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Detection of Neonicotinoids in Northern Leopard Frog (Rana pipiens) Brains

Peyton G. Keller
University of South Dakota

DETECTION OF NEONICOTINOIDS IN NORTHERN LEOPARD FROG (*Rana pipiens*) BRAINS

by

Peyton Keller

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The members of the Honors Thesis Committee appointed
to examine the thesis of Peyton Keller
find it satisfactory and recommend that it be accepted.

Jacob Kerby, Ph.D.

Professor of Biology

Director of the Committee

Jeff Wesner, Ph.D.

Associate Professor of Biology

Cliff Summers, Ph.D.

Professor of Biology

ABSTRACT

Detection of Imidacloprid in Northern Leopard Frog (*Rana pipiens*) Brains

Peyton Keller

Director: Dr. Jacob Kerby, Ph.D.

Neonicotinoids are widespread and commonly used to fight agricultural pests. Unfortunately, these neurotoxic insecticides commonly reach nearby wetlands due to tile drainage systems and agricultural runoff. Non-target organisms, such as amphibians, use wetlands as habitat and are likely exposed to elevated neonicotinoid levels. We collected Northern leopard frogs and water samples from control and tile wetlands to compare imidacloprid brain concentrations and subsequent changes in brain morphology. Additionally, a lab-based experiment was conducted to further analyze the ability of imidacloprid and its metabolite, imidacloprid-olefin, to cross the blood-brain barrier. Tile wetlands had higher aquatic imidacloprid concentrations. Subsequently, amphibians collected from tile wetlands had imidacloprid brain concentrations two times higher than control animals and there were apparent differences in brain length and width measurements of the cerebellum and medulla. Exposure in the lab resulted in a dose-response relationship for imidacloprid and imidacloprid-olefin brain levels. Delayed reaction times to a food stimulus were also noted in the treatment groups. Detection of imidacloprid in neural tissue indicates this contaminant can cross the blood-brain barrier and suggests that tile drainage systems contribute to higher contaminant loads in non-target organisms and the aquatic ecosystem.

Keywords: neonicotinoid, imidacloprid, amphibian, brain, contaminants

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INTRODUCTION

Pesticides are often used to eliminate and control agricultural pests and can be applied in a wide range of ways, such as aerial spraying, seed treatments, and through irrigation systems (Schaafsma et al., 2015). Unfortunately, many pesticides are highly water-soluble and are commonly transported to aquatic habitats through agricultural runoff and drift from aerial spraying (Main et al., 2014). In the United States alone, pesticides can be found in 30-60% of shallow ground water and 60-95% of streams, negatively impacting water quality and the species inhabiting these environments (Buck et al., 2015).

Modern agriculture has implemented artificial drainage systems to remove excess water from the soil and increase crop production (Blann et al., 2009). Midwestern farmers use subsurface tile drains, which are buried underground and can empty straight into nearby wetlands (Blann et al., 2009). Despite increasing crop yields, tile drains can negatively affect wetlands by altering nutrient cycles, impacting aquatic communities, and transporting agricultural contaminants into ephemeral wetlands (Blann et al., 2009). Through tile drains, these pesticides are transported to nearby wetlands and encounter non-target organisms such as amphibians. Pesticides in these habitats have been shown to produce physiological, behavioral and morphological abnormalities in amphibians, which can have lethal effects and result in population declines (Rohr et al., 2017; Jones et al., 2017; Smalling et al., 2015; Mann et al., 2009).

Schwarz & Kerby (2018) previously evaluated agriculture drainage systems in eastern South Dakota and how they modified the wetlands adjacent to them. Within the

study, wetlands were classified by the likelihood to encounter discharge from tile drains. Reference wetland sites were those that did not receive direct discharge from tile drains and were separated from agricultural runoff. Tile wetlands were known to encounter tile discharge directly. Water samples were collected from these wetlands and analyzed for pesticides. Compared to reference wetlands, wetlands connected to subsurface tile drainage systems had elevated levels of contaminants, specifically neonicotinoids and herbicides (Schwarz & Kerby 2018).

Present day agriculture is becoming reliant on a new kind of insecticide, the neonicotinoids, which are used to control pest invasions by targeting the post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS) of invertebrates (Main et al., 2014; Miles et al., 2017). Neonicotinoids were introduced in the 1990s and have become highly favored as the United States applies over 6.7 million pounds of them annually (Bradford et al., 2018; Miles et al., 2017). North American agriculture has rapidly converted to neonicotinoids because of their ability to be applied as a protective seed coating for many popular crops (Douglas & Tooker 2015). Upon germination, the neonicotinoid is absorbed into the crop and distributed to the plant throughout growth (Miles et al., 2017). The most common neonicotinoids are imidacloprid (IMI), clothianidin, and thiamethoxam (Bradford et al., 2018).

Neonicotinoids cause overstimulation, paralysis, and ultimately death for invertebrates as they bind nearly irreversibly to their nAChRs. Neonicotinoids are known to bind more strongly to insect nAChRs than vertebrate nAChRs (Miles et al., 2017; Tomizawa & Casida 2005). In addition, the vertebrate blood-brain barrier (BBB) is thought to block access of imidacloprid to the CNS, which would reduce its toxicity

(Krieger, 2010). Despite claims that neonicotinoids selectively target invertebrates, recent studies showing neonicotinoid affinity for nAChRs in mammal brains have challenged the validity of this concept (Burke et al., 2018). Previous studies have also found imidacloprid in fish brains after exposing them to different concentrations of the pesticide (Iturburu et al., 2017). This suggests that neonicotinoids can cross the BBB in vertebrates and outlines potential harmful effects on the CNS. Additionally, studies have detected changes in *Rana pipiens* brain width after exposure to chlorpyrifos, an insecticide that also targets cholinergic neurotransmission (McClelland et al., 2018). As recent research suggests that neonicotinoids can cross the BBB, then it is possible for the breakdown products to cross the BBB too. A primary breakdown product of imidacloprid is imidacloprid-olefin. The metabolites of neonicotinoids are important to examine as imidacloprid-olefin is said to be more toxic to insects than imidacloprid (Seirtova et al., 2016). To date, no studies have provided evidence of neonicotinoids crossing the BBB in amphibians.

The BBB regulates an organism's neural environment by controlling the access of certain molecules into the brain (O'Brown et al. 2018). For mammals, the BBB consists of tight junctions between endothelial cells that line vessels of brain tissue, which prevent molecules from the circulating blood supply to freely enter fluid of the CNS (Abbott, 1992). Across all vertebrates, a functional endothelial barrier is present (Abbott, 1992; O'Brown et al. 2018). Vertebrate brains are divided into two subtypes based on neuronal complexity. Type 1 brains demonstrate a relatively simple neuronal arrangement and minimal migration of neurons away from ventricular surface (Butler, 2009). Vertebrate taxa belonging to this subtype include amphibians, cartilaginous fish, and lampreys (Butler, 2009). The second subtype, Type 2, has increased neuronal complexity and more neuronal

migration away from surface of ventricle (Butler, 2009). Taxa of this group include mammals, birds, and reptiles (Butler, 2009). While there has been focus on whether commonly used pesticides cross the BBB in mammals, aquatic species with the highest likely exposure to these chemicals have been largely ignored (Burke et al., 2018). Given the difference in makeup of the BBB, it is critical to examine potential uptake in animals such as amphibians.

