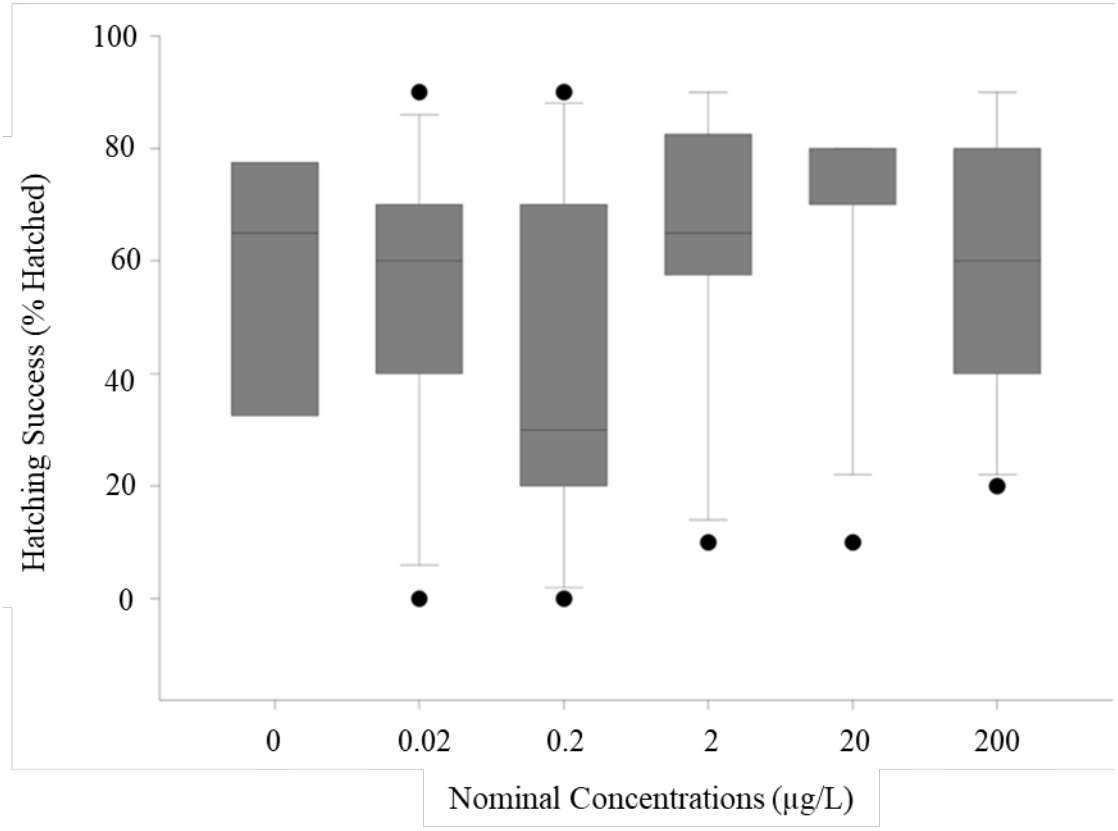


Figure 2. Percent fathead minnow embryos hatched at 6 days post fertilization following chronic exposure to IM.

Effects following 24 hours of IM Exposure on Embryonic Motor Activity

Embryonic motor activity did not change following 24 hours of IM exposure when compared to the control (Figure 3, One Way ANOVA, F = 0.574, df = (5,66), P = 0.679).

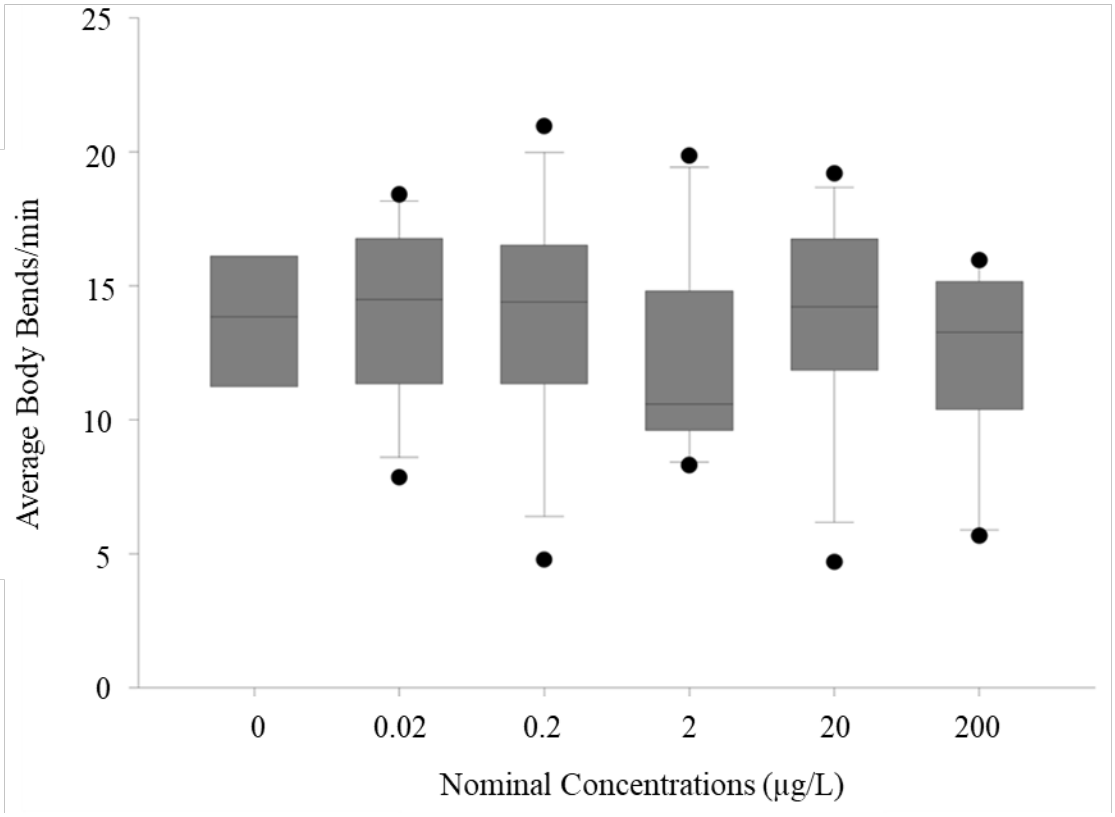


Figure 3. Average movements per minute of fathead minnow embryos exposed to varying concentrations of IM at 1 dpf.

Impact on Predator Escape Response Following 8 Days of IM Exposure

Predator escape response was not impacted by chronic exposure to IM for 8 days (Figure 4). Total escape response (Figure 4, Kruskal-Wallis One Way ANOVA on Ranks, H = 8.035, df = 5, P = 0.154) and latency (Table 1, Kruskal-Wallis One Way ANOVA on Ranks, H = 0.871, df = 5, P = 0.972) were not significantly affected when compared to the controls. Burst speed did show a difference between concentrations, but Dunn's test revealed that the differences were not between the test concentration and the control (Table 1, Kruskal-Wallis One Way ANOVA on Ranks, H = 14.214, df = 5, P = 0.014).

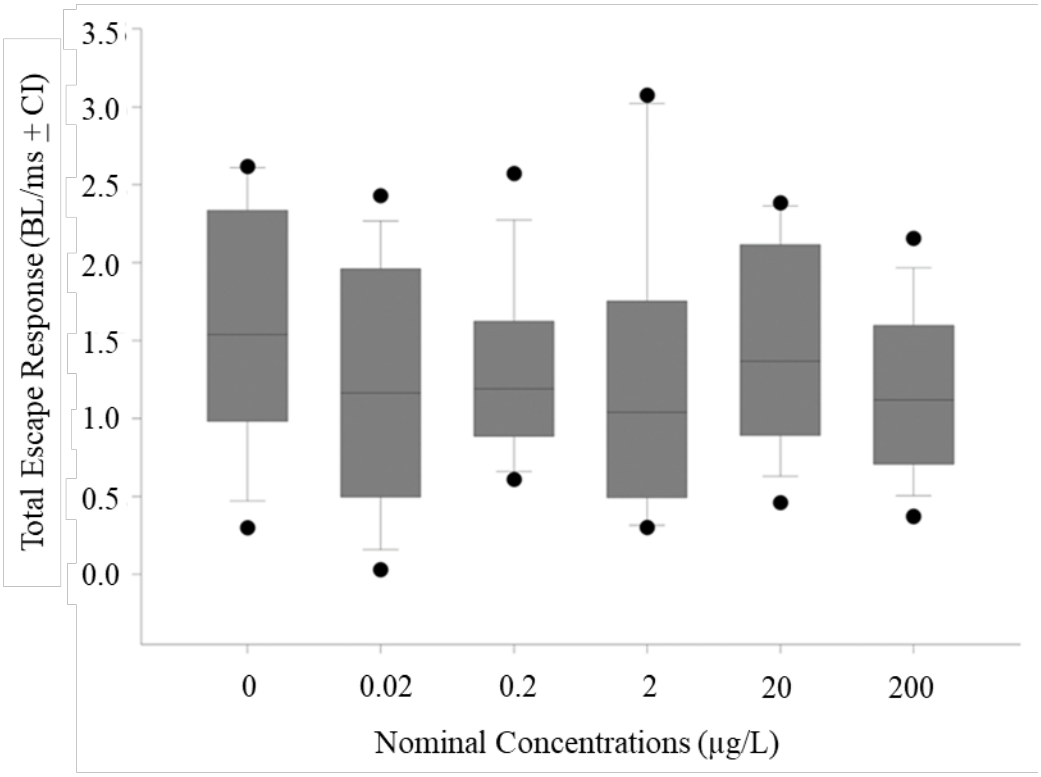


Figure 4. Mean total escape response of fathead minnow larva exposed to increasing concentrations of IM at 8 dpf.

Table 1. Mean values of predator escape response parameters following chronic exposure to IM. Asterisks indicate mean values which differed from each other but not from the controls.

Concentration (µg/L)	Latency (ms ± SE)	Burst Speed (BL/ms ± SE)
0	71.31 ± 30.35	0.03 ± 0.01
0.02	130.5 ± 67.83	0.03 ± < 0.01*
0.2	41.96 ± 22.56	0.30 ± 0.61*
2	51.44 ± 20.82	0.04 ± < 0.01
20	163.6 ± 74.19	0.02 ± <0.01*
200	102.3 ± 59.35	0.22 ± 0.19

Impact of Chronic Exposure to IM on Survival

Fish exposed to 0.2 µg IM/L for 8 days had reduced mean survival when compared to the control at 8 days post fertilization (Figure 5, Gehan-Breslow statistic = 25.262, df = 5, $p < 0.001$).

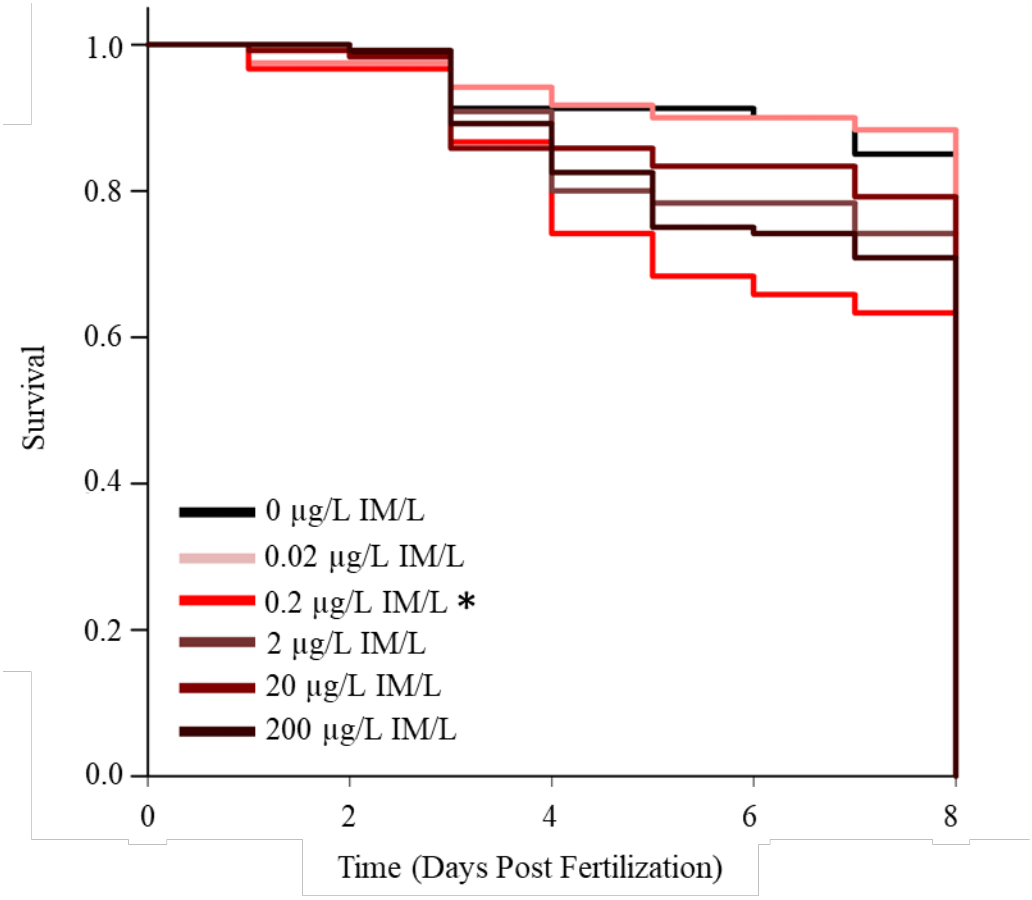


Figure 5. Kaplan-Meier survival analysis of fathead minnows exposed to increasing of IM beginning at 0 dpf and ending at 8 dpf. Asterisks indicate that group differed significantly from the control in pairwise comparisons, according to Gehan-Breslow test.

Impact of IM Chronic Exposure on Growth

Growth was not impacted by chronic exposure to IM for 8 days when compared to the control (Figure 6, One Way ANOVA, F=1.909, df= 5,53, P=0.095).

