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Detection of imidacloprid and metabolites in Northern Leopard frog (*Rana pipiens*) brains

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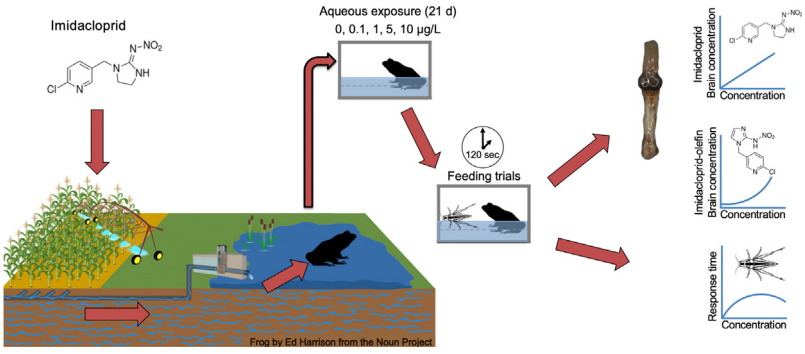
HIGHLIGHTS

- Adult amphibians were exposed to imidacloprid contaminated water.
- Imidacloprid was found in the brain indicating it crossed the blood-brain barrier.
- Imidacloprid-olefin also detected in the brain indicating imidacloprid metabolism.
- Positive relationship between imidacloprid brain levels and slower reaction times.
- Ecologically relevant concentrations of imidacloprid negatively impacted amphibians.

GRAPHICAL ABSTRACT



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ABSTRACT

Neonicotinoids are a new type of highly water-soluble insecticide used in agricultural practices to eliminate pests. Neonicotinoids bind almost irreversibly to postsynaptic nicotinic acetylcholine receptors in the central nervous system of invertebrates, resulting in overstimulation, paralysis, and death. Imidacloprid, the most commonly used neonicotinoid, is often transported to nearby wetlands through subsurface tile drains and has been identified as a neurotoxin in several aquatic non-target organisms. The aim of the present study was to determine if imidacloprid could cross the blood-brain barrier in adult Northern Leopard frogs (*Rana pipiens*) following exposure to 0, 0.1, 1, 5, or 10 µg/L for 21 days. Additionally, we quantified the breakdown product of imidacloprid, imidacloprid-olefin, and conducted feeding trials to better understand how imidacloprid affects foraging behavior over time. Exposure groups had 12 to 313 times more imidacloprid in the brain relative to the control and breakdown products showed a dose-response relationship. Moreover, imidacloprid brain concentrations were approximately 14 times higher in the 10 µg/L treatment compared to the water exposure concentration, indicating imidacloprid can bioaccumulate in the amphibian brain. Reaction times to a food stimulus were 1.5 to 3.2 times slower among treatment groups compared to the control. Furthermore, there was a positive relationship between mean response time and log-transformed imidacloprid brain concentration. These results indicate imidacloprid can successfully cross the blood-brain barrier and bioaccumulate in adult amphibians. Our results also provide insights into the relationship between imidacloprid brain concentration and subsequent altered foraging behavior.

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1. Introduction

Modern day agricultural practices rely heavily on a new class of insecticides, the neonicotinoids (Main et al., 2014). Since the introduction of neonicotinoids in the 1990s, they have become highly favored and used worldwide (Miles et al., 2017). Over 6.7 million pounds of neonicotinoids are annually applied in the United States, with imidacloprid, thiamethoxam, and clothianidin being the most frequently used neonicotinoids (Bradford et al., 2018). Neonicotinoid insecticides are broad-spectrum toxicants and target the post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of invertebrates (Miles et al., 2017). These contaminants bind almost irreversibly to nAChRs, causing overstimulation, paralysis, and ultimately death. Although it has been widely accepted that neonicotinoids have selective toxicity for insects, the validity of this notion has been recently challenged with several studies showing affinity for nAChRs in vertebrate brains (Burke et al., 2018; Roy et al., 2020).