Because neonicotinoids are highly water soluble, they are commonly transported to surface, ground, and drinking waters, where they have been previously detected (Ospina *et al.*, 2019). Elevated levels of neonicotinoids have been detected in South Dakota wetlands that are connected to subsurface tile drainage systems (Schwarz & Kerby 2018). Wetlands connected to tile drain systems offer habitat for amphibians and are largely unprotected (Blann et al., 2009). Amphibians have highly permeable skin, complex life cycles, unshelled eggs, and spend prolonged periods of time in the water, making them very susceptible and sensitive to environmental contaminants that are water-soluble (Brown et al., 2013; Lanctot et al., 2017; Miko et al., 2017). Additionally, amphibians exploit a variety of bodies of water for reproduction; therefore, their eggs and larvae are exposed to any contaminants that may reach these waters (Miko et al., 2017). Amphibians are important in studying ecosystems as they are early indicators for declining water quality and ecosystem health (Hocking & Babbitt, 2014). Additionally, amphibians provide a variety of services to the ecosystem as they distribute nutrients between aquatic and terrestrial habitats and prey on a wide range of invertebrates (Mushet et al., 2014). Due to the potential for high susceptibility to contaminants in amphibians and prior detection of

neonicotinoids in brain tissue of other vertebrates, it is likely that neonicotinoids will be present in amphibian brains after exposure.

One agriculture region that could potentially see these effects is the Prairie Pothole Region (PPR). The PPR is a natural wetland landscape covering Canada and five Midwestern states in America-Iowa, Minnesota, Montana, North Dakota, and South Dakota. Within this area, there is extensive development of drainage systems, both surface and subsurface (Blann et al., 2009). The PPR consists of thousands of shallow wetlands, which are habitats for a diverse group of organisms (Blann et al., 2009). Based on the prevalent use of contaminants and the ability to spread through tile drain systems, it is apparent that wetlands in the PPR are susceptible to alterations. In South Dakota, there is an annualized wetland loss rate of ~0.3% due to agriculture, making it the state's greatest source of wetland loss (Johnston, 2013). When wetlands are damaged, the amphibians who live in the wetland, who are vulnerable to contaminants, may also be affected.

Amphibian populations are rapidly decreasing worldwide as up to 50% of amphibian species face risk of extinction (Miko et al., 2017). This extinction crisis has deemed amphibians as the most threatened class of vertebrates today (Fisher et al., 2009). Several anthropogenic factors, such as habitat destruction, climate change, introduction of invasive species, and contaminant exposure have been linked to these declines (Buck et al., 2015; Jones et al., 2016). Land use for global croplands, plantations, and pastures has led to a reduction of biodiversity through the modification of natural habits (Foley et al., 2005). In hopes of reducing the rapid decline in amphibian populations, it is important to further understand how contaminants are making their way into aquatic habitats. Projects like this one are vital in understanding the prevalence of pesticides and their method of action. By

researching further into pesticides, specifically neonicotinoids, we can learn how amphibians are being affected and use this information to restore their habitats.

The objectives for the field portion of this study were to 1) quantify contaminant load in control and tile wetlands, 2) determine if imidacloprid crosses the BBB and if so, quantify imidacloprid concentration in northern leopard frog brains, and 3) compare these potential concentrations and examine any differences in length and width of brain regions from tile and control wetlands.

The objectives for the laboratory portion of this study were to 1) quantify imidacloprid and its metabolite, imidacloprid-olefin, concentrations in amphibian whole brain samples following exposure to environmentally relevant concentrations, and 2) examine the behavioral effects of neonicotinoid exposure, and 3) compare individual body length and body mass across exposure concentrations.

MATERIALS AND METHODS

Field Experiment

Study Sites

Forty-eight newly metamorphosed Northern Leopard Frogs *(Rana pipiens*) were collected from three reference and three tile Waterfowl Protection Area (WPA) wetland sites throughout eastern South Dakota. Eight individuals (n=8) were collected per site. Unlike tile wetlands, reference wetlands are not directly connected to subsurface tile drainage systems and receive little to no agricultural runoff.

Sampling

At each of the six sites, two surface water grab samples were collected in sterile, 1 L glass amber bottles and stored at -20°C before shipment to the University of Nebraska (Lincoln, NE) for water quality analysis. After collection, animals were anesthetized with benzocaine and euthanized by rapid decapitation. Total length (TL) and body mass were recorded for all individuals. Whole brains were quickly removed, trimmed of cranial nerves, and weighed. Dorsal and ventral surfaces of each whole brain were photographed with a digital microscope (Leica DMS1000) and Image J software (US National Institute of Health) was used to measure brain regions (Figure 1). To ensure accuracy, a minimum of two photographs were taken of dorsal and ventral surfaces and brain region measurements were averaged. Whole brain samples were stored at -20°C until shipment to the University of North Dakota (Grand Forks, ND) for Imidacloprid concentration analysis. All animals were collected under a scientific collector's permit (permit #21) issued by the

South Dakota Game, Fish and Parks and all procedures were carried out with approval from the Institutional Animal Care and Use Committee at the University of South Dakota (Vermillion, SD, USA).

Imidacloprid Analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine imidacloprid concentrations in whole brain and water samples at the University of North Dakota Mass Spectrometry Core and University of Nebraska Lincoln Water Sciences Laboratory, respectively. The two water samples collected from each site were averaged. For the water samples, LC-MS/MS also looked for a variety of contaminants, including azoxystrobin, clothianidin, dimethoate, metalaxyl, thiamethoxam, and others.

Statistical Analysis

All data were analyzed using R (R Core Team, Version 3.6.3) in Rstudio (Rstudio, Inc., Version 1.3.959). The relationship between various response variables (imidacloprid brain concentration, cumulative aquatic contaminant load, brain measurements) and predictor variables (wetland type) was assessed through a generalized linear mixed model with a Gamma likelihood and a log link using Bayesian inference. A gamma distribution was chosen due to the positive nature of our data.

All models were fit using *rstan* (Stan Development Team 2016) via the *brms* (Buerkner 2017) package. Markov chain Monte Carlo (MCMC) was used to obtain the joint posterior distribution. The cumulative water contaminant load model contained four chains and 3000 iterations, 500 of which were used as warm-ups and discarded. The

imidacloprid brain concentration model contained four chains and 2000 iterations, 500 of which were used as warm-ups and discarded. Each brain measurement was analyzed separately with 2000 iterations and 500 warm-ups for all models. Model convergence was visually assessed through trace plots of the posterior distribution and Rhat values (potential scale reduction factor). All models had Rhat values less than 1.1, indicating model convergence. Model fit was inspected through posterior predictive checks, including boxplots and histograms (see Gelman et al. 2013 for more information about posterior predictive checks). For each model, means and 95% credible intervals were estimated for the parameters from the posterior distribution. The *loo* package (Vehtari, Gelman, and Gabry, 2016; Version 2.3.1) was used to compute approximate leave-one-out cross-validation for model comparison.

Response variables were compared over treatments and dates to derive the probability of a difference among means. For the brain measurement models, the difference between two responses was calculated over the 6000 iterations of the posterior distribution and the number of differences greater than zero was divided by the number of samples in the distribution (n = 6000), producing a percent probability of the difference. Alternatively, the cumulative water contaminant load model was analyzed the same way as described previously, but with 10000 iterations instead.

Laboratory Experiment

Sampling

Fifty adult Northern Leopard Frogs (*Rana pipiens*) were collected from reference wetland sites in eastern South Dakota. Reference wetlands receive very little surface run off from nearby agricultural fields and are not connected to subsurface tile drainage systems. Individuals were housed in separate 10-gallon tanks, put on a 12-hour light/dark cycle, and allowed to acclimate for one week. Health checks were recorded daily, and tank locations were rotated biweekly. Total length (TL) and body mass were recorded at onset of experiment and taken every 7 days following initial measurement. The room temperature was set to 26 ° C and never went above this level throughout the experiment.