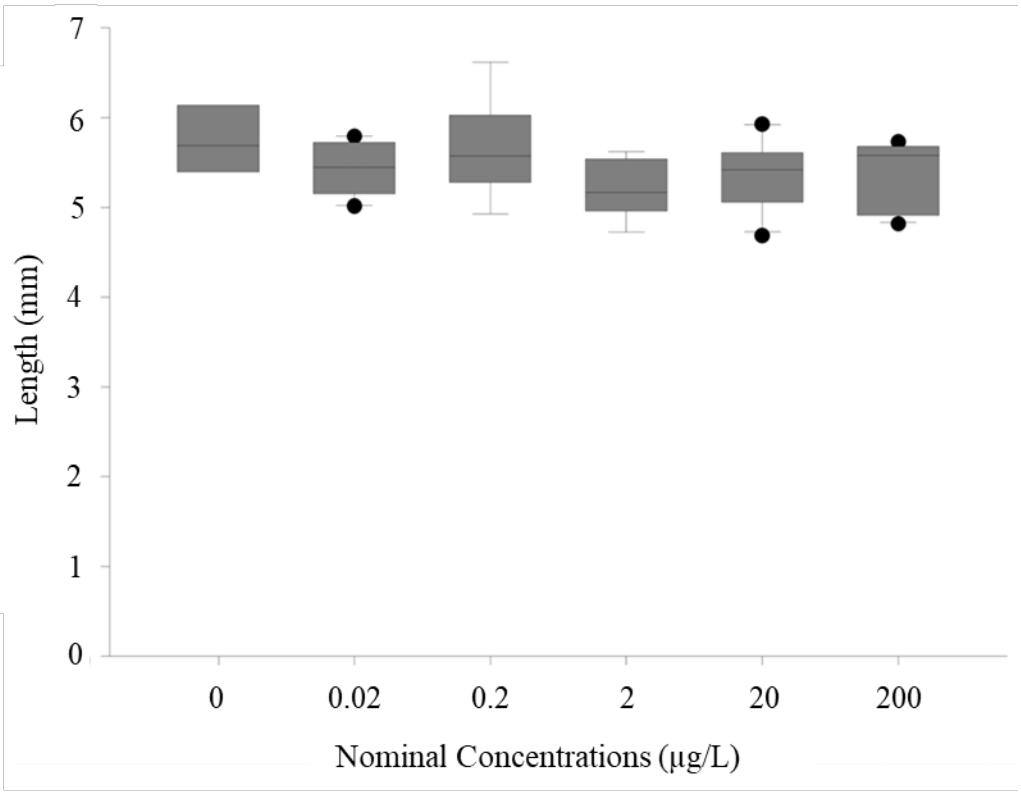


Figure 6. Effects of chronic exposure to IM on growth of fathead minnow larvae.

Discussion

Imidacloprid's highly specific mode of action is meant to reduce risk posed to fish and other vertebrates (Tomizawa & Casida, 2005); however, impacts of chronic exposure to sublethal concentrations of IM on the health and neurobehavioral responses regulated by the nAChR remain largely unknown. Herein, I show that chronic exposure to imidacloprid can decrease survival in fathead minnow larvae, but does not appear to alter growth, hatching success, or embryonic motor activity or predator escape response. This

data suggest that IM has a low affinity for the nAChR as designed but may still cause toxicity through a different mechanism.

Acute Exposure to Imidacloprid Does Not Alter Embryonic Motor Activity

Acute IM exposure did not significantly alter the embryonic motor activity, which was consistent with our current understanding of its mode of action but inconsistent with studies done with other neonicotinoids. Previous work shows that exposure to contaminants, such as nicotine, can lead to alterations in this behavior which have the potential to impact the larva after they have hatched (Mora-Zamorano et al., 2016; Victoria et al., 2022). Exposure to low concentrations of nicotine can increase embryonic motor activity and high concentration can cause paralysis, consistent with dose-dependent hyper- and hypo-activity found previously in fish exposed to other nAChR agonists (Tierney et al., 2011; Victoria et al., 2022). Neonicotinoids are expected to have a low affinity for binding with the vertebrate nAChR; however, acute exposure of thiamethoxam to fathead minnows induced subtle alterations in embryonic motor activity (Tomizawa & Casida, 2005; Victoria et al., 2022). Stinson et al. (2022) found that IM did not bind significantly to the fathead minnow nAChR under non-specific binding conditions, which may explain why IM did not induce increased embryonic motor activity in fathead minnow embryos while thiamethoxam did. However, the isolation technique used in Stinson et al. (2022) likely resulted in the isolation of nAChR subtypes associated with the skeletal muscles rather than the central nervous system.

24-hour IM Exposure Does Not Impact Embryonic Motor Activity or Hatching Success

Exposure to IM for 24 hours also did not significantly alter the embryonic motor activity, consistent with our current understanding of the expected low affinity of IM for the nAChR (Tomizawa & Casida, 2005) and with tests done with thiamethoxam (Victoria et al., 2022). Additionally, chronic exposure to IM did not alter hatching success. Embryonic motor activity is associated with hatching because process of hatching requires successful coordination of the nervous system and biochemical functions (Fraysse et al., 2006). Motor activity increases before hatching, likely aid in the process of breaking out of the chorion (Woynarovich & Horvath, 1980). Common carp exposed to IM showed decreased hatching as concentrations of IM increased, with 29% hatching success at 1000 µg IM/L compared to 80% hatching success in the controls (Islam et al., 2019). As the fathead minnows did not experience any impacts from chronic exposure to IM, it follows that the hatching success at environmentally relevant concentrations would not be affected as well.

Chronic Embryo Exposure to IM Does Not Impact Predator Escape Response

This study did not see any impact on any aspect of the predator escape response, which aligns with our embryonic motor activity results which were also insignificant. IM concentrations above those used in this study have been shown in previous work to both increase the predator escape response of zebrafish, while decreasing larval swimming activity overall and decrease arousal and limit burst speed (Crosby et al., 2015; Faria et al., 2019). This could be due the low concentrations used in this study, which may not have been sufficient to interfere with the nAChR and alter the response.

Chronic Exposure to Sublethal Concentrations of IM Decreases Survival but Does Not Impact Growth

Chronic exposure to 0.2 µg IM/L resulted in decreased survival compared to the control, but mortality was not affected following exposure to higher concentrations and fish did not display any developmental malformations. The lack of mortality in the higher dosing solution groups could be due to the variability in the survival of control fish or could indicate a non-monotonic dose-response to chronic exposure to IM. The decrease in survival of fathead minnows exposed to 0.2 µg IM/L and the lack of effect on higher concentrations indicates that fathead minnows may be at risk of individual mortality at environmentally relevant concentrations of IM in natural habitats with environmental stressors, the decrease in survival of larval fish may impact recruitment and population sizes (Garrido et al., 2015). It may be a hormesis dose response, where there is a higher effect at low concentrations than high concentrations. Fathead minnows may have mechanisms to cope with the presence of IM, but those mechanisms do not activate until the concentrations they are exposed to reach above 0.2 µg IM/L. Further research into how IM impacts survival in fathead minnow embryos and larvae is necessary to understand the dose-response curve.

Fathead minnow larvae exposed to IM concentrations <5000 µg/L were not observed to exhibit adverse effects on survival (Lanteigne et al., 2015), a concentration 4 orders of magnitude above detected concentrations of IM in the environment (Hladik & Kolpin, 2016; Senger et al., 2019). However other fish species, Japanese medaka and zebrafish, show toxicity at lower concentrations. Recent studies suggest that the 96-hr LC_{50}, is 611 µg IM/L (Islam et al., 2019). Japanese medaka showed embryonic and larval

such as scoliosis, oedema, hemorrhage, and jaw/skull/tail deformities and zebrafish showed thickened muscle tissues due to IM exposure at concentrations in the range of µg/L (Vignet et al., 2019). The higher toxicity due to exposure to IM in these fish species when compared to fathead minnows may be due to the thickness of the chorion or the length of time spent in the embryonic stage. Japanese medaka and zebrafish hatch within about 3 days, compared to the fathead minnow's 6 days, and have thinner chorions. Fathead minnows may be less sensitive to IM than Japanese medaka and zebrafish or it may be possible that fathead minnow chorions limit IM uptake compared to Japanese medaka and zebrafish during embryonic development.

IM was not expected to impact growth as thiamethoxam and clothianidin are not believed to have developmental effects (Gibbons et al., 2015). Our work supports this study that chronic exposure to environmentally relevant concentrations does not likely impact growth of larvae. While growth was not measured, common carp (*Cyprinus carpio*) exposed as embryos to >200 µg/L IM were observed to have deformed notochords, lordosis, and body arcuation (Islam et al., 2019), which could affect total length. This disparity in toxicity could be due to a difference in the structure between fathead minnow and common carp nAChRs or IM may not be able to pass through the fathead minnow chorion as easily as the common carp chorion.

Limitations

Although the sample size for each test was small, it was consistent with existing literature which explores multiple endpoints (Diamond et al., 2016; Nair et al., n.d.; Thomas et al., 2009). Sample size was limited by the time required to complete each assay and studies which focused on fewer endpoints were able to have greater sample

sizes (McGee et al., 2009; Painter et al., 2009). The expected adverse effects due to IM would have been subtle due to its low binding specificity to the invertebrate nAChR, so the high variability may have prevented effects from being identified. Finally, concentrations have not yet been confirmed which limits the accuracy of conclusions drawn from the results. Confirmation of the dosing solutions will provide greater confidence in the results.

Conclusions

Chronic embryonic exposure to sublethal concentrations of IM may not cause overt neurotoxic effects, but concentrations of approximately 0.2 µg/L may impact survival in fathead minnows after 8 days of exposure, possibly due to a non-monotonic dose response or hormesis. Our study suggests that toxic effects may be observed below the no observable effect concentration (NOEC) of 1.099 µg IM/L, calculated by Kienzler et al., (2016). Further studies are necessary to determine how and if IM interacts with the fathead minnow nAChR. Overall, we observed no neurobehavioral or developmental effects in fathead minnow embryos or larvae due to exposure to IM. IM has been shown to have a risk of chronic toxicity to freshwater macroinvertebrate and vertebrate species, resulting in effects such as delayed emergence of adult macroinvertebrates, developmental deformities of vertebrates, and loss of species richness (Maloney et al., 2018; Morrissey et al., 2015; Schmidt et al., 2022; Tišler et al., 2009; Vignet et al., 2019; Wang et al., 2022). Current work indicates that other species may be more sensitive to IM exposure than fathead minnows, highlighting the importance of comparative studies exploring different species sensitivities to environmental contaminants (Stinson et al., 2022; Tišler et al., 2009; Vignet et al., 2019; Wang et al., 2022).

CHAPTER II

EFFECTS OF CHRONIC EXPOSURE TO BINARY MIXTURES OF NEONICOTINOID PESTICIDES

Introduction

It is common when assessing the toxicity of various environmental contaminants to focus on individual chemicals. However, aquatic contaminants exist as mixtures in the environment which may cause greater adverse effects than following exposure to individual contaminants (Carvalho et al., 2014; Junghans et al., 2006; Kortenkamp & Altenburger, 1999; Lydy et al., 2004). One of the most widely sold insecticides are neonicotinoids, making up approximately 80% of seed treatments sold worldwide, and are often applied in agriculture as a seed coating (Byrne & Toscano, 2006; Jeschke et al., 2011). They are systemic pesticides, meaning they can be found in all parts of the plant as it grows (Byrne & Toscano, 2006). Their high specificity to the invertebrate nAChR is due to their electron rich nitroethylene, nitroimine, or cyanoimine groups which are meant to limit their effects on nontarget organisms (Yamamoto et al., 1998). However, their high water solubility and frequent application has allowed them to leach into the environment through agricultural runoff and become pseudo-persistent in streams and groundwater (Anderson et al., 2015; Hladik & Kolpin, 2016; Huseth & Groves, 2014; Thompson, 2020). Over half of the streams sampled by the United States Geological Survey in 2016 contained neonicotinoids, with imidacloprid (IM) and thiamethoxam

(TM) being found most often (Hladik & Kolpin, 2016). Groundwater and streams in the Central Sands region of Wisconsin were found to be contaminated with imidacloprid, thiamethoxam, and clothianidin, sometimes simultaneously, with IM detected surface waters in the range 0.05 to 0.08 µg IM/L and TM detected in surface waters at 0.057-2.78 µg TM/L (Senger, 2018). Since neonicotinoid pesticides occur in aquatic ecosystems as mixtures, it is important to compare adverse effects observed following exposure to individual neonicotinoids with adverse effects observed following exposure to mixtures found in the environment.

Existing literature regarding the toxicity of mixtures of neonicotinoids in aquatic organisms has focused on the effects to invertebrates. For example non-biting midges (*Chironomus dilutus*) exposed to binary mixtures of IM,TM or clothianidin at ratios of 1:0, 3:1, 1:1, 1:3, 0:1 exhibited additive toxicity depending on the concentration level of each pesticide in the mixtures (Maloney et al., 2017). Non-biting midges exposed to a mixtures composed concentrations ranges of 0.03-1.49 µg/L IM and 0.56-25.73 µg/L TM exhibited decreased emergence as concentrations increased when compared to populations exposed to either neonicotinoid alone (Maloney et al., 2018). Amphipods (*Hyalella azteca*) exhibited decreased swimming in 50% of amphipods sampled after 96 hours of exposure (EC_{50}) when exposed to mixtures of IM and another pesticide called cyfluthrin (a pyrethroid) at concentrations of 0.0015 µg cyfluthrin/L:33.5 µg IM/L (Lanteigne et al., 2015). These studies suggest that mixtures of neonicotinoid pesticides can cause additive toxicity in invertebrates.

Due to the conservation of portions of the nAChR subtypes between invertebrates and vertebrates it may be possible for chronic exposure to sublethal concentrations of

mixtures of IM and TM to have a greater impact development and neurobehavior of vertebrates compared to exposure of individual pesticides. (Tomizawa, 2013). While IM and TM occur together within surface waters, there are no studies to date that have looked at the toxicity of binary mixtures to fish. Chronic exposure to $\geq$ 1.57 µg TM/L reduced survival and increased embryonic motor activity (tail bends) at $\geq$ 1.57 µg TM/L, increased latency (time taken to respond to external stimuli) in their predator escape response behavior at and decreased burst speed at $\geq$ 155 µg TM/L of fathead minnow larvae (Victoria et al., 2022). Concurrent work indicates that chronic exposure to similar concentrations of IM do not significantly impact the survival or behavior of fathead minnow larvae (Unpublished data). Based upon work in invertebrates, it is possible that exposure to binary mixtures of IM and TM could cause additive toxicity in fathead minnow (*Pimephales promelas*) larvae.

Sublethal toxic effects of neonicotinoids to fish may be due to the conservation of portions of the nAChR subtypes between invertebrates and vertebrates, particularly in the area of the protein where the ligand binds also known as the binding pocket (Tomizawa, 2013). The structural similarities between neonicotinoids and nicotine may allow the possibility for chronic exposure to sublethal concentrations of IM and TM to cause developmental and neurobehavioral toxicity in vertebrates, particularly those associated with the nAChR (Tomizawa, 2013), however the binding affinity may not be sufficient to cause paralysis and death as has been shown in TM (Victoria et al., 2022). Behavioral responses to exposure, such as embryonic motor activity or avoidance behaviors like the predator escape response, can link the physiological and ecological consequences of chronic, low concentration exposure to pesticides (Shuman-Goodier & Propper, 2016).

The goal of this project is to use fathead minnows as a model organism to test the hypothesis that exposure to environmentally relevant mixtures of IM and TM would be more toxic than exposure to either pesticide alone. Additionally, I sought to determine whether length of exposure influenced the type and extent of effects caused by chronic exposure to IM and TM mixtures. Developmental effects were characterized by growth and hatching, and I used embryonic motor activity and predator escape response behaviors to characterize potential motor activities disrupted by exposure to nAChR agonists. *In-silico* molecular docking was performed to help characterize ligand-protein interactions in both vertebrates and invertebrates to better understand how IM and TM interact with the nAChR of each group.