Recently, neonicotinoids have warranted concern due to widespread use, high water solubility ($\log_{kow}$ 0.57 to 1.26) and potential toxicity to non-target organisms (Bradford et al., 2018; Schwarz et al., 2018; Özdemir et al., 2018; Katić et al., 2020). Neonicotinoids and other agricultural contaminants are often transported to nearby bodies of water through subsurface drainage systems, agricultural runoff, and drift from aerial spraying (Main et al., 2014). Neonicotinoids, specifically imidacloprid, have been detected in bodies of water all over the world, including Canada (Struger et al., 2017), Bangladesh (Sumon et al., 2018), and the Netherlands (Morrissey et al., 2015). In the United States alone, pesticides can be found in 30–60% of shallow ground water and 60–95% of streams (Buck et al., 2015). Previous studies have detected clothianidin concentrations up to 0.257 μg/L in Iowa (Hladik et al., 2014), thiamethoxam concentrations of 2.49 μg/L in South Dakota (Schwarz et al., 2018), and imidacloprid concentrations up to 41.1 μg/L in California (Mineau, 2020) and 1.59 μg/L in Wisconsin drinking water (Bradford et al., 2018). Additionally, elevated levels of neonicotinoids have been detected in South Dakota wetlands that are connected to subsurface tile drainage systems (Schwarz et al., 2018).

These largely unprotected, ephemeral wetlands provide habitat for amphibians, which are currently the most threatened vertebrate class (Stuart et al., 2004). Amphibians play an important role in the ecosystem by serving as early indicators for declining water quality and overall ecosystem health (Hocking and Babbitt, 2014). Additionally, amphibians are also known to provide a wide range of ecosystem services, such as consuming a large variety of agricultural pests and distributing nutrients between aquatic and terrestrial habitats (Mushet et al., 2014).

Many pesticides found in agricultural wetlands, presumably from tile drains, have been shown to contribute to physiological, behavioral and morphological abnormalities in amphibians that can ultimately lead to population declines (Mann et al., 2009; Smalling et al., 2015; Jones et al., 2017; Rohr et al., 2017). Amphibians' highly permeable skin and extended period of time spent in water make them exceptionally susceptible to chronic exposure of water-soluble contaminants (Lanctôt et al., 2017). Previous studies have detected the presence of neonicotinoids, specifically imidacloprid, in fish brain tissue after exposure to various concentrations (Iturburu et al., 2017; Özdemir et al., 2018). Detection of imidacloprid in fish brain tissue indicates that this contaminant can cross the blood-brain barrier (BBB) in organisms other than insects, thus contributing to potential negative impacts on the central nervous system. Furthermore, studies have also found changes in amphibian brain widths in the optic tectum (region responsible for vision), medulla oblongata (respiratory and auditory function), and the diencephalon (regulates the endocrine system, homeostasis, motor function control, and relays sensory information) in *Rana pipiens* after exposure to the insecticide, chlorpyrifos, which also targets cholinergic neurotransmission (McClelland et al., 2018). These changes in brain widths are likely due to apoptosis and neural swelling, providing a probable cause for negative neural impacts and consequent behavioral changes in amphibians (Woodley et al., 2015; McClelland et al., 2018). Studies have also shown that imidacloprid can reduce action potential amplitude, area and latency of the sciatic nerve in *Rana ridibunda* (Akbas et al., 2014) and cause DNA damage in other amphibian species (Feng et al., 2004; Ruiz de Arcaute et al., 2014). Due to previously reported abnormal behavior, physiological disruptions, and detections of neonicotinoids in the tissue of other vertebrate taxa, there is a high likelihood that imidacloprid can cross the BBB in amphibians and alter behavioral performance following exposure.

Despite widespread studies showing the negative impacts of imidacloprid on non-target organisms, such as various bee species (Tasman et al., 2020), this neurotoxic chemical is still widely used throughout the United States. Moreover, there is a general lack of knowledge about the uptake and distribution of imidacloprid in vertebrate brains, particularly aquatic organisms. Therefore, research focusing on the neurotoxicity of imidacloprid in amphibians is highly warranted and overdue considering imidacloprid was first introduced over 30 years ago. To fill this important research gap, we investigated the effects of imidacloprid in adult Northern Leopard frogs (*Rana pipiens*) in a laboratory based study. The aims of this study were to 1) quantify imidacloprid and the metabolite, imidacloprid-olefin, enrichment in adult Northern Leopard frog brains; 2) reveal behavioral effects of aquatic exposure to imidacloprid at environmentally relevant concentrations; 3) determine differences in total length and body mass between treatment groups; and 4) establish if there is a relationship between imidacloprid brain concentration and feeding response times.