Experimental Design

Individuals were randomly exposed to treatments of analytical standard imidacloprid (CAS no.138261-41-3) at 0, 0.5, 5, 25, or 50 μg/L (control=reverse osmosis water and dimethyl sulfoxide (DMSO)) for 21 consecutive days. For each concentration, 10 individuals were exposed. Before exposure, imidacloprid was diluted with DMSO to create a stock solution. Every 7 days, water was changed and re-dosed with the corresponding contaminant concentration.

After the exposure period, individuals were anesthetized via benzocaine and euthanized via rapid decapitation. Decapitated heads were immediately flash frozen and stored at -80°C for future brain extraction.

Imidacloprid Analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine imidacloprid and imidacloprid-olefin concentrations in whole brain samples at the University of North Dakota (Grand Forks, ND). Additionally, protein concentration was measured in all brain samples and used to standardize imidacloprid brain concentrations (IMI ng/mg protein).

Feeding Trials

During the 21-day exposure to varying imidacloprid concentrations, three feeding trials were conducted 24 hours after water changes were made. Feeding trials consisted of randomly placing one cricket at the front of each housing tank. The time to consumption was recorded for each trial. Timing was stopped after two minutes if individuals did not consume the cricket and this failure to consume was noted. Individuals were fed in a randomized order. To minimize distractions from other individuals, dividers were placed between each tank while feeding trials were conducted.

Statistical Modeling

All data were analyzed using R (R Core Team, Version 3.6.3) in RStudio (RStudio, Inc., Version 1.3.959). The relationship between various response variables (imidacloprid brain concentration, imidacloprid-olefin brain concentration, food response time) and predictor variables (treatment, date) was assessed through a generalized linear mixed model with a Gamma likelihood and a log link using Bayesian inference. A gamma distribution was chosen due to the positive nature of our data. All models were fit using

rstan (Stan Development Team 2016) via the *brms* (Buerkner 2017) package as described above.

Response variables were compared over treatments and dates to derive the probability of a difference among means. The difference between two responses was calculated over the 6000 iterations of the posterior distribution and the number of differences greater than zero was divided by the number of samples in the distribution (n = 6000), producing a percent probability of the difference.

RESULTS

Field Experiment

Aquatic Neonicotinoid Load

Mean cumulative neonicotinoid load (μg/L) found in tile wetlands, 0.44 μg/L (95% CrI: 0-2.07), was approximately triple the mean cumulative neonicotinoid load found in control wetlands, 0.14 μg/L (95% CrI: 0-0.53) (Figure 2). Based on our samples,there was a greater than 80% probability that tile wetlands have higher neonicotinoid loads compared to control wetlands. Clothianidin, a neonicotinoid, was primarily detected only at tile wetlands, except for extremely low detection (0.001 μg/L) at one control site; however, this concentration was below the method detection limit (0.002 μg/L). At one tile site, clothianidin concentrations were above the Environmental Protection Agency's (EPA) chronic toxicity benchmark for aquatic invertebrates (0.05 μg/L) ("Aquatic life benchmarks and ecological risk assessments for registered pesticides", 2021). Imidacloprid was only detected at one tile site and at two control sites. All imidacloprid detection concentrations were above the EPA's chronic toxicity benchmark for aquatic invertebrates (0.01 μg/L) ("Aquatic life benchmarks and ecological risk assessments for registered pesticides", 2021). Thiamethoxam, a neonicotinoid, was detected at all tile sites; however, only one tile site had concentrations that were above method detection limits. All detections of thiamethoxam were below the EPA's chronic detection limit for aquatic invertebrates.

Imidacloprid Brain Concentration

Mean imidacloprid brain concentration (ng/mg protein) in Northern leopard frogs from tile wetlands, 4.69 ng/mg protein (95% CrI: 1.37-11.7), was approximately double the mean imidacloprid brain concentration found in brains from control wetlands, 2.23 ng/mg protein (95% CrI: 0.69-6.24) (Figure 3). Based on our samples, there was a greater than 87% probability that the average concentration difference between wetland types was greater than zero.

Brain Measurements

Two measurements that demonstrated a notable difference were cerebellum width (cm) and medulla oblongata length (cm). The widths of the cerebellums from control wetland brains were an average of 0.045 cm wider than frog brains collected from tile sites (Table 1). There was a greater than 95% probability that the difference between widths was greater than zero (Figure 4). Likewise, tile wetland frog brains had medulla oblongata lengths that were 0.077 cm longer than control brains (Table 1). There was a greater than 92% probability that brains from tile sites had longer medulla oblongata regions (Figure 5). These compare to the estimated 50% probability that all other measured brain regions were different between wetland types (Table 1).

Laboratory Experiment

Imidacloprid Brain Concentrations

Imidacloprid concentration (ng/mg protein) in brain tissue increased linearly with the concentration of analytical standard imidacloprid assigned in treatment (Figure 6). Individuals exposed to 5 µg/L had imidacloprid brain concentrations that were almost 25 times higher compared to the control (Pr=95%), while individuals exposed to 50 µg/L had brain levels that were almost 12 times higher than the 5 µg/L treatment (Pr=95%). Average IMI brain concentration ranged from 4.67 ng/mg protein in the control group to 1376 in the 50 µg/L group. There was a greater than 99% probability that the difference between treatments was greater than zero.

Imidacloprid-Olefin Brain Concentrations

The breakdown product of imidacloprid is imidacloprid-olefin. Analysis found imidacloprid-olefin brain concentrations that ranged from 3.47 to 22.1 ng/mg protein (Figure 7). With exception of the control group, imidacloprid-olefin brain concentrations followed a dose-response relationship. Although the control group was never exposed to imidacloprid during the experiment, whole brain samples had an average of 3.47 ng imidacloprid-olefin/mg protein (Figure 7). This is likely due to natural exposures in the field prior to collection for this study.

Morphological Changes

All treatment groups experienced a decrease in overall body mass (g) (Figure 8) during the course of the experiment. Average initial body mass at the start of the

experiment ranged from 15.4 to 20.4 g in all treatments, while final body mass ranged from 13.2 to 18 g. Average body mass loss (initial-final mass) varied among treatments and ranged from 2 to 2.4 g, in which the control group lost the most body mass while the 0.5 µg/L group lost the least amount of body mass (Figure 8). The 10 µg/L group experienced a 180% decrease in body mass. There was a greater than 73% probability that overall changes in body mass between the control and 0.5 µg/L group was greater than zero. There was less than a 69% probability that overall changes in body mass between the control and 5 µg/L, 25 µg/L, and 50 µg/L group was greater than zero.

Average change in growth (final – initial TL) among treatments ranged from 5.8 to 21.3 mm. The 50 µg/L group experienced the least amount of growth while the 0.5 µg/L group demonstrated the greatest increase in TL (Figure 9). There was a greater than 99.99% probability that the 0.5 µg/L group experienced greater gains in TL compared to the control group. There was a greater than 82% probability that the control group experienced greater gains in TL compared to the 50 µg/L group.