Materials and Methods

Chemicals

Thiamethoxam (>99% purity) and imidacloprid (>99% purity) were purchased from Sigma-Aldrich, Inc. TM and IM dosing solutions were prepared through serial dilutions of 20,000 µg/L stock solutions with moderately hard water (US EPA, 80-100 mg/L $CaCO_3$, pH 7.4-7.8, US EPA, 2002). Binary mixtures were prepared using a fixed ratio design, using 1:1 ratios of concentrations previously shown to cause toxicity in fish for both pesticides as well as ratios that represent observed environmental concentrations (Senger, 2018). The first experiment utilized 1:1 ratios ranging from 0, 0.02, 0.2, 2, 20, or 200 µg/L each, and the environmentally relevant mixtures were 1:3 (0.05 µgIM/L : 0.15 µgTM/L), 1:4 (0.05 µgIM/L : 0.2 µgTM/L), and 1:5 (0.05 µgIM/L : 0.25 µgTM/L) based on reported detections (Senger, 2018).

Test Species and Animal Protocols

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) Animal Use Protocol (Protocol 3-19) and tests followed standard toxicity protocols outlined by the US EPA and Organization for Economic Co-operation and Development (OECD, 2012; US EPA, 2002). Fathead minnow eggs were provided by the Wisconsin State Lab of Hygiene (Madison, WI). Embryos were removed from spawn tiles, sorted by age under a dissecting microscope and raised at 25º C with a 16-hour light cycle.

Potential for IM and TM Mixtures to Activate the nAChR on Motor Activity in Fathead minnow Embryos

Eggs were collected from 3 different spawn groups and were sorted so that there were 10 embryos in each well of a 24 well plate. When the embryos display the tail bend behavior, 1 day post fertilization, they were placed in 1 mL of standard culture water under a dissecting microscope attached to a Nikon 80i camera (Nikon Instruments, Inc., Mellville, NY, USA) set at 43 fps (frames per second, maximum possible speed) with the NIS Elements software (Nikon Instruments, Inc., Mellville, NY, USA). Embryos were given 1 minute to acclimate and then were dosed with the appropriate concentrations of IM and TM to produce the target dosing solutions (0.05 µg IM/L : 0.15 µg TM/L, 0.05 µg IM/L : 0.2 µg TM/L, 0.05 µg IM/L : 0.25 µg TM/L) and then a 1 minute video was recorded. Video recordings were taken in a cycle pattern by (1 well per dose for each n) to eliminate time as a source of variability. Video analysis was performed blind and embryonic motor activity evaluated by counting movements (tail clench and release) per minute after being dosed with test solutions. The number of tail bends/min for all fish

within the same dose were averaged before statistical analysis (n = 4/dosing solution with 3 experiments, total n = 12).

Effects of Chronic Exposure to IM and TM Mixtures in Fathead Minow Embryos and Larvae

Eggs were collected from different spawn groups and were sorted by age. Embryos were randomly placed into 24-well culture plates in groups of 10 embryos per well. Each well had 2 mL of dosing solutions and a 100% complete renewal of the solutions was performed every day. At 6 days post fertilization after all the embryos had hatched, they were transferred to corresponding 50 mL beakers to accommodate their larger size with 30 mL of the same solution which is also 100% renewed each day. There were 2 separate exposure periods, one being shorter with minnows exposed for 3 days post fertilization and then raised to 8 days post fertilization in moderately hard water. The other exposure lasted for 8 days post fertilization. In the 3-day exposure test with 1:1 IM:TM mixtures followed by 5 days of depuration in moderately hard water, experiments were repeated 2 times with 4 wells/beakers per dosing solution, creating a total of n=8. There were 2 different dosing regimens exposed for the 8-day exposure period, 1:1 IM and TM and environmentally relevant ratios of IM and TM. In the 8-day 1:1 IM and TM exposure tests mixture experiments were repeated 3 times with 4 dosing solutions per concentration, creating a total of n=12. In the 8-day environmentally relevant IM:TM exposure tests mixture experiments were repeated 2 times with 6 wells/beakers per concentration, creating a total of n=12. One well was removed from the environmentally relevant control exposures due to unrelated mortality. Environmentally relevant mixture

experiments were only run for the 8-day exposure period due to time constraints. Effects on growth, development and behavior were assessed as described below.

Impacts on Survival, Hatching and Growth After Exposure to IM for 8 days

Mortality and hatching were recorded daily throughout the IM:TM exposure period to calculate survival and the proportion of fish that hatched at 6 dpf. At the end of each exposure, total length (TL) of 2 fish from each dosing solution group was measured and used as an indicator of growth. ImageJ software (National Institute of Health and Laboratory for Optical and computational Instrumentation, Madison, Wisconsin, USA) was used to measure it.

Behavioral indicators of neurotoxicity

Behavioral indicators of neurotoxicity were analyzed with two assays, the embryonic motor activity assay performed on day 1 and the predator escape response assay performed on day 8. The embryonic motor activity assay was set up and analyzed in the same method as described above (1:1 mixtures n = 12; both environmentally relevant ratio mixtures n = 12).

The predator escape response assay, composed of latency, burst speed, length, and total escape response was assessed according to the methods described in Painter et al., (2009) and McGee et al., (2009). A dissecting microscope was set up with a 1 by 1 mm grid attached to a stimulus plate with a vibrating buzzer in place of the glass base piece. A Phantom MiroC210 camera (Vision Research, Wayne, NJ, USA) was attached to the dissecting microscope and set at 1000 fps. A ring light and two-headed external light source were arranged on the microscope to increase visibility. Videos were recorded

using the Phantom Camera Control Application software (Vision Research, Wayne, NJ, USA). The camera and the buzzer on the vibrational stimulus plate were activated simultaneously by a trigger. Individual larva, subsampled from each well within each dosing solution (1:1 mixtures n = 12; environmentally relevant mixtures n = 12), were placed in a plastic dish with 2 mL of standard culture water, with no neonicotinoids. This dish was placed on the vibrational stimulus plate and the larva was allowed 1 minute to acclimate prior to the trigger for the camera and buzzer being activated and the predator escape response being recorded. If the larva failed to produce a predator escape response after 2 stimuli attempts, a new larva from the same beaker was selected and tested in the original larva's place. Videos were recorded in cycles for all experiments (1 larva/beaker in each n) to remove time a source of variability. Video analysis was performed blind using the ImageJ software (National Institute of Health and Laboratory for Optical and Computational Instrumentation, Madison, Wisconsin, USA) following methods described by Diamond et al., (2016). Length data was obtained during this analysis, described above, as well as Latency (amount of time between initial stimulus and initial response movement), burst speed (speed of initial 40 ms of response), and total escape response (combination of latency, length of larva, and burst speed).

Molecular Docking

To predict the interactions of IM, TM, and nicotine with the nAChR of a vertebrate model (*Homo sapiens*) and an invertebrate model (*Lymnaea stagnalis*) *in-silico* molecular docking models were produced using the Maestro software program (Schrodinger, LLC, New York, NY, 2022). Chemical structures of imidacloprid, thiamethoxam, and nicotine were found in the ZINC database (Sterling & Irwin, 2015)

and the 3-dimensional structures of both nAChR proteins used (PDB IDs: 1UW6 and 6 PV7) were acquired from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDb) (Berman et al., 2000). Schrodinger's cheat sheets were referenced to complete protein and ligand preparation in Maestro using Protein Preparation Wizard (Schrodinger, LLC, New York, NY, 2022) and LigPrep (Schrodinger, LLC, New York, NY, 2022). Grid generation completed using the binding site of crystallized nicotine as a reference and glide ligand docking was done for each compound in both nAChR proteins. Once docking was modeled state and ionization penalties (quantification of the energetic favorability the docked states), docking and Glide gscores (approximate binding affinity and strength), and ligand interaction diagrams or LIDs (interaction of the ligand and specific amino acids) were generated through the Maestro program.

Data Analysis

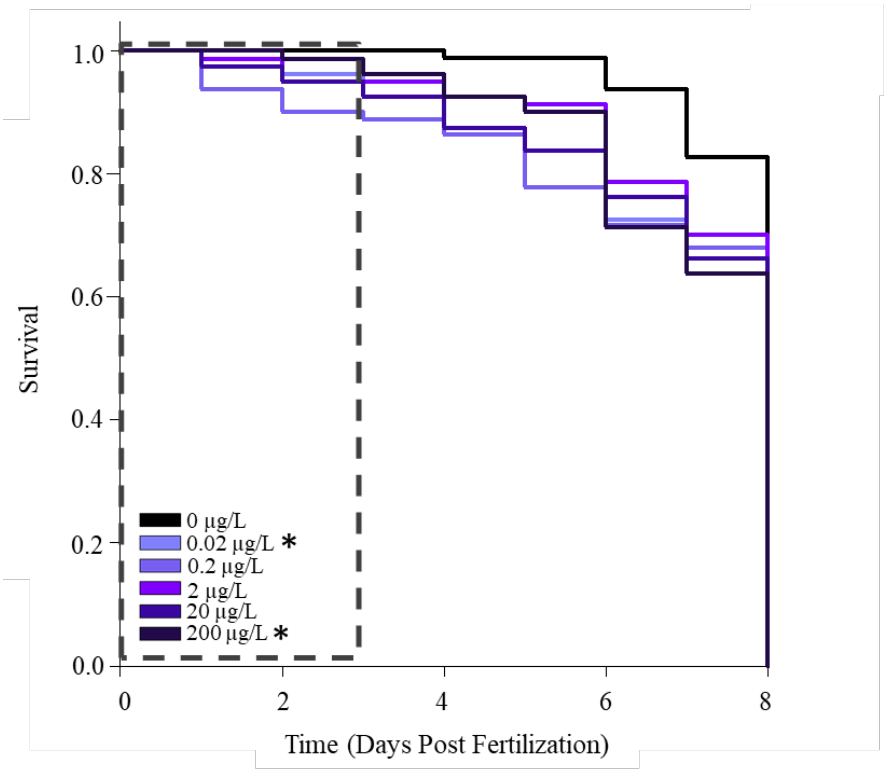
Data analyses were conducted using SigmaStat (Ver 4 integrated with SigmaPlot11, Spss Inc, Chicago, IL, USA). All experimental data except survival data were analyzed using the one-way analysis of variance (ANOVA) with Tukey's post-hoc tests at 95% confidence comparing each dosage group (0, 0.02, 0.2, 2, 20, 200 1:1 µg IM/L:µg TM/L, 0.05 µg IM/L : 0.15 µg TM/L, 0.05 µg IM/L : 0.2 µg TM/L, 0.05 µg IM/L : 0.25 µg TM/L) to the control (0 µg IM/L:µg TM/L) and assumptions of normality and homoscedasticity were checked using Shapiro-Wilk and Levene's test respectively. If data failed to pass normality, then ranked ANOVA was used to complete analysis. Survival data were analyzed using the Kaplan-Meier survival analysis with the Gehan-Breslow significance test.

Results

Impacts of Different Embryonic Exposure Periods of 1:1 IM:TM Mixtures on Survival

Chronic exposure to 1:1 IM:TM mixtures for 3 days post fertilization followed by 5 days depuration in moderately hard water resulted in significantly decreased survival at 0.02 µg IM/L: 0.02 µg TM/L and 200 µg IM/L: 200 µg TM/L (Figure 7A, Gehan-Breslow statistic = 11.520, df= 5, $p < 0.042$), while chronic exposure to 1:1 IM:TM mixtures for 8 days post fertilization with no depuration resulted in significantly increased survival at 2 µg IM/L: 2 µg TM/L and 20 µg IM/L: 20 µg TM/L (Figure 7B, Gehan-Breslow statistic = 31.359, df = 5, $p < 0.001$).

A.)



B.)

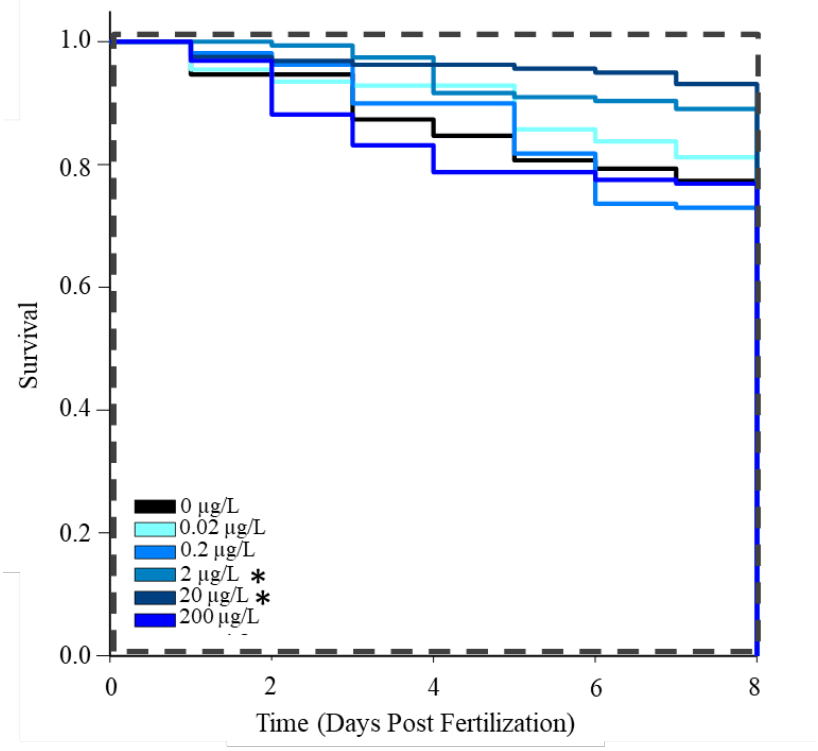


Figure 7. Kaplan-Meier survival analysis of fathead minnows exposed to increasing concentrations (µg/L) of 1:1 IM and TM beginning at 0 dpf and ending at either (A) 3 or (B) 8 dpf. Dotted boxes indicate IM:TM mixture exposure period. Asterisks indicate that groups differed significantly from the control according to Gehan-Breslow tests.

Impacts of Environmentally Relevant IM:TM Mixtures on Survival

Chronic exposure to environmentally relevant IM:TM mixtures for 8 days post fertilization significantly increased survival at the 1:4 (0.05 µg IM/L: 0.20 µg TM/L) and 1:5 (0.05 µg IM/L: 0.25 µg TM/L) mixtures (Figure 8, Gehan-Breslow statistic = 21.914, df = 3, $p < 0.001$).