2. Materials and methods

2.1. Field collection

Fifty adult Northern Leopard frogs were collected from a single reference U.S. Fish and Wildlife Service Waterfowl Production Area in eastern South Dakota (Lost Lake WPA: 43.67738, −97.05740). This site is part of a long-term monitoring study and previous data support this wetland as a reference site (Schwarz et al., 2018). Collected individuals were housed in standard 10-gallon tanks at 26 °C (±0.48 °C) with a photoperiod set at 12:12-h light:dark and allowed to acclimate for one week prior to experimental exposure. Following the acclimation period, animals were transferred to individual plastic containers containing 1000 mL of dosed water, which roughly came up to the animal's "mid-section", for the remainder of the study. 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).

2.2. Experimental design

To investigate the ability of imidacloprid to cross the blood brain barrier in amphibians, adult Northern Leopard frogs were randomly exposed to treatments of analytical standard imidacloprid (Sigma-Aldrich; CAS no.138261-41-3) at nominal concentrations of 0, 0.1, 1, 5, and 10 μg/L (control = reconstituted reverse osmosis water and 0.001% dimethyl sulfoxide (DMSO)) for 21 consecutive days (n = 10 per treatment). These concentrations are considered environmentally relevant and were based on results from a long-term study in eastern South Dakota and prior detections throughout the United States and several other countries (Sánchez-Bayo et al., 2016; Schwarz et al., 2018; Mineau, 2020). Water was changed and re-dosed with the appropriate imidacloprid concentration every 7 days. Tank locations were rotated at random bi-weekly to avoid potential micro-environmental effects. Mass and total length were documented at the beginning and end of the experiment and mass was subsequently recorded every 7 days with water changes.

Prior to exposure, a stock solution was prepared by diluting imidacloprid in DMSO. Previous studies have indicated DMSO concentrations at or below 20 μL/L (0.02% DMSO) can be used in amphibian ecotoxicology studies (Young et al., 2020). This agrochemical was selected because imidacloprid is widely used throughout the United States and has

been previously detected at high concentrations in tile wetlands in South Dakota (Schwarz et al., 2018). Individuals were anesthetized via benzocaine and euthanized via rapid decapitation. Decapitated heads were immediately flash frozen and stored at −80 °C. Flash frozen Northern Leopard frog heads were removed from −80 °C at a later date and whole brains were quickly and carefully excised, trimmed of cranial nerves, and weighed. All excised whole brain samples were stored in micro-centrifuge tubes at −20 °C prior to shipment to the University of North Dakota (Grand Forks, ND) for imidacloprid and imidacloprid-olefin tissue concentration analysis. Imidacloprid and imidacloprid-olefin brain concentrations were normalized to protein concentration (pg/mg protein) to account for varying water loss among brain samples during sample storage and shipping (Appendix A).

2.3. Imidacloprid and imidacloprid-olefin analysis

Liquid chromatography-tandem mass spectrometry was used to determine imidacloprid and imidacloprid-olefin concentrations in whole brain samples at the University of North Dakota's Mass Spectrometry Core facility (Grand Forks, ND) (Appendix A). Liquid chromatography mass spectrometry was used to confirm experimental stock solution concentrations at the University of Nebraska-Lincoln Water Science Laboratory (Lincoln, NE). Verified stock solutions were within 68–82% of nominal stock concentrations.

2.4. Feeding trials

Three feeding trails were conducted during the 21-day exposure to varying concentrations of imidacloprid. One cricket was placed at the front of each container and 'time to consumption' was recorded in seconds. Timing was stopped after 2 min (120 s) and individuals that did not consume the cricket within this time were noted. Crickets that were not consumed within 120 s were left in the container to ensure all animals were fed the same amount. Individuals were fed in a random order and dividers were placed between each tank during feeding trials to prevent distractions and agitation of adjacent amphibians. All feeding trials occurred 24 h after water changes and re-dosing of imidacloprid concentrations.