Feeding Trials

Across all three feeding trial dates, the 5 µg/L treatment had the slowest response times to a food stimulus (Figure 10). Feeding response times were particularly slow across all treatments during the first trial (July 4), which occurred approximately 24 hours after the first water change and re-dosing of imidacloprid (all feeding trials occurred 24 hours after re-dosing) (Figure 11). Interestingly, feeding response times in all treatments were faster during the second feeding trial (July 8) compared to the first (July 4) and last (July 11) feeding trial. This trend was particularly pronounced in the 50 µg/L treatment, in which

there was a greater than 99% probability that feeding response times were slower during the first (July 4) and last (July 11) feeding trial compared to the second (July 8). Additionally, mean response times were similar in the control and 50 µg/L treatment during the second feeding trial, with mean response times of 12.9 and 17.8 seconds, respectively.

DISCUSSION

This study is the first to provide evidence of neonicotinoids crossing the amphibian BBB. Additionally, this study verified previous findings that neonicotinoid concentrations were different in control and tile wetlands. These contaminant concentrations, which were elevated in tile wetlands, are important to investigate as they are traversing into the brains of amphibians within the wetlands. Subsequently, higher concentrations of imidacloprid were detected in the brains of frogs from tile wetlands. Further confirming the results from the field study, this project found imidacloprid and imidacloprid-olefin brain concentration to increase linearly with exposure concentration. Lastly, imidacloprid in the brain appeared to exhibit differences in behavior and brain morphology.

Imidacloprid crosses the blood brain barrier

Although previous research has reported that the vertebrate BBB blocks access of imidacloprid to the CNS (Krieger, 2010), the findings of this study directly contradict this notion and suggest that imidacloprid can cross the BBB in amphibians. Amphibians collected from tile wetlands had imidacloprid brain concentrations that were more than twice as high as individuals collected from control wetlands. In a laboratory setting, this project found imidacloprid and imidacloprid-olefin brain concentrations up to 1376 and 24 ng/mg protein in amphibian brains. Furthermore, both imidacloprid and imidacloprid-olefin exhibited a concentration dependent relationship between brain levels and exposure concentration in a laboratory setting. As individuals were exposed to higher concentrations of imidacloprid, higher levels of imidacloprid and imidacloprid-olefin in the brain were detected.

Although imidacloprid-olefin was detected at much lower concentrations than imidacloprid in brain tissue, metabolites are often more toxic and persistent than the parent compound itself (Thompson et al., 2020; Hussain et al., 2016). In previous research, Hussain et al. (2016) and Honda et al. (2006) have found imidacloprid-olefin to be up to ten times more toxic to insects and mammals. A recent study by Wang et al. (2018) found imidacloprid-olefin in lizard brains at much higher levels than the parent imidacloprid compound that the organisms were exposed to. The metabolites of imidacloprid appear to be more toxic in amphibians, mammals, insects, and reptiles (Honda et al., 2006; Wang et al., 2018). This neurotoxic prevalence of neonicotinoids and their metabolites could represent a more widespread issue.

This is the first time that imidacloprid has been detected in amphibian brains. The understanding of the uptake and bioaccumulation of imidacloprid in neural tissue of vertebrates, particularly aquatic species, is extremely limited. Other recent studies have detected imidacloprid in fish brains and demonstrated neonicotinoid affinity for nAChRs in mammal brains (Burke et al., 2018; Iturburu et al., 2017). Neonicotinoids are known to bind more strongly to insect nAChRs than vertebrate nAChRs and are unable to cross the vertebrate BBB (Miles et al., 2017; Tomizawa & Casida, 2005; Krieger, 2010). The results of recent studies and this project demonstrate the presence of neonicotinoids in vertebrate brains and directly contradict the assumption that neonicotinoids are selectively toxic to insects.

The assumption that neonicotinoids are selectively toxic to insects is critically important to refute. It has been widely accepted that neonicotinoids are only toxic to insects and have low toxicity to vertebrate species; therefore, modern agriculture believed that

neonicotinoids were a viable crop treatment. As a result, neonicotinoids are extremely popular in modern agriculture and have become the most widely used insecticide (Miles et al., 2017). In South Dakota, upwards of 94% of corn and 50% of soybeans were treated with neonicotinoids (Berhiem et al., 2019; Stockstad, 2013) Despite their excessive use, only a small quantity of the active ingredients in neonicotinoids are absorbed by the plant and the rest is presumably taken up by the soil and water (Sur & Stork, 2003). Neonicotinoids can also persist in the soil for months to years under the right conditions (Bradford et al., 2018; Bonmatin et al., 2014). Non-target organisms are being exposed to increasing levels of neonicotinoids, which are crossing the blood brain barrier and accumulating at higher levels under elevated concentrations.

Imidacloprid alters behavior and brain morphology

A recent study examined the effects of neonicotinoids on the simulated escape behaviors of frogs that were chronically exposed to imidacloprid or thiamethoxam as tadpoles (Lee-Jenkins & Robinson, 2018). Lee Jenkins et al. (2018) found that frogs that were chronically exposed to neonicotinoids were less likely to escape simulated predator attacks. Similarly, we found individuals experimentally exposed to imidacloprid portrayed delayed reaction times to food stimuli, particularly in the 5 µg/L treatment. Considering the altered behavior demonstrated in both studies after exposure to imidacloprid, these results suggest that imidacloprid exposure may negatively impact perception and cognitive function.

Additionally, amphibians collected from tile drains during the field portion of this study had larger medulla oblongata lengths and smaller cerebellum widths compared to

individuals collected from control wetlands. The medulla oblongata is a vital center responsible for respiratory and auditory function while the cerebellum helps maintain balance and is responsible for muscle coordination. Although we cannot definitively state that differences in the medulla oblongata and cerebellum were a direct result of exposure to imidacloprid in the field, these differences highlight the importance of further examining how commonly used pesticides may alter brain regions in non-target organisms and potentially lead to physiological effects.

Variable growth responses to imidacloprid

All treatment groups experienced a decrease in overall body mass, but differences between treatments were minimal. Regarding total body length, all treatment groups experienced growth, but the 0.5 µg/L group demonstrated the greatest increase in total body length while the 50 µg/L group experienced the least amount of growth. These results demonstrate the hormetic effects that neonicotinoids can have. Hormesis is an adaptive response that an organism can have after exposure to stress, which can be anything from environmental contaminants to low oxygen conditions (Mattson, 2008). It is characterized by a biphasic dose response, where low doses are stimulating, and beneficial and high doses are toxic or inhibitory (Mattson, 2008). At low doses, neonicotinoids have been found to increase reproduction, fecundity, and developmental rates (Berry & Lopez-Martinez, 2020). Neonicotinoids have recently been found to have an adverse range of effects on non-target organisms such as rats, mice, rabbits, and fish (Gibbons et al., 2014). It is important to further study environmental contaminants and how organisms respond to them.

Higher neonicotinoid load in tile wetlands

This project found higher concentrations of neonicotinoids in water samples collected from tile wetlands compared to control wetlands. Specifically, clothianidin was detected at all tile sites. In addition, imidacloprid was detected in both tile and control wetland sites. These results validate a recent report that detected elevated levels of neonicotinoids in South Dakota wetlands that are connected to subsurface tile drainage systems (Schwarz & Kerby 2018). Additionally, the findings of this project corroborate previous studies that have detected neonicotinoids in surface, ground, and drinking waters (Miles et al., 2017; Ospina et al., 2019). Overall, these findings suggest that tile drains may contribute to higher contaminant body burdens in aquatic and semi-aquatic species. Considering the higher detection of neonicotinoids in tile wetlands and their adverse effects, it is important to understand more about these contaminants and their mode of action.

Why should we care about water quality?