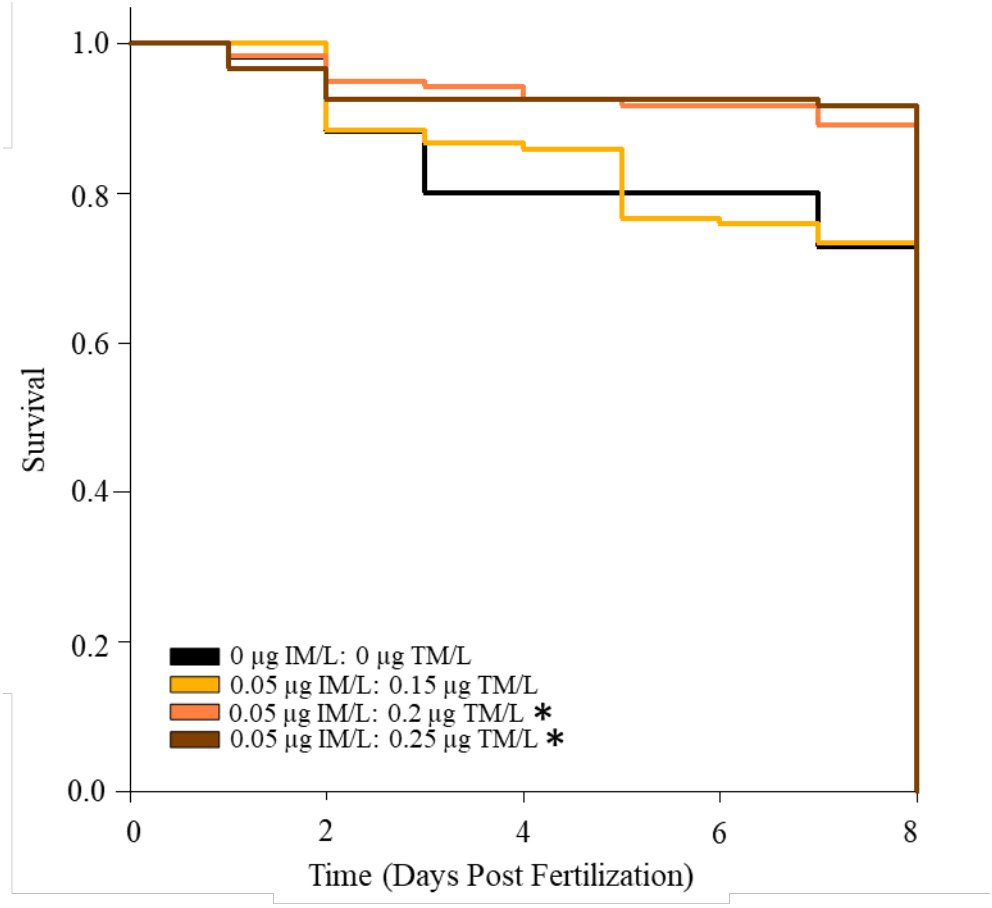
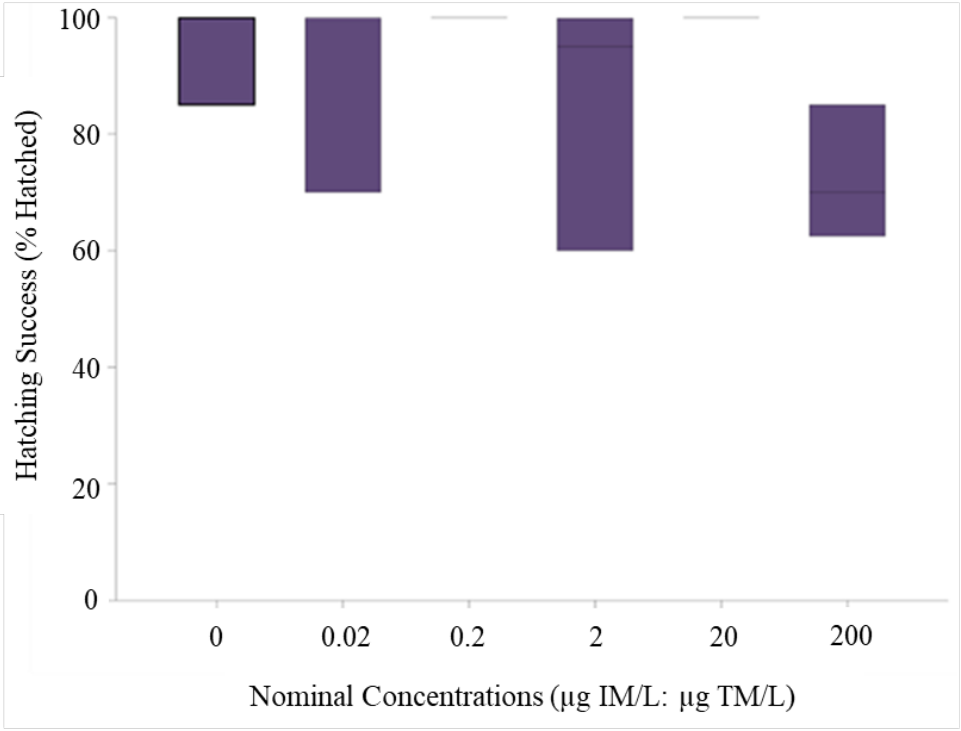


Figure 8. Kaplan-Meier survival analysis of fathead minnows exposed to environmentally relevant mixtures of IM & TM beginning at 0 dpf and ending at 8 dpf. Asterisks indicate that groups differed significantly from the control according to Gehan-Breslow tests.

Hatching Success Following Exposure to Different Embryonic Exposure Periods of 1:1 IM:TM Mixtures

Hatching success at 6 days post fertilization of fathead minnow embryos was not impacted by chronic exposure to 1:1 IM:TM mixtures for 3 days followed by 3 days depuration; there was a difference in hatching between groups but Tukey's post-hoc tests revealed no groups differed significantly from the controls (Figure 9A, Kruskal-Wallis ANOVA on Ranks with Tukey's post-hoc tests, $H = 11.233$, $df = 5$, $P = 0.047$). Likewise, no significant impact on hatching success at 6 days post fertilization was seen in fathead minnow embryos exposed to 1:1 IM:TM mixtures for 6 days (Figure 9B, Kruskal-Wallis ANOVA on Ranks with Tukey's post-hoc tests, $H = 8.3685$, $df= 5$, $P = 0.122$).

A.)



B.)

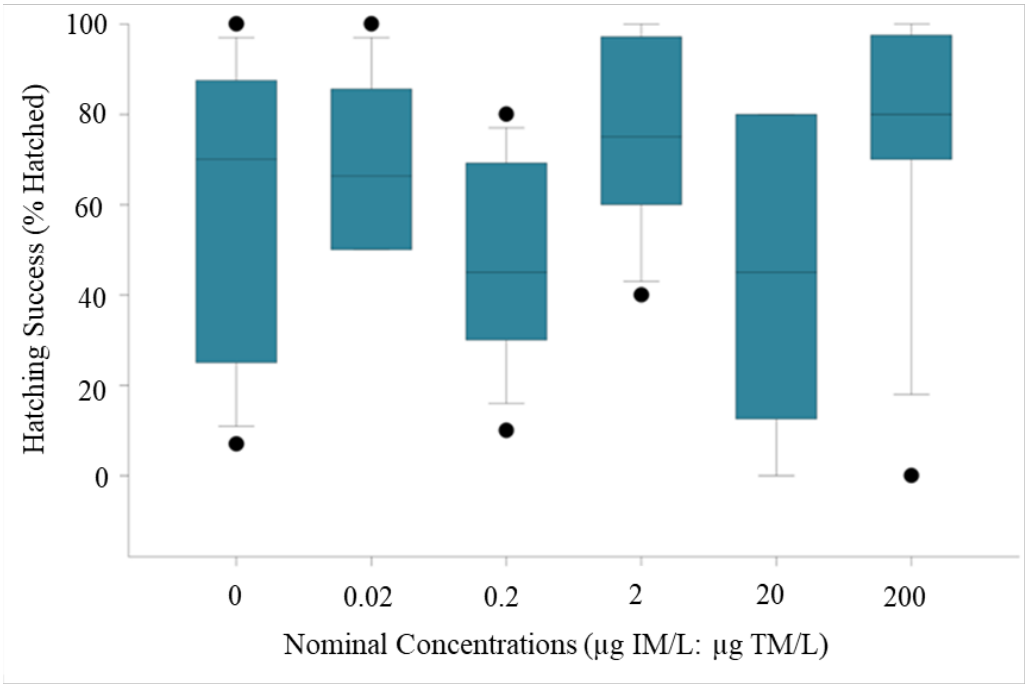


Figure 9. Percent fathead minnow embryos hatched at 6 days post fertilization following chronic exposure to 1:1 ratios of IM & TM for (A) 3 or (B) 6 days.

Hatching Success Following Exposure to Environmentally Relevant IM:TM Mixtures

Chronic exposure to environmentally relevant IM:TM mixtures for 6 days did not impair hatching success of fathead minnow embryos at 6 dpf (Figure 10, One Way ANOVA, $F = 1.697$, $df = 3, 42$, $P = 0.182$).

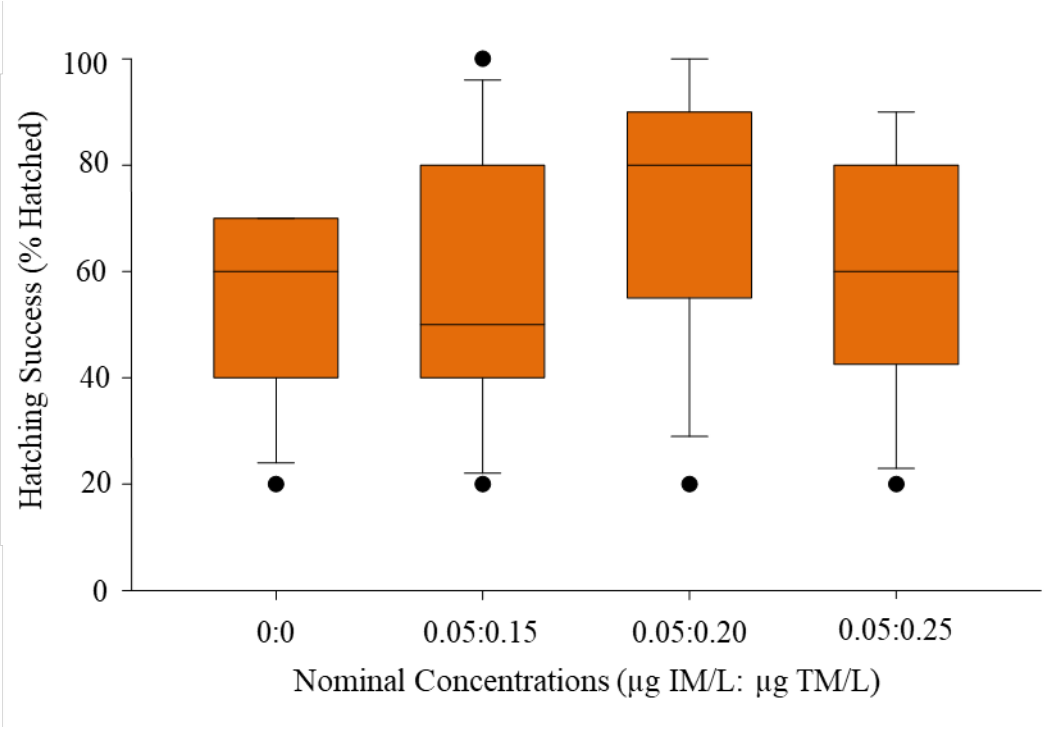
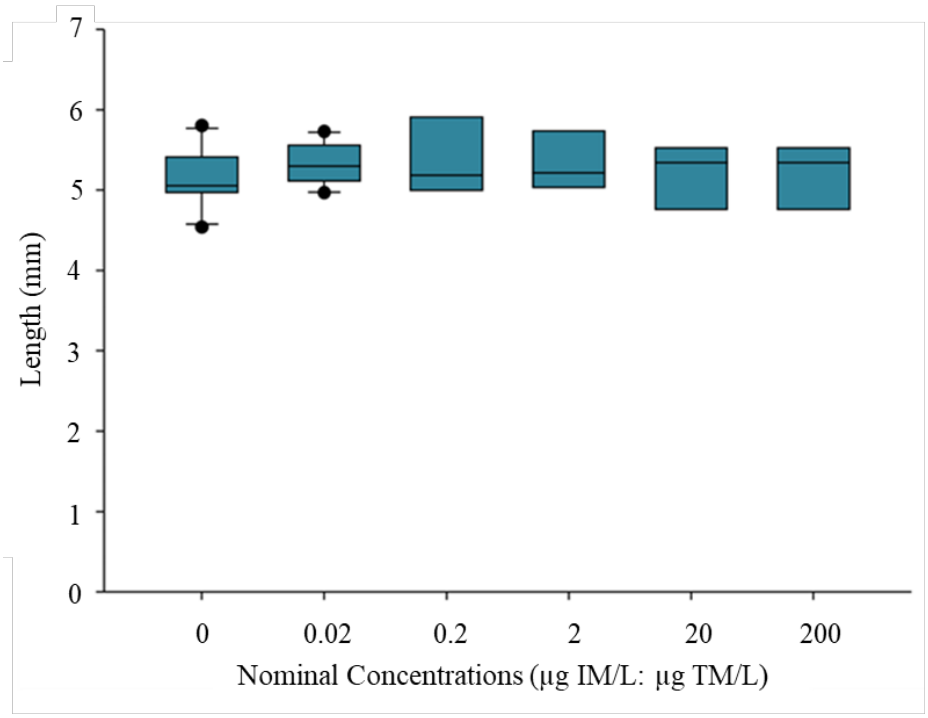


Figure 10. Percent hatched on day 6 post fertilization of fathead minnows exposed to environmentally relevant mixtures of IM & TM.

Impacts of Exposure to different Ratios of IM:TM Mixtures for 8 Days on Growth

Growth was not impacted by exposure to 1:1 IM:TM (Figure 11A, One Way ANOVA, $F = 0.574$, $df = 5, 80$, $P = 0.719$) or environmentally relevant mixtures of IM:TM (Figure 11B, One Way ANOVA, $F = 1.035$, $df = 3, 42$, $P = 0.387$).

A.)



B.)