2.5. Statistical modeling

All data were analyzed using R (R Development Core Team, 2017) in RStudio (RStudio Team, 2020). Relationships between response and predictor variables were assessed through generalized linear and generalized linear mixed models using Bayesian inference. A gamma distribution with a log link was chosen for most models due to the positive nature of our data. A skew normal distribution was used to model the relationship between imidacloprid-olefin brain concentration and treatment group due left-skewness and small values in this dataset. Priors were determined through prior simulation (Wesner and Pomeranz, 2020).

Models were fit using *rstan* (Stan Development Team, 2020) via the *brms* (Bürkner, 2017) package. Markov chain Monte Carlo (MCMC) was used to obtain the joint posterior distribution. All models contained four chains and a minimum of 2000 iterations, 500–800 of which were used as warm-ups and discarded (Table B1). Model convergence was visually assessed through trace plots of the posterior distribution and r-hat values (potential scale reduction factor). All models had r-hat values less than 1.1, indicating model convergence. Model fit was inspected through posterior predictive checks, including boxplots and histograms (Gelman and Shalizi, 2013). For each model, means and 95% credible intervals were estimated for the parameters from the posterior distribution. The *loo* package (Yao et al., 2018) 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. The difference between two responses was calculated over the number of iterations from the posterior distribution and then the number of differences greater than zero was divided by the number of samples in the distribution, producing a percent probability of the difference. We highlight below when mean differences were different between groups when the 89% posterior credible interval did not contain zero. All data and code are available through GitHub (user: kscampbell, repository: imi_brain_lab).

3. Results

3.1. Imidacloprid brain concentrations

Imidacloprid brain levels were concentration dependent and increased linearly (Fig. 1). Average imidacloprid brain concentrations ranged from 4.36 pg/mg protein in the control group to 1365 in the 10 μg/L group. There was a greater than 99% probability that each treatment group had higher imidacloprid brain concentrations compared to the control (Table 1).

3.2. Imidacloprid-olefin brain concentrations

Average imidacloprid-olefin brain concentrations followed a dose-response relationship and ranged from 2.18 to 15 pg/mg protein (Fig. 2). The control group had an average 2.18 imidacloprid-olefin pg/mg protein (±0.66 SD; 89% CrI [1.0, 3.24]), despite never being exposed to contaminants in a lab setting. Based on our data and the model, imidacloprid-olefin brain concentrations in the 0.1 and 1 μg/L treatment groups were not different from the control (Table 2). However, there was a >99% probability that the 5 and 10 μg/L exposure groups differed in imidacloprid-olefin brain levels compared to the control (Table 2).

3.3. Feeding response times

Average response times across all feeding trials and treatments ranged from 10.4 to 32.9 s. Reaction times followed a biphasic dose-response relationship (hormesis), in which the 1 μg/L exposure group experienced the slowest reaction times (32.9 ± 17 SD; 89% CrI [12.3, 63.5]) (Fig. 3). Reaction times varied by treatment and trial date. On average, response times