As pesticides are making their way into unintentional locations, it is vital to understand how they affect the ecosystem. Amphibians are important figures in learning about water quality and ecosystem health (Hocking & Babbitt, 2014). These organisms are particularly susceptible and sensitive to water-soluble contaminants like neonicotinoids (Brown et al., 2013; Lanctot et al., 2017; Miko et al., 2017). This project brings attention to the ability for contaminants to influence non-target organisms in ways that are believed to be impossible. The implementation of tile drainage systems also appears to contribute to higher contaminant loads in non-target organisms and the aquatic ecosystem. Wetlands,

which house a variety of organisms, are being deteriorated by agriculture, tile drains, and contaminants. By modifying and modernizing natural habitats, land is being used for industrialized agriculture, which has led to a global reduction in biodiversity (Foley et al., 2005). Amphibian populations are rapidly declining worldwide and human caused factors like habitat destruction and contaminant exposure are associated with these declines (Miko et al., 2017; Buck et al., 2015; Jones et al., 2017). If we can better understand how chemicals are affecting ecosystems, we can learn more about overall ecosystem decline and attempt to preserve the species that are suffering because of it.

Future work should examine stress and how it affects the permeability of the blood brain barrier. In addition to neonicotinoids, amphibians are exposed to a variety of other contaminants. Research should attempt to understand the interaction of these contaminants together and how they impact the individual they encounter.

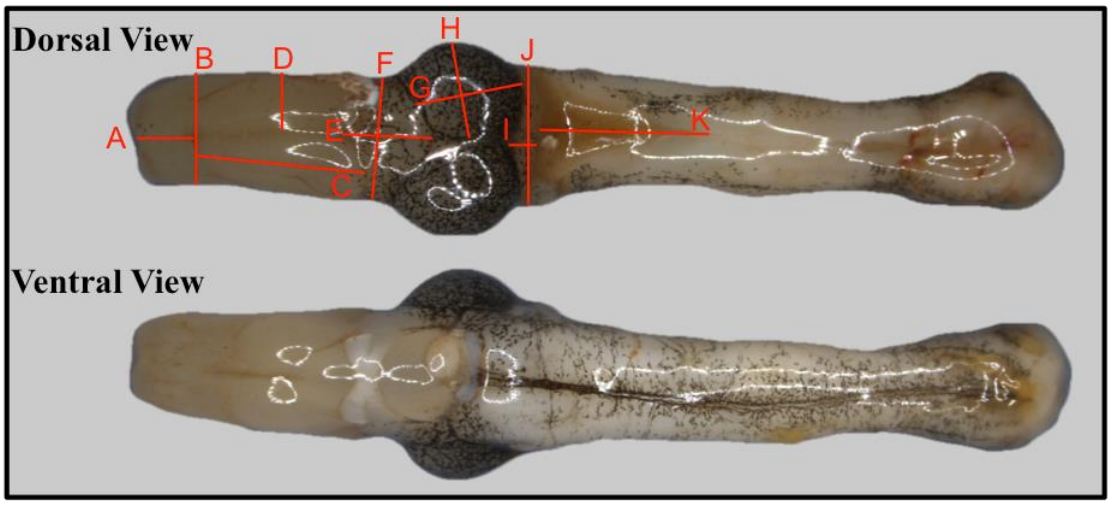

Figure 1: Brain regions measured with ImageJ software. A) Olfactory bulb length, B) olfactory bulb width, C) telencephalon length, D) telencephalon width, E) diencephalon length, F) diencephalon width, G) optic tectum length, H) optic tectum width, I) cerebellum length, J) cerebellum width, K) medulla oblongata length.

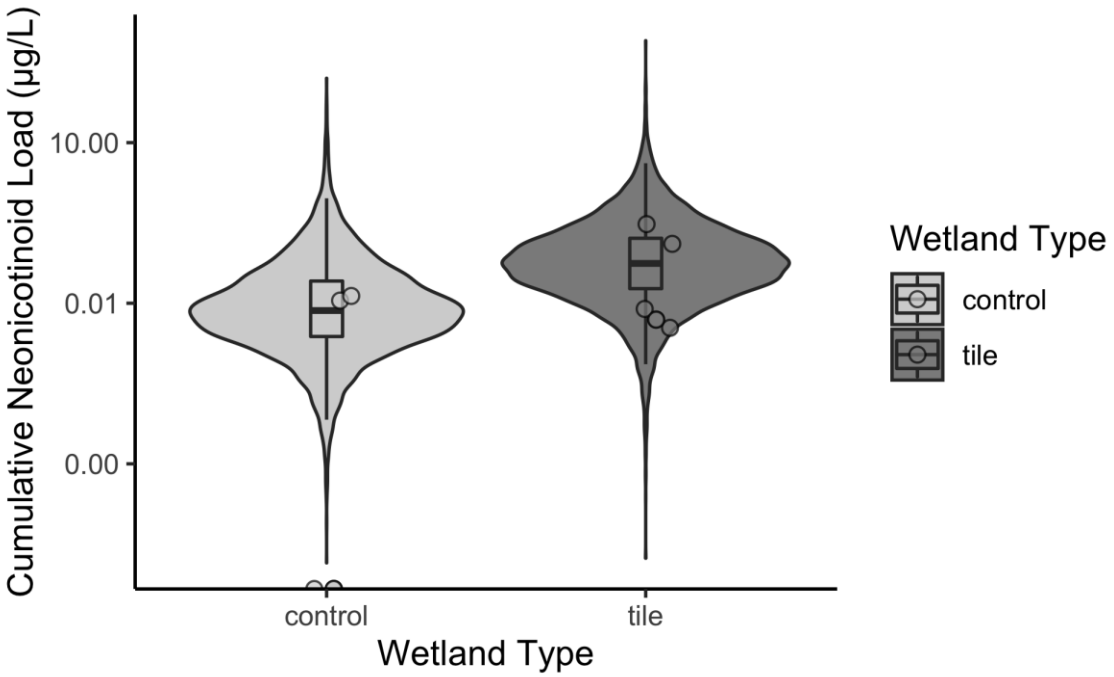

Figure 2: Comparison of cumulative neonicotinoid load (µg/L) by wetland type. Results are averages and 95% credible intervals from the posterior distribution of a Bayesian generalized linear mixed model. Y axis is on the log scale.