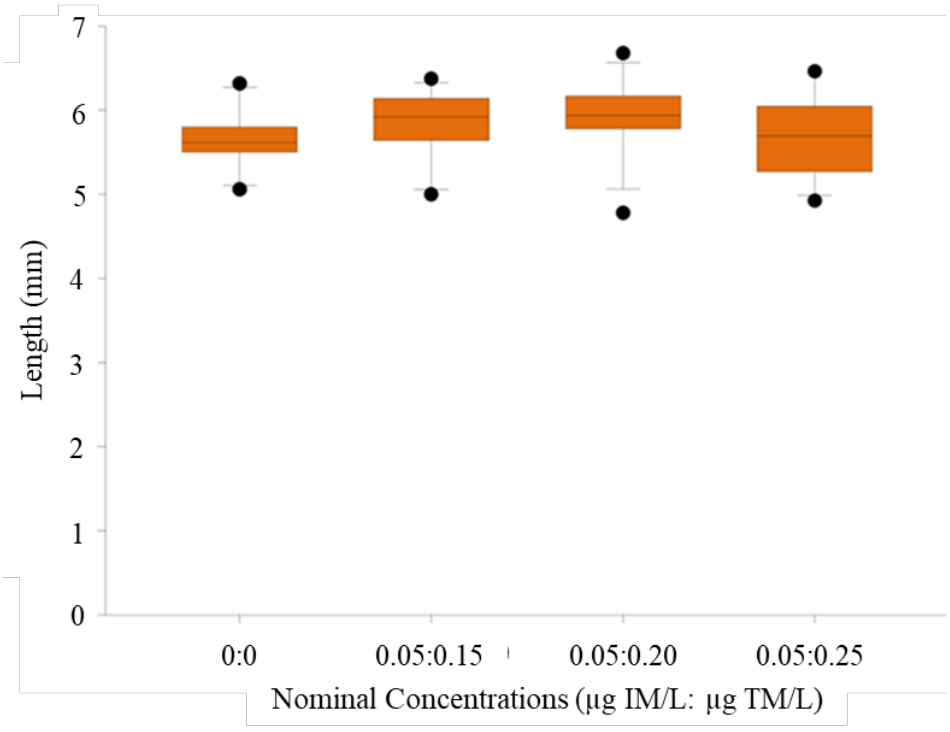
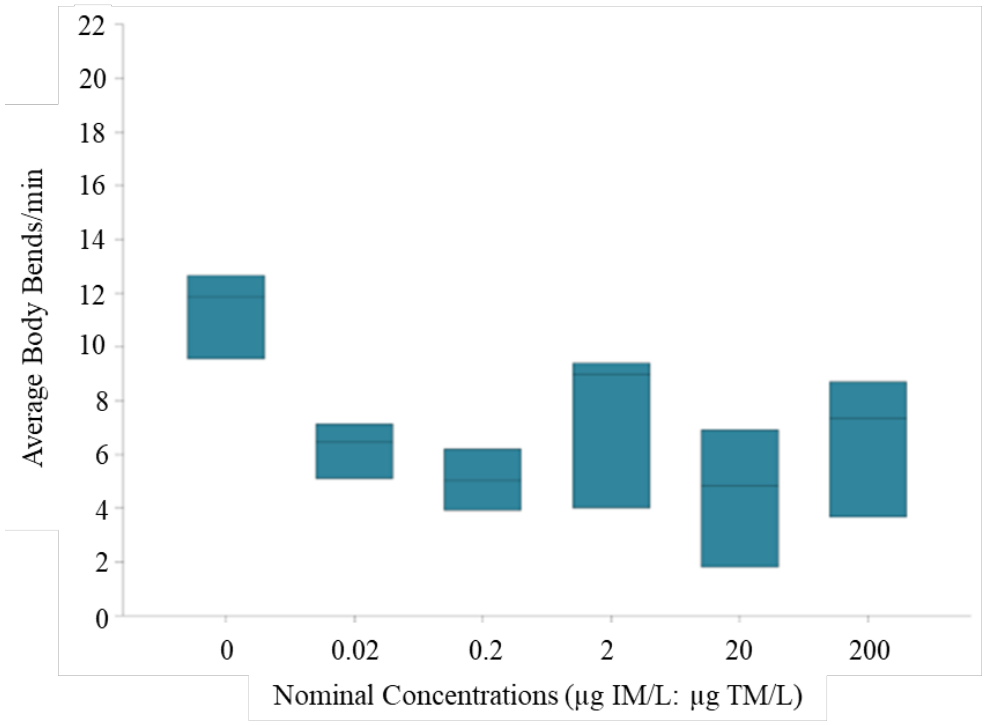


Figure 11. Effects of chronic exposure to increasing concentrations (A) 1:1 IM & TM mixtures or (B) environmentally relevant mixtures of IM for 8 days.

Embryonic Motor Activity Following 24 hours of Exposure to Different Ratios of IM:TM Mixtures

Exposure to 1:1 mixtures of IM and TM at concentrations up to 200 µg IM/L: µg TM/L (Figure 12A, Kruskal-Wallis ANOVA on Ranks, $H = 10.967$, $df = 5$, $P = 0.052$) or the environmentally relevant ratios of IM and TM mixtures (Figure 12B, One Way ANOVA, $F = 2.013$, $df = 3, 44$, $P = 0.126$) for 24 hours did not alter embryonic motor activity.

A.)



B.)

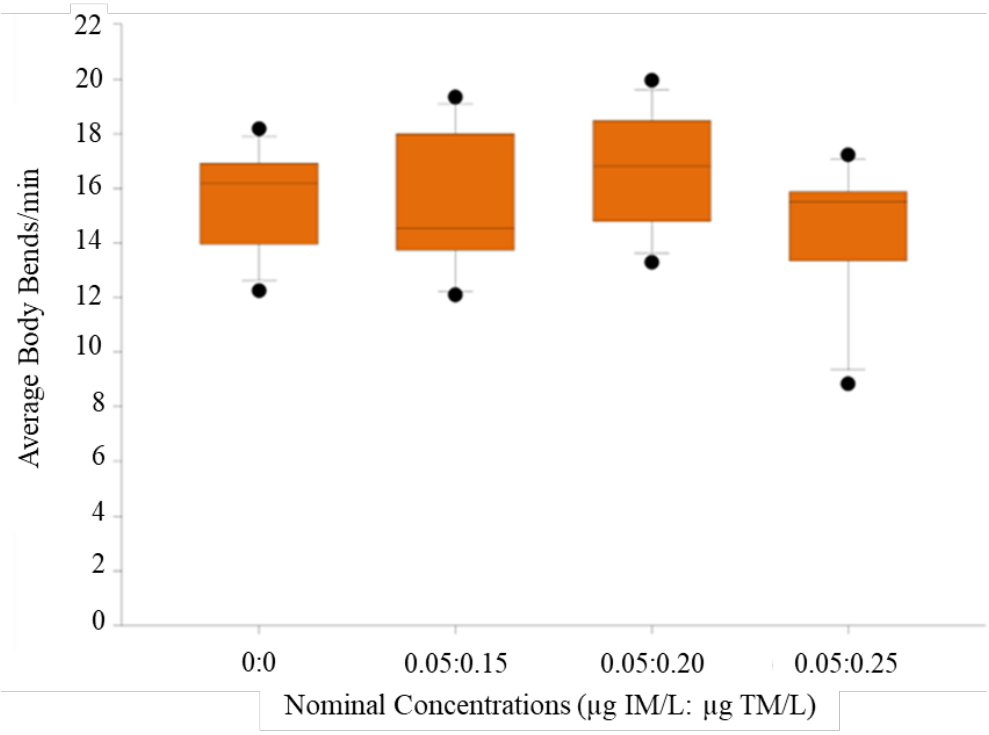


Figure 12. Average movements per minute of fathead minnow embryos exposed to (A) increasing or (B) environmentally relevant mixtures of IM and TM for 24 hours.

Impact of Acute Exposure to Environmentally Relevant Ratios of IM:TM Mixtures

Acute exposure of environmentally relevant ratios of IM:TM mixtures did not alter the embryonic motor activity of fathead minnow embryos (Figure 13, One Way ANOVA, F = 0.437, df = 3,20, P = 0.729).

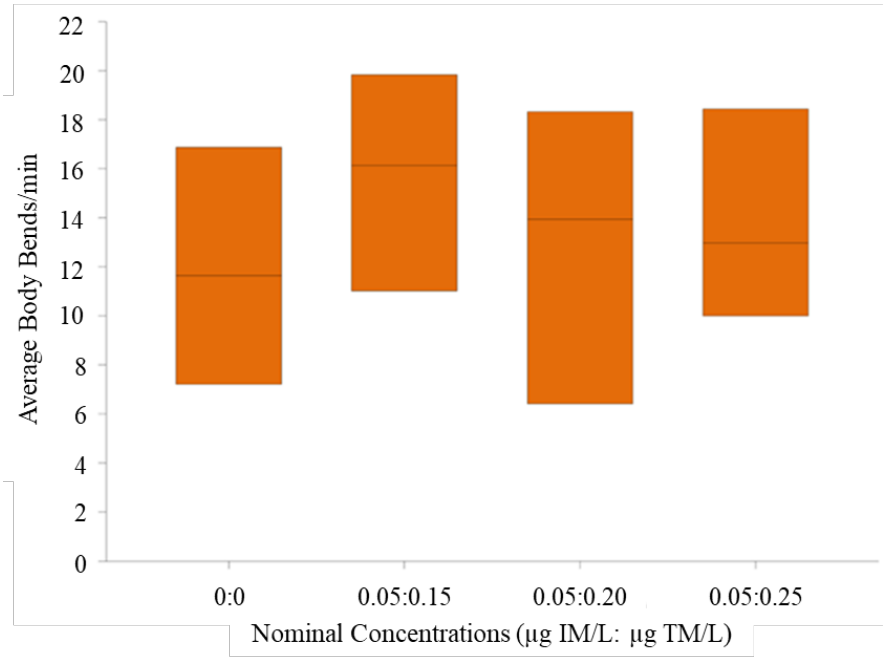


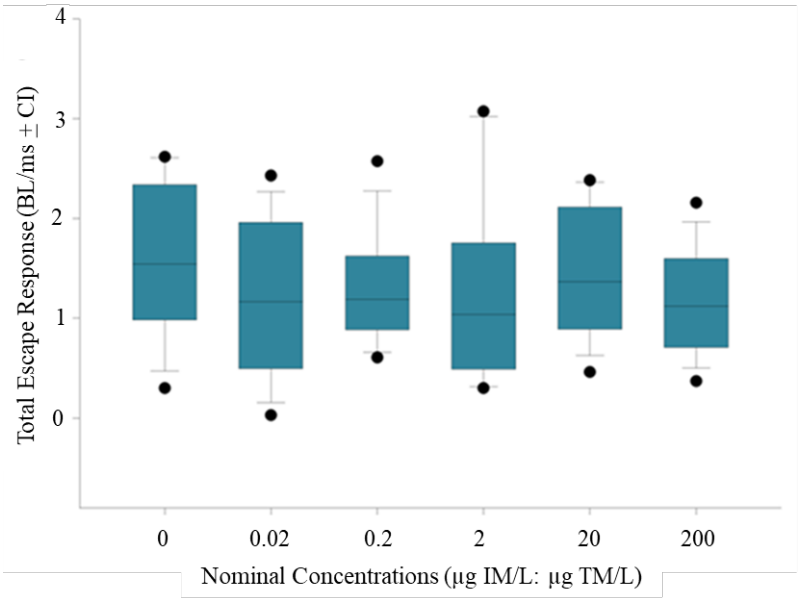
Figure 13. Average movements per minute of fathead minnow embryos spiked with environmentally relevant mixtures of IM.

Predator Escape Response Following Exposures to Different IM:TM Mixtures for 8 days

The predator escape response was not significantly affected by exposure to 1:1 IM:TM mixtures (Figure 14A, Kruskal-Wallis ANOVA on Ranks, H = 3.704, df = 5, P = 0.593), with neither latency (Table 2, Kruskal-Wallis ANOVA on Ranks, H = 5.241, df = 5, P = 0.387) nor burst speed (Table 2, Kruskal-Wallis ANOVA on Ranks, H = 6.240, df = 5, P = 0.284) exhibiting significant differences from the control. There was no impact

due to exposure to environmentally relevant ratios of IM:TM mixtures on predator escape response (Figure 14B, Kruskal-Wallis ANOVA on Ranks, H = 1.235, df= 3, P = 0.745), latency (Table 3, Kruskal-Wallis ANOVA on Ranks, H = 3.177, df = 3, P = 0.365), and burst speed (Table 3, One Way ANOVA, F = 2.59, df = 3,41, P = 0.066).

A.)



B.)

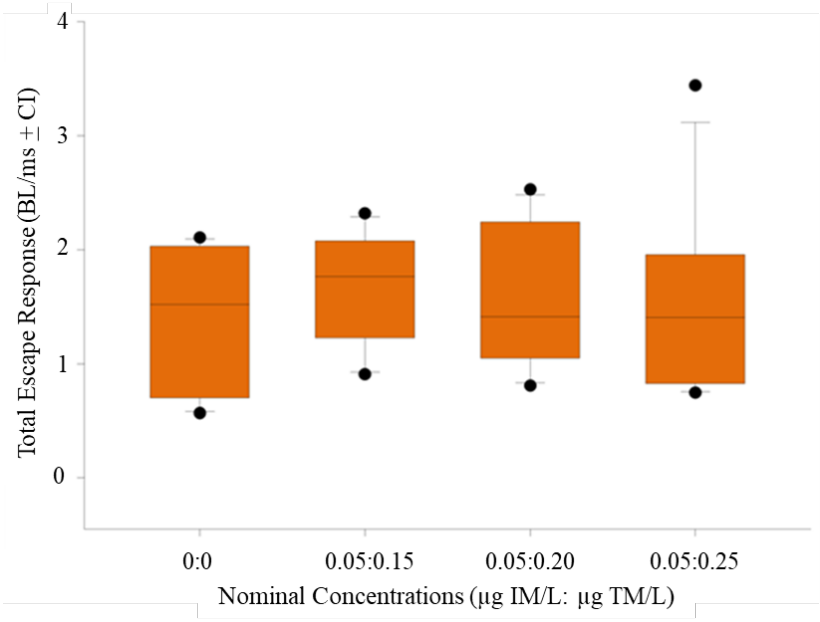


Figure 14. Mean total escape response of fathead minnow larvae exposed to (A) increasing concentrations or (B) environmentally relevant mixtures of IM and TM for 8 days.

Table 2. Mean values of predator escape response parameters of 0-8 day exposure 1:1 IM and TM measured after 8 days.

Concentration (µg IM/L: µg TM/L)	Latency (ms ± SE)	Burst Speed (BL/ms ± SE)
0:0	54.0 ± 23.4	0.040 < 0.01
0.02	53.1 ± 19.8	0.031 < 0.01
0.2	73.5 ± 29.4	0.038 < 0.01
2	71.8 ± 21.8	0.033 ± 0.01
20	83.2 ± 28.1	0.034 ± 0.01
200	86.3 ± 22.8	0.037 < 0.01

Table 3. Mean values of predator escape response parameters of 0-8 day exposure environmentally relevant ratios of IM and TM measured after 8 days.

Concentration (µg IM/L: µg TM/L)	Latency (ms ± SE)	Burst Speed (BL/ms ± SE)
0:0	54.0 ± 23.4	0.040 < 0.01
0.05:0.15	53.1 ± 19.8	0.031 < 0.01
0.05:0.20	73.5 ± 29.4	0.038 < 0.01
0.05:0.25	71.8 ± 21.8	0.033 < 0.01

Molecular Docking

Molecular modeling techniques allow us to predict interactions of IM and TM with an invertebrate (*Lymnaea stagnalis*) and vertebrate (*Homo sapiens*) nAChR compared with a positive control (nicotine). Binding affinity and strength were quantified as docking scores and Glide gscores, with scores < 0 indicating greater binding affinity and strength. Docking scores were calculated using the ionization penalty, referring to the protonation of the ligand, while Glide gscores were calculated using the state penalty, which is the same as an ionization penalty but it also takes the energy of the protonation state on the binding affinity. Glide gscores were considered to be more accurate because of the state penalty. The invertebrate docking scores for IM and TM bound in the nAChR were greater than that of nicotine and the Glide gscores of the IM and TM bound in the invertebrate nAChR were also greater than nicotine, but neither approximate score was different from each other for IM or TM. The same was true in the vertebrate nAChR (Table 2). The nAChR proteins for the *H. sapiens* and *L. stagnalis* were not made by the same person and thus data cannot be compared across receptors.

Table 4. Molecular docking calculations of the Docking score, Glide gscore, and ionization and state penalties of nicotine, imidacloprid, and thiamethoxam to the nAChR of *Lymnaea stagnalis* and *Homo sapiens.*

Species Compound	Ionization Penalty (Kcal/mol)	State Penalty (Kcal/mol)	Docking Score	Glide gscore
L. stagnalis				
Nicotine	0.033	-0.03	-10.332	-10.362
Imidacloprid	< 0.001	< 0.001	-6.495	-6.495
Thiamethoxam	< 0.000	< 0.001	-5.720	-5.720
H. Sapiens				
Nicotine	0.0329	0.03	-9.116	-9.146
Imidacloprid	< 0.001	< 0.001	-6.675	-6.675
Thiamethoxam	< 0.001	< 0.001	-4.519	-4.519

Glide was used to predict ligand interactions which were visualized by ligand interaction diagrams (LIDs). In the invertebrate positive control, nicotine-*L. stagnalis*, complex the TYR C:192 and TRP D:53 was bound by pi-cation interactions. TRP C:143 was bound to the nicotine by pi-cation and H-bond interactions to the methyl group and nitrogen of the pyrrolidine respectively, while also bonded with the pyridine by pi-pi stacking (Figure 15A). In the vertebrate positive control, nicotine-*H. sapiens*, complex the TRP A:149, TRP B:59, and TYR A:197 were bound to the pyrrolidine, additionally TRP A:149 is bound to the nitrogen of the pyrrolidine by H-bond interactions (Figure 15B).

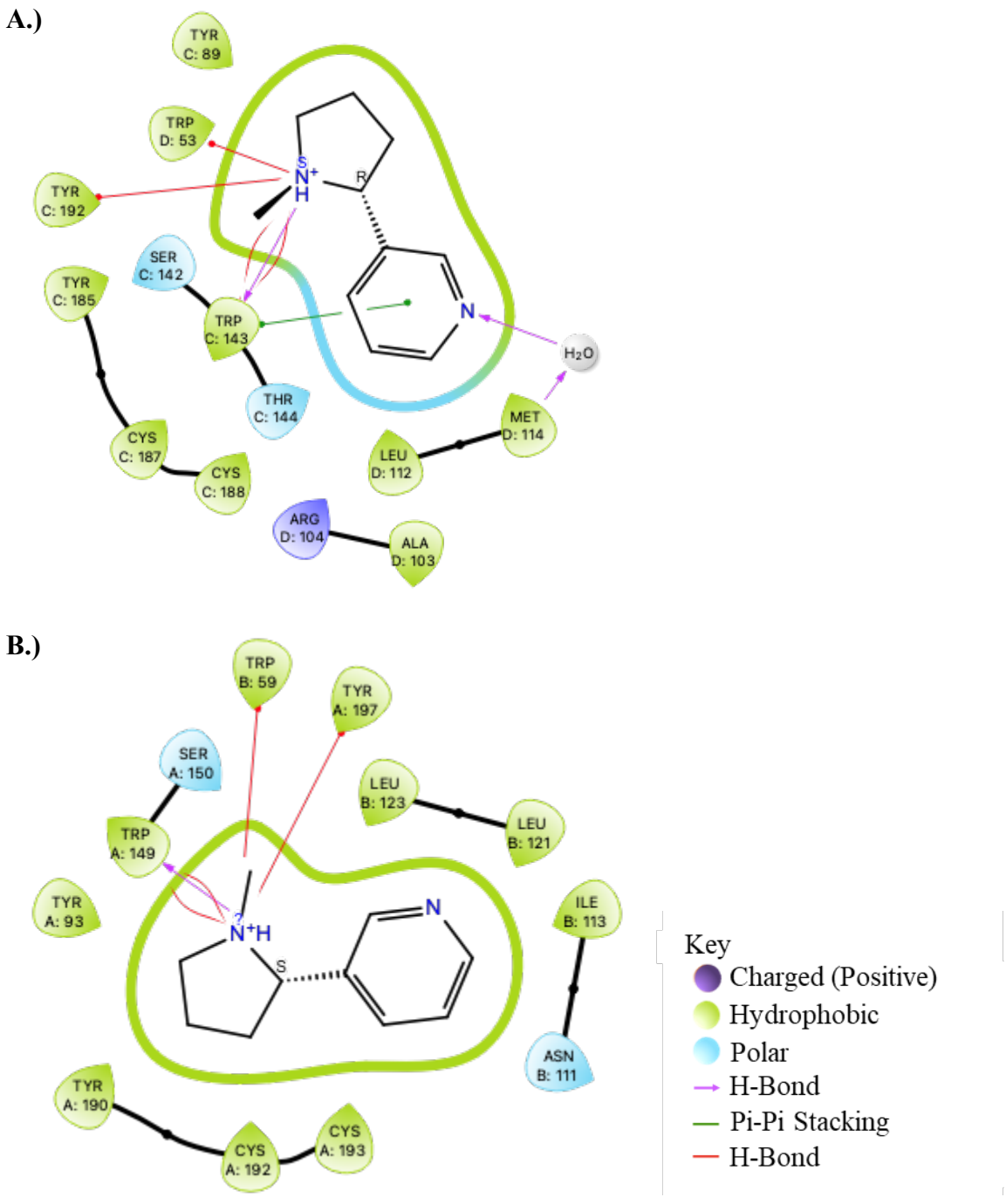


Figure 15. Ligand interaction diagrams generated by Schrödinger's Glide (Release 2021: Glide, Schrödinger, LLC, New York, NY, 2021). (A) Nicotine bound to the nAChR of *Lymnaea stagnalis* and (B) nicotine bound to the nAChR of *Homo sapiens*.

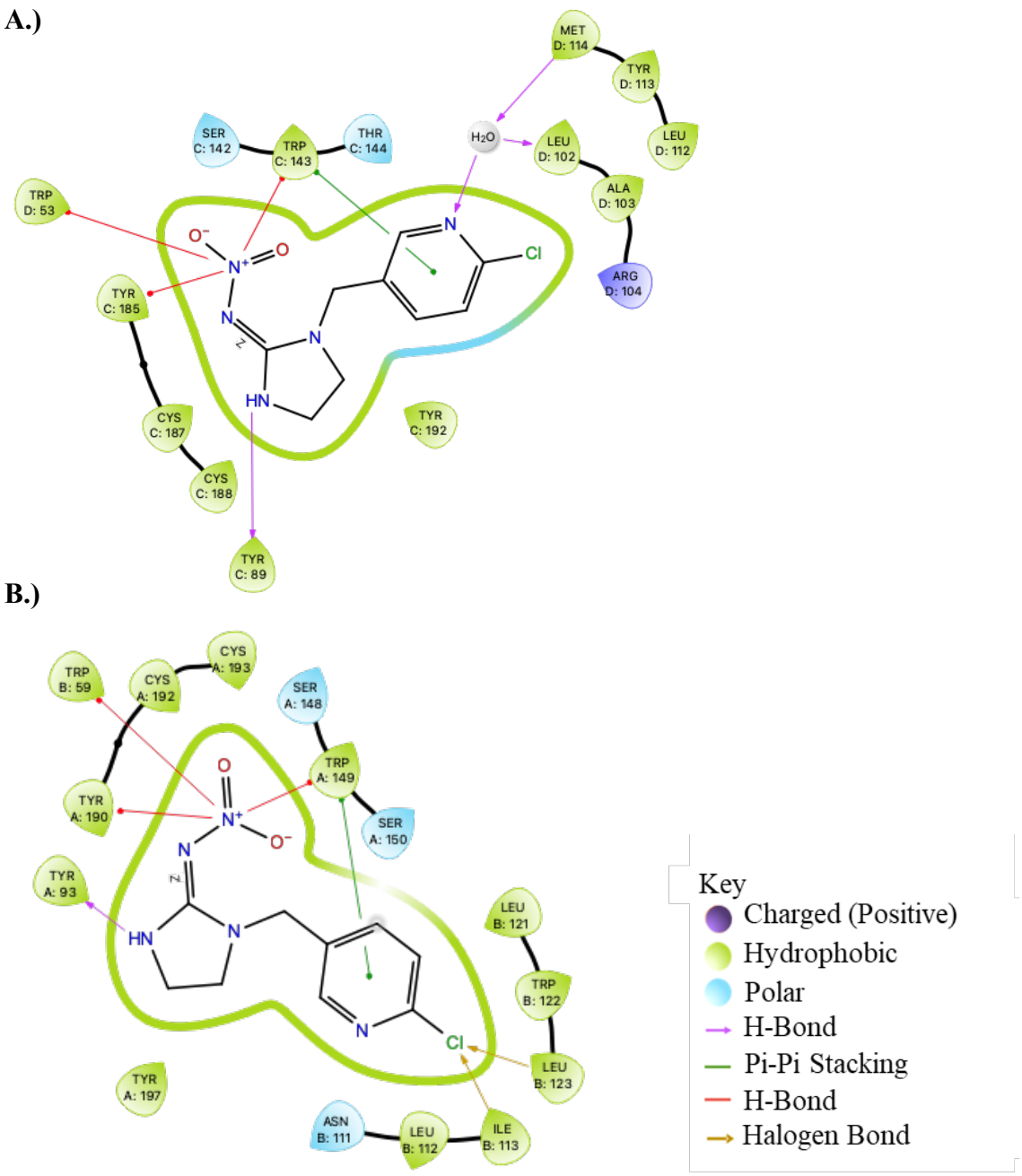


Figure 16. Ligand interaction diagrams generated by Schrödinger's Glide (Release 2021: Glide, Schrödinger, LLC, New York, NY, 2021). (A) Imidacloprid bound to the nAChR of *Lymnaea stagnalis* and (B) Imidacloprid bound to the nAChR of *Homo sapiens*.

In the IM-*L. stagnalis* complex TYR C:185, TRP D:53, and TRP C:143 were bound to the nitrogen of the nitroguanidine, and TRP C:143 was also bound with the pyridine. TYR A:89 was bound to the methyl group, LEU B: 123 and ILE B:113 were both bound by H-bonds to a H_2O molecule which was bound by another H-bond to the nitrogen of the pyridine (Figure 16A). In the IM-*H. sapiens* complex TYR A:190, TRP B:59, and TRP A:149 were bound to the nitrogen of the nitroguanidine, and TRP A:149 was also bound with the pyridine. TYR A:93 was bound to the methyl group, LEU B: 123 and ILE B:113 were both bound to the chlorine off the pyridine by halogen bonds (Figure 16B).

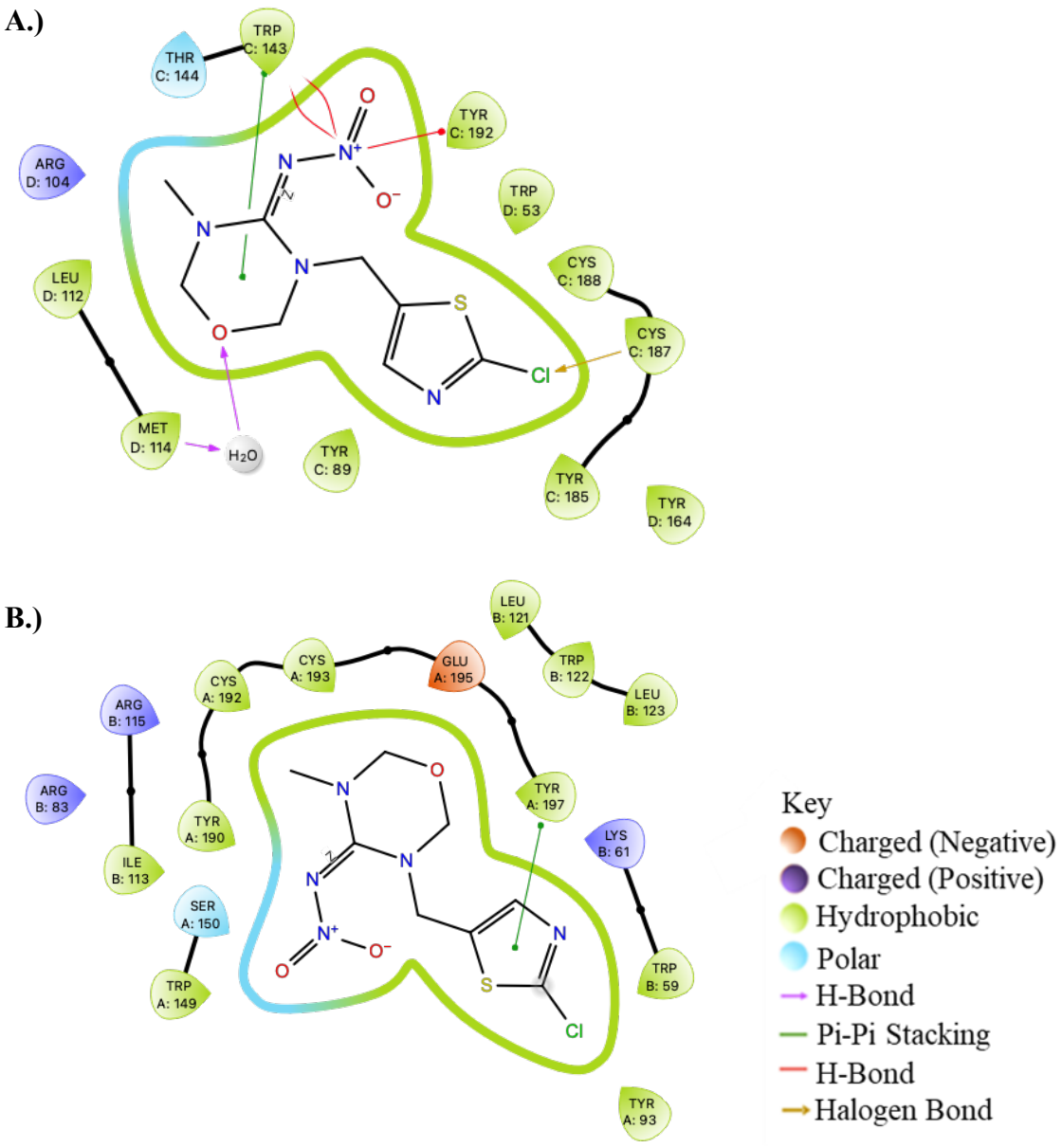


Figure 17. Ligand interaction diagrams generated by Schrödinger's Glide (Release 2021: Glide, Schrödinger, LLC, New York, NY, 2021). (A) Thiamethoxam bound to the nAChR of *Lymnaea stagnalis* and (B) thiamethoxam bound to the nAChR of *Homo sapiens*.