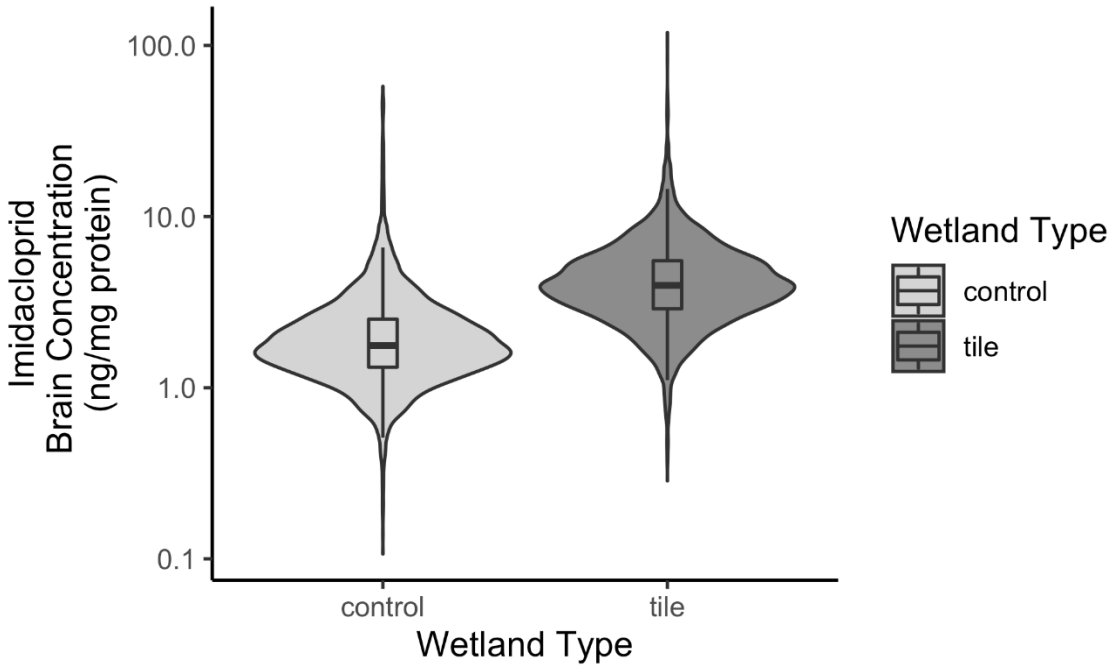

Figure 3: Comparison imidacloprid brain concentrations (ng/mg protein) in Northern Leopard Frogs by wetland type. Results are averages and 95% credible intervals from the posterior distribution of a Bayesian generalized linear mixed model. Y axis is on the log scale.

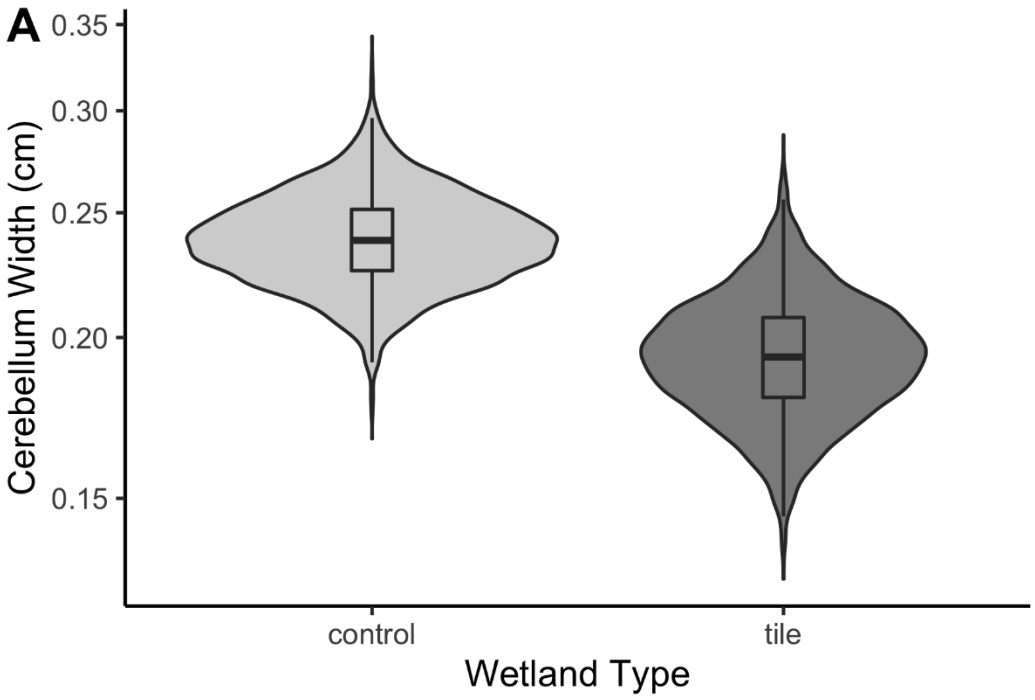

Figure 4: Comparison of Cerebellum Width (cm) of brains collected from control and tile wetlands. Results are averages and 95% credible intervals from the posterior distribution of a Bayesian generalized linear mixed model.

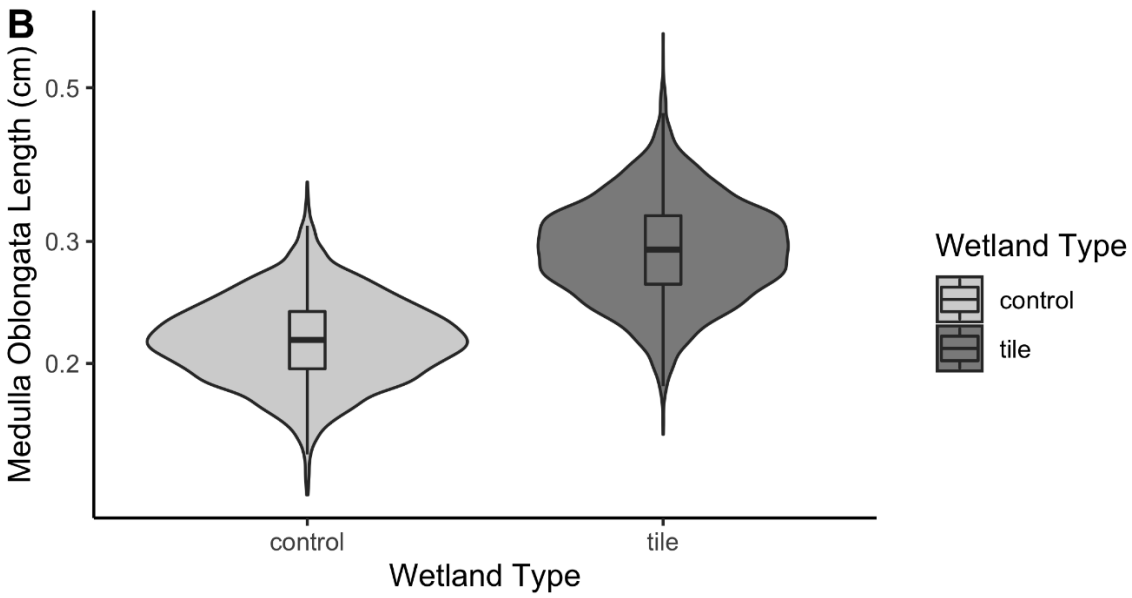

Figure 5: Comparison of Medulla Oblongata Length (cm) of brains collected from control and tile wetlands. Results are averages and 95% credible intervals from the posterior distribution of a Bayesian generalized linear mixed model.

Table 1. Difference in brain region measurements across wetland type. Brain regions measured were those indicated in Figure 1.

Brain Region	Measurement	Wetland Type	Average difference (cm)	95% Credible Interval of difference	Probability that difference is greater than zero
Olfactory Bulb	Length	Control-Tile	0.004	(-0.01474259, 0.02403117)	69%
Olfactory Bulb	Width	Control-Tile	0.001	(-0.08674222, 0.08032727)	53%
Telencephalon	Length	Tile-Control	0.017	(-0.0793634, 0.1225809)	62%
Telencephalon	Width	Tile-Control	0.011	(-0.02279107, 0.04872885)	73%
Diencephalon	Length	Tile-Control	4.00E-04	(-0.08321026, 0.08529498)	50%
Diencephalon	Width	Tile-Control	4.00E-04	(-0.08321026, 0.08529498)	50%
Optic Tectum	Length	Tile-Control	0.005	(-0.05045341, 0.06463182)	56%
Optic Tectum	Width	Control-Tile	0.003	(-0.02698044, 0.03356214)	58%
Cerebellum	Length	Tile-Control	0.028	(-0.06611897, 0.14474686)	70%
Cerebellum	Width	Control-Tile	0.045	(-0.01034035, 0.10208170)	95%
Medulla Oblongata	Length	Tile-Control	0.077	(-0.03186541, 0.19723859)	92%