The TM-*L. stagnalis* complex had pi-cation interactions between the nitrogen of the nitroguanidine and TYR C:192 as well as TYR C:143. TYR C:143 was also bound to the nitrogen heterocyclic ring by pi-pi stacking. MET D:114 was bound by H-bonds to a

H_2O molecule which was bound by another H-bond to the nitrogen of the pyridine. CYS C:187 was bound by halogen bonds to the chlorine off the pyridine (Figure 17A). The TM-*H. sapiens* had only one interaction where thiazole was bound by pi-pi stacking to TYR A:197 (Figure 17B).

Discussion

Neonicotinoids are common contaminants in the environment and have been detected in mixtures at concentrations at or above regulatory guidelines in freshwater systems nationwide (Hladik & Kolpin, 2016; Morrissey et al., 2015; Senger, 2018; Wolfram et al., 2018). There is little information regarding the effects of exposure to mixtures of neonicotinoids on neurobehavioral endpoints. In this study, I demonstrated that chronic sublethal exposure to varying mixtures of IM and TM can both decrease and increase the survival of fathead minnows; however, hatching, embryonic motor activity, and predator escape response behaviors did not appear to be altered due to exposure. The in-silico findings indicated that IM and TM may bind and activate the nAChR of another vertebrate species, however laboratory findings did not appear to support those predictions. Taken together, my data suggests that IM and TM binary mixtures may not be as toxic as single TM exposures and have low affinity for the nAChR, but may cause toxicity when interacting with a different mechanism.

Exposure Period and Environmentally Relevant Mixtures of IM:TM Alters Survival, but Hatching Success and Growth Unaffected

Fathead minnows exposed to 1:1 mixture of IM and TM mixtures for 8 days exhibited increased survival at 2 and 20 µg IM/L: µg TM/L. This differs from our IM

only exposure, in which survival decreased at 0.2 µg IM/L (Chapter 1), and previous work done on TM only exposures on fathead minnows, which found that concentrations >1.57 µg TM/L decreased survival (Victoria et al., 2022). Fathead minnows exposed to the environmentally relevant ratios of 0.05 µg IM/L: 0.20 µg TM/L and 0.05 µg IM/L: 0.25 µg TM/L also exhibited increased survival when compared to the controls, again differing from the IM only exposure (Chapter 1) and the TM only exposures (Victoria et al., 2022). The differences between the mixture toxicity and the toxicity expressed when exposed to each pesticide individually has implications for toxicity testing, as standard practice for pesticide testing, and regulation targets compounds individually rather than in mixtures (Weisner et al., 2021). As pesticides are detected as mixtures in the environment (Hladik & Kolpin, 2016; Schmidt et al., 2022; Senger, 2018; Wolfram et al., 2018), without more study of the effects of pesticide mixtures we cannot accurately predict ecological risks (Weisner et al., 2021). Therefore, when assessing the toxicity of IM and TM in the laboratory and natural habitat settings, it is beneficial to consider how they behave in a mixture.

Interestingly, fish exposed to 1:1 mixtures of IM:TM for 3 days had greater mortality following depuration compared with fish exposed to a similar 1:1 mixture and environmentally relevant mixtures for 8 days without depuration. It should be noted that the survival of control fish was reduced compared with other experiments (see Figure 9B) so it is possible that the observed difference could be attributed to the overall quality of that batch of eggs used for those experiments. Fathead minnows exposed to TM during their larval stage only did not express an impact on survival (Victoria et al., 2022). The

increased survival in the 8 day exposures may indicate that there is a different mode of action being used by the neonicotinoids than the nAChR.

Hatching and growth were not significantly affected by exposure period or varying concentrations of IM and TM mixtures. These results are consistent with our results from the IM only (Chapter 1) exposure as well as previous work focusing on TM only (Victoria et al., 2022). Exposure to similar concentrations of IM and TM alone have been shown to cause malformations such as Japanese medaka exposed to IM in the µg/L range as embryos and larvae exhibited scoliosis, oedema, hemorrhage, and jaw/skull/tail deformities while zebrafish exposed to IM showed thickened muscle tissues. Chinese rare minnows exposed to TM in the range of µg/L were shown to have decreased body length at 50 µg TM/L (Zhu et al., 2019). That IM and TM mixtures did not show decreased growth or malformations may indicate that IM and TM may disrupt the other's ability to bind to the nAChR, or a receptor in another system, and decrease observed adverse developmental effects.

Acute and 24-hour exposure to Mixtures of IM:TM Does Not Impact Embryonic Motor Activity or Predator Escape Response

Embryonic motor activity was not impacted in both acute and 24 hour tests at varying concentrations of IM:TM mixtures, which was consistent with our IM only results but not with TM or nicotine result from existing literature (Victoria et al., 2022). Nicotine increased the embryonic motor activity when spiked into solution with fathead minnow embryos at high concentrations which indicates that the nAChR can be stimulated through this assay, while chronic exposure of fathead minnow embryos to TM increased embryonic motor activity at concentrations >155 µg TM/L (Victoria et al.,

2022). As this result was not observed in the difference in toxicity between these compounds may indicate a possible different mode of action for the neonicotinoids compared to the nicotine, as the lack of increased embryonic motor activity and no effect on the predator escape response indicate that the nAChR was likely not stimulated. This difference in toxicity when compared to nicotine may also be due to a difference in binding affinity, which could indicate the possibility of antagonistic effects when binding to the same receptor. IM and TM may disrupt each other's ability to bind to the fathead minnow nAChR and decrease observed adverse effects when present in a mixture.

Molecular Docking

IM, TM and other neonicotinoids are known to have stronger interactions with the nAChR of invertebrates than vertebrates (Tomizawa, 2013). However, molecular docking models did not predict lower binding affinity of IM and TM for the nAChR in the vertebrate model (*H. sapiens*) compared to the invertebrate model (*L. stagnalis*), conversely nicotine was reported to have high binding affinity for both species. IM was shown to have higher binding affinity than TM in both species as well, but not enough to truly consider the compounds to be different from each other. Docking scores and Glide gscores between the 3 compounds were closer in the invertebrate model than the vertebrate, as expected and which suggests that the IM and TM-*L. stagnalis* nAChR interactions are closer in strength to the nicotine-*L. stagnalis* nAChR interactions than interactions in the *H. sapiens* nAChR. These differences in the type and quantity of interactions that IM and TM form with the nAChR in both species may explain why each compound exhibits subtler or no behavioral and biochemical responses in fish, however IM and an IM and TM mixture being less toxic than TM in the same assay is not

supported by findings here. Recent studies have suggested that IM does not bind the nAChR of fathead minnows and that IM may be an antagonist for the GABA receptor in invertebrates (Stinson et al., 2022; Taylor-Wells et al., 2015). TM has been documented to disrupt the endocrine system of Chinese rare minnows (*Gobiocypris rarus*) at 50 µg TM/L after 90 days of exposure (Zhu et al., 2019). While not understood at the time of writing, the biotransformation of IM and TM within the fathead minnows may also play a role in the toxicity of mixtures. IM and TM are water soluble and in Sprague Dawley (SD) rats (*Rattus norvegicus*) exposed to IM and TM, the neonicotinoids were processed by the kidneys and excreted along with several metabolites in the urine (Xu et al., 2021), should a similar system exist in fathead minnows this biotransformation and elimination of the neonicotinoids could influence the dose response seen in the embryos and larvae. Further research is needed to elucidate the interactions between IM and TM and the fathead minnow nAChR and the possible modes of action of these neonicotinoids in other systems within fathead minnows, such as the limbic or endocrine systems.

Limitations

The sample size for each test was small and allowed for high variability, but consistent with existing literature which explores multiple endpoints (Diamond et al., 2016; Nair et al., n.d.; Thomas et al., 2009). Sample size was limited by the time required to complete each assay and studies which focused on fewer endpoints were able to have greater sample sizes (McGee et al., 2009; Painter et al., 2009). The expected adverse effects due to IM would have been subtle due to its low binding specificity to the invertebrate nAChR, so the high variability may have prevented effects from being identified. Our controls had unexpected mortality; abnormal control wells were removed

while remaining control data was compared to previous control data using a One Way ANOVA and was not found to be significantly different. Our dosing solution concentrations have not yet been confirmed which limits the accuracy of conclusions drawn from the results. Confirmation of our dosing solutions will provide greater confidence in our results. And finally, the proteins used to model our vertebrate nAChRs was not a fathead minnow nAChR. As biological systems are conserved the *H. sapiens* nAChR may serve as a proxy but cannot be used to derive specific information about fathead minnow binding affinity.

Conclusions

This work investigated the possible toxicological risks posed to the model organism, fathead minnow, by mixtures of the neonicotinoids imidacloprid and thiamethoxam. These mixtures significantly impacted survival, both increasing and decreasing it, at and above environmentally relevant concentrations, while other physiological and behavioral endpoints were not impacted. Preliminary molecular docking gave insight into predicted interactions for imidacloprid and thiamethoxam in the nAChR vertebrate and invertebrate model organisms, which could begin to explain the lack of sublethal effects seen in fathead minnow larvae exposed to mixtures. The failure of mixture exposures to produce neurobehavioral toxicity as well as the increase noted in survival may indicate that there is another mode of action for IM and TM in fathead minnows. These results raise questions about how these sublethal concentrations may impact fish survival in the environment where mixtures are detected, as well as questions about other modes of action for neonicotinoids in fathead minnows. In summary,

continued study of the effects of imidacloprid an thiamethoxam mixtures is necessary to determine the mode of action.

Summary Conclusion

The overarching goal of this project was to learn more about the effects of exposure to IM and mixtures of IM and TM on fathead minnow embryos and larval development and behavior. When assessing the toxicity of environmental contaminants it is common to focus on chemicals individually; however, existing research indicates that there may be greater adverse effects following exposure to mixtures rather than to contaminants individually (Carvalho et al., 2014; Junghans et al., 2006; Kortenkamp & Altenburger, 1999; Lydy et al., 2004). I found significant effects on survival in both the single and binary exposure, however the effect itself differed. IM alone and exposure of IM:TM 1:1 mixture for 3 days decreased survival, while the exposure of IM:TM 1:1 for 8 days and the environmentally relevant mixtures of IM:TM increased it. This may be due to hormesis, possibly indicating the presence of a coping mechanism in fathead minnows for processing neonicotinoids which requires a certain threshold concentration to activate or a different mode of action for IM and TM in fathead minnows. The results of this study suggest that the nAChR is not activated by IM or TM, despite *in silico* molecular modeling predicting that they would bind to this receptor. Neither embryonic motor activity nor predator escape response, two behavioral assays associated with the activation of nAChR in fish (McGee et al., 2009; Painter et al., 2009; Victoria et al., 2022), were affected by exposure to neonicotinoids for any length of time, acute or chronic exposure. While further insight into where and how IM and TM interact with fathead minnow physiology is needed, the negative effects on survival due to exposure at 0.2 µg IM/L for 8 days and 0.02 µg IM/L: µg TM/L for 3 days may have implications for populations of fathead minnows in the environment. The embryonic and early larval

stage is important for the recruitment of fathead minnows into the greater population and also a period of high risk for fathead minnow larvae mortality due to predators or starvation (Garrido et al., 2015). Compounding existing stressors with possible mortality from neonicotinoids may increase risk of mortality to fathead minnows in the environment, necessitating continued research into the effects of single and binary exposures of IM and TM on fathead minnows.

REFERENCES

Anderson, J. C., Dubetz, C., & Palace, V. P. (2015). Neonicotinoids in the Canadian aquatic environment: A literature review on current use products with a focus on fate, exposure, and biological effects. *Science of The Total Environment*, *505*, 409–422. https://doi.org/10.1016/j.scitotenv.2014.09.090

Beggel, S., Werner, I., Connon, R. E., & Geist, J. P. (2010). Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval fathead minnow (Pimephales promelas). *Science of The Total Environment*, *408*(16), 3169–3175. https://doi.org/10.1016/j.scitotenv.2010.04.004

Beketov, M. A., & Liess, M. (2008). Acute and delayed effects of the neonicotinoid insecticide thiacloprid on seven freshwater arthropods. *Environmental Toxicology and Chemistry*, *27*(2), 461. https://doi.org/10.1897/07-322R.1

Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S., & Krystal, J. H. (2000). Antidepressant effects of ketamine in depressed patients. *Biological Psychiatry*, *47*(4), 351–354. https://doi.org/10.1016/S0006-3223(99)00230-9

Borsuah, J. F., Messer, T. L., Snow, D. D., Comfort, S. D., & Mittelstet, A. R. (2020). Literature Review: Global Neonicotinoid Insecticide Occurrence in Aquatic Environments. *Water*, *12*(12), 3388. https://doi.org/10.3390/w12123388

Butler, D. (2018). Scientists hail European ban on bee-harming pesticides. *Nature*. https://doi.org/10.1038/d41586-018-04987-4

Byrne, F. J., & Toscano, N. C. (2006). Uptake and persistence of imidacloprid in grapevines treated by chemigation. *Crop Protection*, *25*(8), 831–834. https://doi.org/10.1016/j.cropro.2005.11.004

Carvalho, R. N., Arukwe, A., Ait-Aissa, S., Bado-Nilles, A., Balzamo, S., Baun, A., Belkin, S., Blaha, L., Brion, F., Conti, D., Creusot, N., Essig, Y., Ferrero, V. E. V., Flander-Putrle, V., Fürhacker, M., Grillari-Voglauer, R., Hogstrand, C., Jonáš,

A., Kharlyngdoh, J. B., … Lettieri, T. (2014). Mixtures of Chemical Pollutants at European Legislation Safety Concentrations: How Safe Are They? *Toxicological Sciences*, *141*(1), 218–233. https://doi.org/10.1093/toxsci/kfu118

Cavallaro, M. C. (2019). Neonicotinoids and other agricultural stressors collectively modify aquatic insect communities. *Chemosphere 226*, 945-955. https://doi.org/10.1016/j.chemosphere.2019.03.176

Crosby, E. B., Bailey, J. M., Oliveri, A. N., & Levin, E. D. (2015). Neurobehavioral impairments caused by developmental imidacloprid exposure in zebrafish. *Neurotoxicology and Teratology*, *49*, 81–90. https://doi.org/10.1016/j.ntt.2015.04.006

Diamond, K. M., Schoenfuss, H. L., Walker, J. A., & Blob, R. W. (2016). Flowing water affects fish fast-starts: Escape performance of the Hawaiian stream goby, *Sicyopterus stimpsoni*. *The Journal of Experimental Biology*, *219*(19), 3100–3105. https://doi.org/10.1242/jeb.137554

Faria, M., Bedrossiantz, J., Prats, E., Rovira Garcia, X., Gómez-Canela, C., Piña, B., & Raldúa, D. (2019). Deciphering the mode of action of pollutants impairing the fish larvae escape response with the vibrational startle response assay. *Science of The Total Environment*, *672*, 121–128. https://doi.org/10.1016/j.scitotenv.2019.03.469

Fraysse, B., Mons, R., & Garric, J. (2006). Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. *Ecotoxicology and Environmental Safety*, *63*(2), 253–267. https://doi.org/10.1016/j.ecoenv.2004.10.015

Garrido, S., Ben-Hamadou, R., Santos, A. M. P., Ferreira, S., Teodósio, M. A., Cotano, U., Irigoien, X., Peck, M. A., Saiz, E., & Ré, P. (2015). Born small, die young: Intrinsic, size-selective mortality in marine larval fish. *Scientific Reports*, *5*(1), 17065. https://doi.org/10.1038/srep17065

Gibbons, D., Morrissey, C., & Mineau, P. (2015). A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environmental Science and Pollution Research*, *22*(1), 103–118. https://doi.org/10.1007/s11356-014-3180-5

Gill, R. J., Ramos-Rodriguez, O., & Raine, N. E. (2012). Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature*, *491*(7422), 105–108. https://doi.org/10.1038/nature11585

Goulson, D. (2013). Review: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology*, *50*(4), 977–987. https://doi.org/10.1111/1365-2664.12111

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., & Decourtye, A. (2012). A common pesticide decreases foraging success and survival in honeybees. *Science*, *336*(6079), 348–350. https://doi.org/10.1126/science.1215039

Hladik, M. L., & Kolpin, D. W. (2016). First national-scale reconnaissance of neonicotinoid insecticides in streams across the USA. *Environmental Chemistry*, *13*(1), 12. https://doi.org/10.1071/EN15061

Huseth, A. S., & Groves, R. L. (2014). Environmental Fate of Soil Applied Neonicotinoid Insecticides in an Irrigated Potato Agroecosystem. *PLoS ONE*, *9*(5), e97081. https://doi.org/10.1371/journal.pone.0097081

Ihara, M., & Matsuda, K. (2018). Neonicotinoids: Molecular mechanisms of action, insights into resistance and impact on pollinators. *Current Opinion in Insect Science*, *30*, 86–92. https://doi.org/10.1016/j.cois.2018.09.009

Islam, Md. A., Hossen, Md. S., Sumon, K. A., & Rahman, M. M. (2019). Acute toxicity of imidacloprid on the developmental stages of common carp *Cyprinus carpio*. *Toxicology and Environmental Health Sciences*, *11*(3), 244–251. https://doi.org/10.1007/s13530-019-0410-8

Jeschke, P., Nauen, R., Schindler, M., & Elbert, A. (2011). Overview of the Status and Global Strategy for Neonicotinoids. *Journal of Agricultural and Food Chemistry*, *59*(7), 2897–2908. https://doi.org/10.1021/jf101303g

Junghans, M., Backhaus, T., Faust, M., Scholze, M., & Grimme, L. (2006). Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. *Aquatic Toxicology*, *76*(2), 93–110. https://doi.org/10.1016/j.aquatox.2005.10.001

Kienzler, A., Halder, M., & Worth, A. (2016). Waiving chronic fish tests: Possible use of acute-to-chronic relationships and interspecies correlations. *Toxicological & Environmental Chemistry*, 1–23. https://doi.org/10.1080/02772248.2016.1246663

Kortenkamp, A., & Altenburger, R. (1999). Approaches to assessing combination effects of oestrogenic environmental pollutants. *Science of The Total Environment*, *233*(1–3), 131–140. https://doi.org/10.1016/S0048-9697(99)00228-4

Lanteigne, M., Whiting, S. A., & Lydy, M. J. (2015). Mixture toxicity of imidacloprid and cyfluthrin to two non-target species, the fathead minnow *Pimephales promelas* and the amphipod *Hyalella azteca*. *Archives of Environmental Contamination and Toxicology*, *68*(2), 354–361. https://doi.org/10.1007/s00244-014-0086-7

Liu, X., Zhang, Q., Li, S., Mi, P., Chen, D., Zhao, X., & Feng, X. (2018). Developmental toxicity and neurotoxicity of synthetic organic insecticides in zebrafish (Danio rerio): A comparative study of deltamethrin, acephate, and thiamethoxam. *Chemosphere*, *199*, 16–25. https://doi.org/10.1016/j.chemosphere.2018.01.176

Lydy, M., Belden, J., Wheelock, C., Hammock, B., & Denton, D. (2004). Challenges in Regulating Pesticide Mixtures. *Ecology and Society*, *9*(6), art1. https://doi.org/10.5751/ES-00694-090601

Ma, X., Li, H., Xiong, J., Mehler, W. T., & You, J. (2019). Developmental Toxicity of a Neonicotinoid Insecticide, Acetamiprid to Zebrafish Embryos. *Journal of Agricultural and Food Chemistry*, *67*(9), 2429–2436. https://doi.org/10.1021/acs.jafc.8b05373

Maloney, E. M., Morrissey, C. A., Headley, J. V., Peru, K. M., & Liber, K. (2017). Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios: Acute neonicotinoid mixture toxicity to *C. dilutus*. *Environmental Toxicology and Chemistry*, *36*(11), 3091–3101. https://doi.org/10.1002/etc.3878

Maloney, E. M., Morrissey, C. A., Headley, J. V., Peru, K. M., & Liber, K. (2018). Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in Chironomus dilutus? *Ecotoxicology and Environmental Safety*, *156*, 354–365. https://doi.org/10.1016/j.ecoenv.2018.03.003

McGee, M. R., Julius, M. L., Vajda, A. M., Norris, D. O., Barber, L. B., & Schoenfuss, H. L. (2009). Predator avoidance performance of larval fathead minnows (Pimephales promelas) following short-term exposure to estrogen mixtures. *Aquatic Toxicology*, *91*(4), 355–361. https://doi.org/10.1016/j.aquatox.2008.12.002

Mora-Zamorano, F. X., Svoboda, K. R., & Carvan, M. J. (2016). The Nicotine-Evoked Locomotor Response: A Behavioral Paradigm for Toxicity Screening in Zebrafish (Danio rerio) Embryos and Eleutheroembryos Exposed to Methylmercury. *PLOS ONE*, *11*(4), e0154570. https://doi.org/10.1371/journal.pone.0154570

Morrissey, C. A., Mineau, P., Devries, J. H., Sanchez-Bayo, F., Liess, M., Cavallaro, M. C., & Liber, K. (2015). Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. *Environment International*, *74*, 291–303. https://doi.org/10.1016/j.envint.2014.10.024

Nair, S. G., Radha, R., & Nair, C. R. (2017). Studies on acute toxicity to pesticide stress in a freshwater fish Cirrhinus mrigala. *Int. J. Fish. Aquat, 5(4),* 355-358.

OECD. (2012). Test No. 210: Fish, Early-life stage toxicity test. OECD Guidelines for the testing of chemicals, section *2*. *OECD Publishing*.

Painter, M. M., Buerkley, M. A., Julius, M. L., Vajda, A. M., Norris, D. O., Barber, L. B., Furlong, E. T., Schultz, M. M., & Schoenfuss, H. L. (2009). Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, *28*(12), 2677. https://doi.org/10.1897/08-556.1

Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B. K., Pedersen, T. R., Yourstone, J., & Smith, H. G. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*, *521*(7550), 77–80. https://doi.org/10.1038/nature14420

Schmidt, T. S., Miller, J. L., Mahler, B. J., Van Metre, P. C., Nowell, L. H., Sandstrom, M. W., Carlisle, D. M., Moran, P. W., & Bradley, P. M. (2022). Ecological consequences of neonicotinoid mixtures in streams. *Science Advances*, *8*(15), eabj8182. https://doi.org/10.1126/sciadv.abj8182\

Senger, S. (2018). 2018 Targeted Sampling Summary Report. *Wisconsin Department of Agriculture, Trade, and Consumer Protection* 1-24.

Senger, S., Cook, C., Bowman, L., Graham, R., Mccolloch, M., Oemig, O., Blanchard, D., Debaker, A., Kelley, G., & Shafiee, F. (2019). *A summary of groundwater and surface water test results for neonicotinoid insecticides from 2008 through 2016*. 1-49.

Shuman-Goodier, M. E., & Propper, C. R. (2016). A meta-analysis synthesizing the effects of pesticides on swim speed and activity of aquatic vertebrates. *Science of The Total Environment*, *565*, 758–766. https://doi.org/10.1016/j.scitotenv.2016.04.205

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D. W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C. H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E. A. D., Morrissey, C. A., … Wiemers, M. (2015). Systemic insecticides (neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research*, *22*(1), 5–34. https://doi.org/10.1007/s11356-014-3470-y

Starner, K., & Goh, K. S. (2012). Detections of the Neonicotinoid Insecticide Imidacloprid in Surface Waters of Three Agricultural Regions of California, USA, 2010–2011. *Bulletin of Environmental Contamination and Toxicology*, *88*(3), 316–321. https://doi.org/10.1007/s00128-011-0515-5

Sterling, T., & Irwin, J. J. (2015). Zinc 15 – Ligand Discovery for Everyone. *Journal of Chemical Information and Modeling*, *55*(11), 2324–2337. https://doi.org/10.1021/acs.jcim.5b00559

Stinson, S. A., Hasenbein, S., Connon, R. E., Deng, X., Alejo, J. S., Lawler, S. P., & Holland, E. B. (2022). Agricultural surface water, imidacloprid, and chlorantraniliprole result in altered gene expression and receptor activation in Pimephales promelas. *Science of The Total Environment*, *806*, 150920. https://doi.org/10.1016/j.scitotenv.2021.150920

Taylor-Wells, J., Brooke, B. D., Bermudez, I., & Jones, A. K. (2015). The neonicotinoid imidacloprid, and the pyrethroid deltamethrin, are antagonists of the insect Rdl GABA receptor. *Journal of Neurochemistry*, *135*(4), 705–713. https://doi.org/10.1111/jnc.13290

Thomas, L. T., Welsh, L., Galvez, F., & Svoboda, K. R. (2009). Acute nicotine exposure and modulation of a spinal motor circuit in embryonic zebrafish. *Toxicology and Applied Pharmacology*, *239*(1), 1–12. https://doi.org/10.1016/j.taap.2008.08.023

Thompson, D. A. (2020). A critical review on the potential impacts of neonicotinoid insecticide use: Current knowledge of environmental fate, toxicity, and implications for human health. *Environmental Science*, 32.

Tierney, K. B., Sekela, M. A., Cobbler, C. E., Xhabija, B., Gledhill, M., Ananvoranich, S., & Zielinski, B. S. (2011). Evidence for behavioral preference toward environmental concentrations of urban-use herbicides in a model adult fish. *Environmental Toxicology and Chemistry*, *30*(9), 2046–2054. https://doi.org/10.1002/etc.588

Tišler, T., Jemec, A., Mozetič, B., & Trebše, P. (2009). Hazard identification of imidacloprid to aquatic environment. *Chemosphere, 76,* 907-914.

Tomizawa, M. (2013). Chemical Biology of the Nicotinic Insecticide Receptor. *Advances in Insect Physiology*, 44, 63–99. Elsevier. https://doi.org/10.1016/B978-0-12-394389-7.00002-8

Tomizawa, M., & Casida, J. E. (2005). Neonicotinoid Insecticide Toxicology: Mechanisms of Selective Action. *Annual Review of Pharmacology and Toxicology*, *45*(1), 247–268.https://doi.org/10.1146/annurev.pharmtox.45.120403.095930

Topal, A., Alak, G., Ozkaraca, M., Yeltekin, A. C., Comaklı, S., Acıl, G., Kokturk, M., & Atamanalp, M. (2017). Neurotoxic responses in brain tissues of rainbow trout exposed to imidacloprid pesticide: Assessment of 8-hydroxy-2-deoxyguanosine activity, oxidative stress and acetylcholinesterase activity. *Chemosphere*, *175*, 186–191. https://doi.org/10.1016/j.chemosphere.2017.02.047

US EPA. (2002). Method 1000.0: Fathead Minnow, Pimephales promelas, Larval Survival and Growth: Chronic Toxicity. *Office of Research and Development and Office of Water. EPA*/660/3-75009.

Victoria, S., Duffy, S., Harrahy, E., & King-Heiden, T. (2022). Embryonic exposure to thiamethoxam reduces survival and alters neurobehavior of fathead minnows. *Environmental Toxicology and Chemistry*, *41*(5), 1276-1285. https://doi.org/10.1002/etc.5301

Vignet, C., Cappello, T., Fu, Q., Lajoie, K., De Marco, G., Clérandeau, C., Mottaz, H., Maisano, M., Hollender, J., Schirmer, K., & Cachot, J. (2019). Imidacloprid induces adverse effects on fish early life stages that are more severe in Japanese medaka (Oryzias latipes) than in zebrafish (Danio rerio). *Chemosphere*, *225*, 470–478. https://doi.org/10.1016/j.chemosphere.2019.03.002

Wang, Y. Y. L., Xiong, J., Ohore, O. E., Cai, Y.-E., Fan, H., Sanganyado, E., Li, P., You, J., Liu, W., & Wang, Z. (2022). Deriving freshwater guideline values for neonicotinoid insecticides: Implications for water quality guidelines and ecological risk assessment. *Science of The Total Environment*, *828*, 154569. https://doi.org/10.1016/j.scitotenv.2022.154569

Weisner, O., Frische, T., Liebmann, L., Reemtsma, T., Roß-Nickoll, M., Schäfer, R. B., Schäffer, A., Scholz-Starke, B., Vormeier, P., Knillmann, S., & Liess, M. (2021). Risk from pesticide mixtures – The gap between risk assessment and reality. *Science of The Total Environment*, *796*, 149017. https://doi.org/10.1016/j.scitotenv.2021.149017

Whitehorn, P. R., Cook, N., Blackburn, C. V., Gill, S. M., Green, J., & Shuker, D. M. (2015). Sex allocation theory reveals a hidden cost of neonicotinoid exposure in a parasitoid wasp. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150389. https://doi.org/10.1098/rspb.2015.0389

Whitehorn, P. R., O'Connor, S., Wackers, F. L., & Goulson, D. (2012). Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production. *Science*, *336*(6079), 351–352. https://doi.org/10.1126/science.1215025

Wolfram, J., Stehle, S., Bub, S., Petschick, L. L., & Schulz, R. (2018). Meta-Analysis of Insecticides in United States Surface Waters: Status and Future Implications. *Environmental Science & Technology*, *52*(24), 14452–14460. https://doi.org/10.1021/acs.est.8b04651

Woynárovich, & Horváth, L. (1980). The artificial propagation of warm-water finfishes : a manual for extension / by E. Woynarovich, L. Horváth. *Food and Agriculture Organization of the United Nations*.

Xu, L., Xu, X., Guo, L., Wang, Z., Wu, X., Kuang, H., & Xu, C. (2021). Potential Environmental Health Risk Analysis of Neonicotinoids and a Synergist. *Environmental Science & Technology*, *55*(11), 7541–7550. https://doi.org/10.1021/acs.est.1c00872

Yamamoto, I., Tomizawa, M., Saito, T., Miyamoto, T., Walcott, E. C., & Sumikawa, K. (1998). *Structural factors contributing to insecticidal and selective actions of neonicotinoids*. 9.

Zhu, L., Li, W., Zha, J., Li, N., & Wang, Z. (2019). Chronic thiamethoxam exposure impairs the HPG and HPT axes in adult Chinese rare minnow (Gobiocypris rarus): Docking study, hormone levels, histology, and transcriptional responses. *Ecotoxicology and Environmental Safety*, *185*, 109683. https://doi.org/10.1016/j.ecoenv.2019.109683