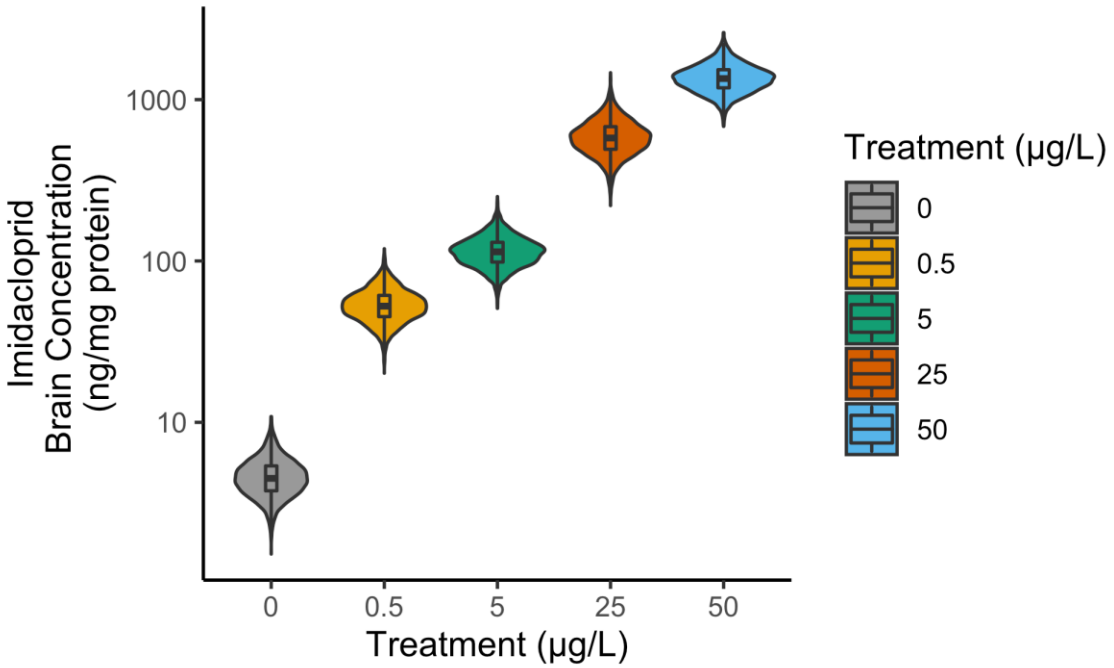

Figure 6: Comparison of imidacloprid brain concentration (ng/mg protein) between treatments. Results are averages from the posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals. Y-axis is on the log scale.

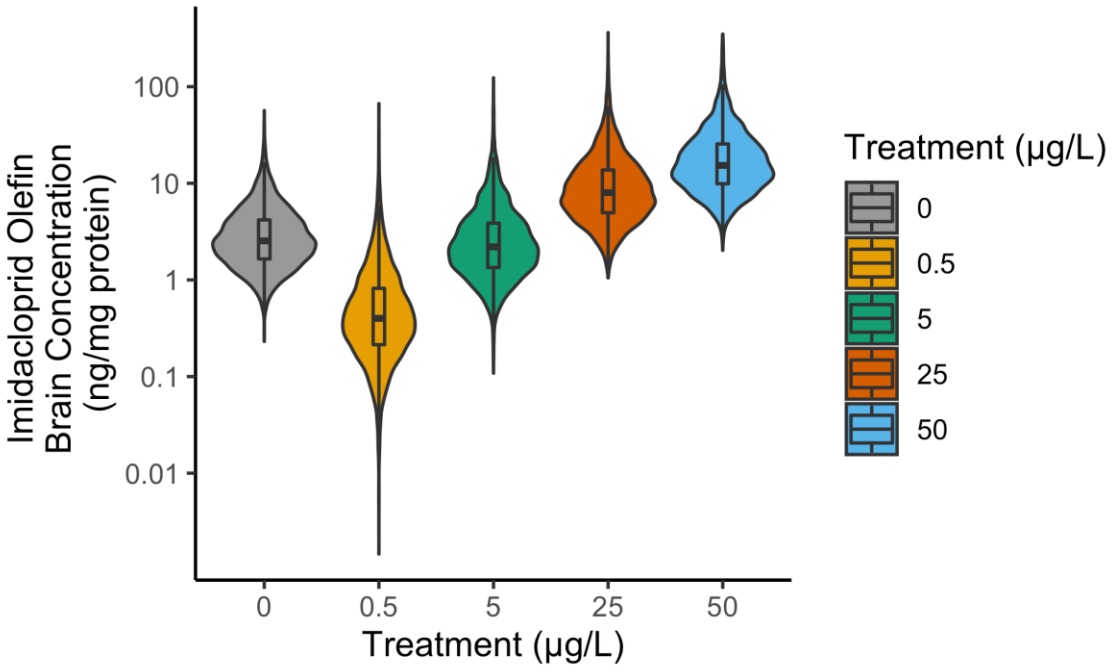

Figure 7: Comparison of imidacloprid olefin brain concentration (ng/mg protein) between treatments. Results are averages from the posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals. Y-axis is on the log scale.

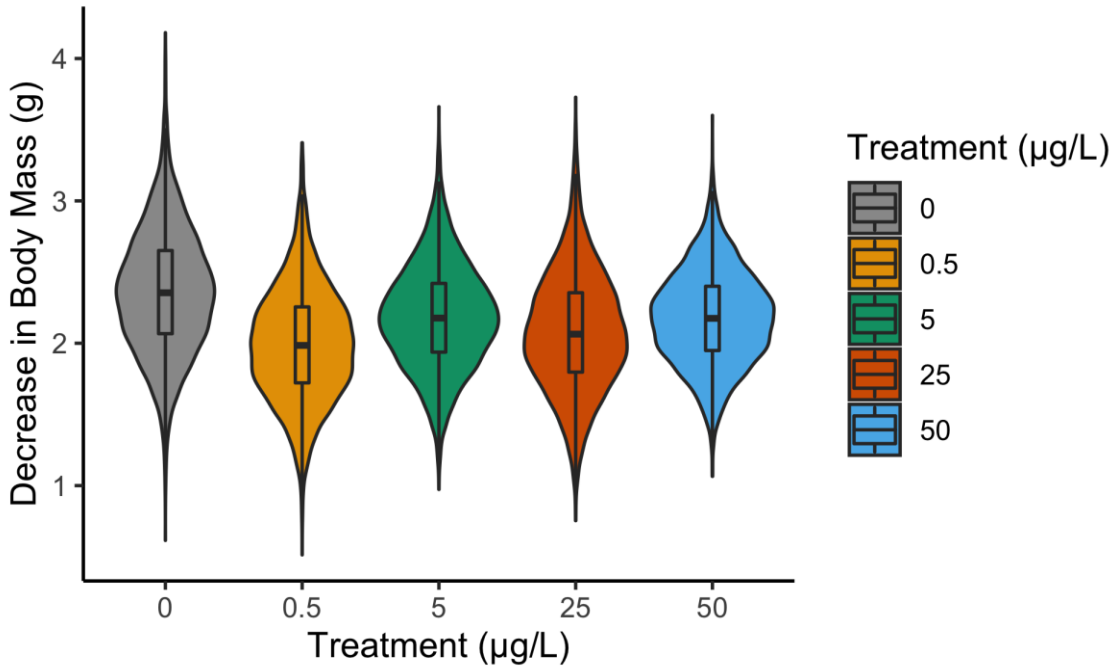

Figure 8: Comparison of change in body mass (g) across experimental period. Results are averages from posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals.

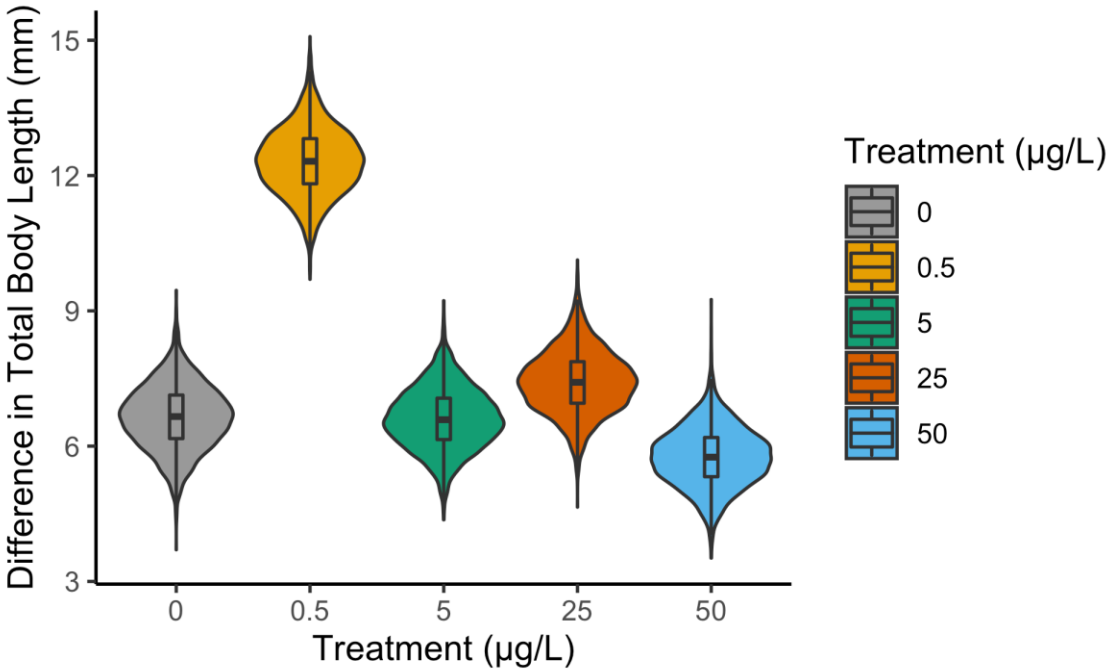

Figure 9: Comparison of change in total body length (mm) across experimental period. Results are averages from a posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals.

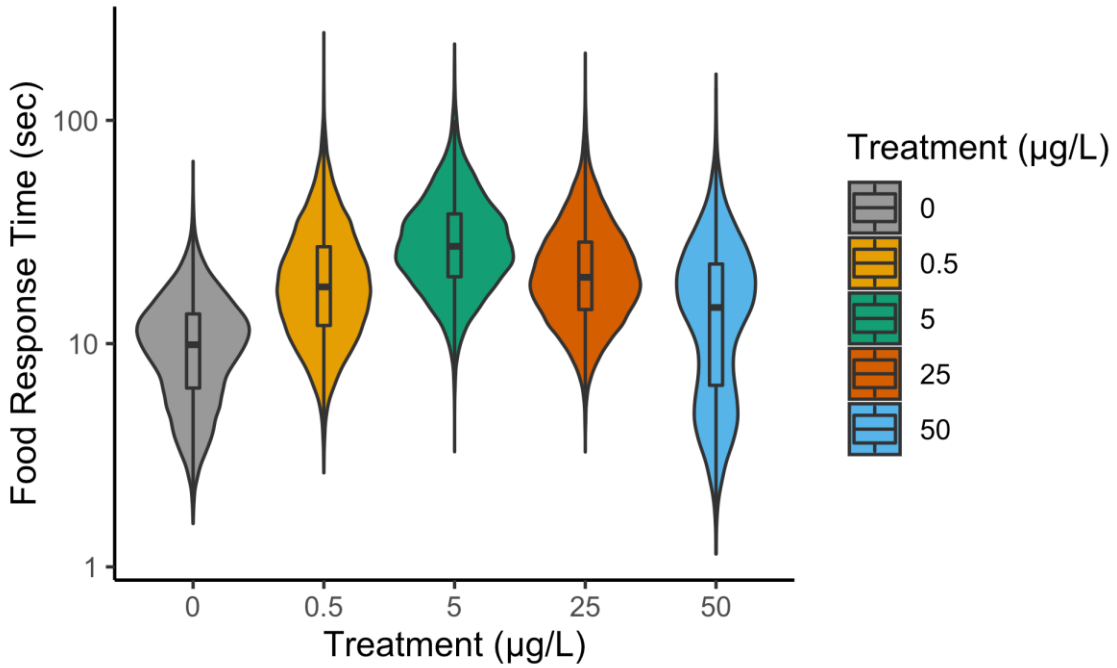

Figure 10: Comparison of food response time (sec) between treatments across all three feeding trials. Results are averages from the posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals.

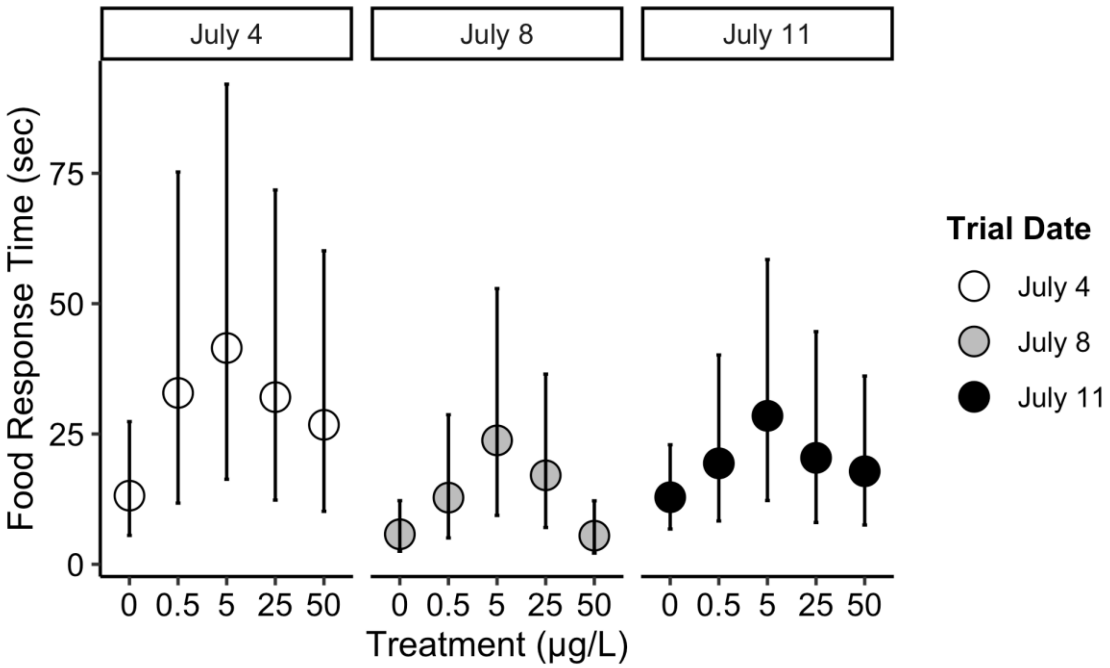

Figure 11: Comparison of food response time (sec) between treatments for each of the three feeding trials. Results are averages from the posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals.

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