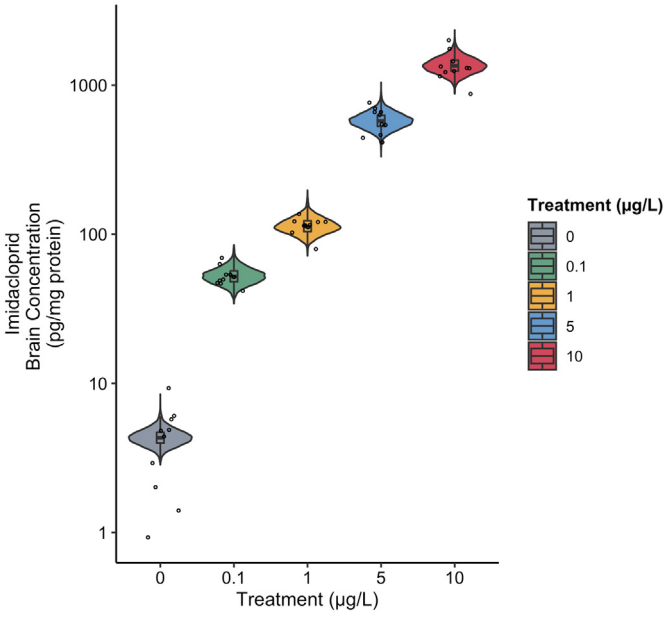

Fig. 1. Comparison of imidacloprid brain concentrations (pg/mg protein) in Northern Leopard frogs by treatment (n = 10 per treatment). Points represent raw imidacloprid brain concentrations per individual. Results are averages and 95% credible intervals from the posterior distribution of a Bayesian generalized linear mixed model. Y-axis is on the log scale.

Table 1
Average difference in imidacloprid brain concentration between treatment groups. Averages were extracted from the posterior distribution of a Bayesian generalized model with 89% credible intervals.

Treatment-pair	Average difference in imidacloprid brain (pg/mg protein)	89% credible interval of difference	Probability difference is greater than zero
0–0.1	48.37	(38.72, 59.71)	>99.99%
0–1	109.99	(88.98, 134.3)	>99.99%
0–5	577.32	(468.74, 701.68)	>99.99%
0–10	1360.75	(1111.46, 1649.62)	>99.99%

Table 2
Average difference in imidacloprid-olefin brain concentration between treatment groups. Averages were extracted from the posterior distribution of a Bayesian generalized model with 89% credible intervals.

Treatment-pair	Average difference in imidacloprid-olefin brain (pg/mg protein)	89% credible interval of difference	Probability difference is greater than zero
0–0.1	0.46	(−0.82, 1.75)	72.80%
0–1	0.18	(−1.36, 1.72)	58.10%
0–5	5.12	(3.4, 6.86)	>99.99%
0–10	12.83	(11.1, 14.64)	>99.99%

were slowest during the 1st (28.8 s ± 15.5 SD; 89% CrI [10.5, 57]) and 3rd (22 s ± 11.9 SD; 89% CrI [8.18, 43.7]) feeding trials, while reaction times were fastest during the 2nd trial (11.5 s ± 6.1 SD; 89% CrI [4.2, 22.3]). When comparing mean differences in reaction time by treatment, the 89% credible intervals for the 0.1, 1, and 5 μg/L groups did not contain zero, indicating reaction times were different from the control (Table 3). Conversely, average reaction times in the 10 μg/L treatment were 4.8 s (±6.5 SD; 89% CrI [−3.5, 16.2]) slower than the control and the credible interval for the difference of means contained zero, indicating reaction times in 10 μg/L treatment were not different from the control group (Table 3). Additionally, the 1 and 10 μg/L treatments were different from each other, in which response times were 17.6 s slower in the 1 μg/L group (89% CrI [1.5, 43.1]).

3.4. Interaction between response time, treatment, and imidacloprid brain concentrations

There was a positive relationship between log-transformed imidacloprid brain concentration and mean feeding response times (Fig. 4; slope: 1.1 (1.0 to 1.2); median (89% CrI)). There was a >99.99% probability that the slope was greater than zero. Additionally, the relationship between mean response time and log-transformed imidacloprid brain concentration was asymptotic but was linear on the log-log scale (Fig. 4).

3.5. Morphological changes

Average initial body mass ranged from 15.4 to 20.3 g in all treatments, while final body mass ranged from 13.2 to 17.9 g. The control group had higher initial body mass compared to the 1 and 10 μg/L groups, but was not considered different from the 0.1 or 5 μg/L treatments. Similarly, final body mass was highest in the control group and was different from the 1 and 10 μg/L treatments, but not the 0.1 or 5 μg/L groups (Table B2). Total loss of body mass ranged from 2.1 to 2.4 g, in which there was a greater than 99.97% probability that final body mass was lower than initial body mass among all treatments (Table B2). Although all treatments experienced a loss of body mass, these differences were not considered significant between treatments (Fig. B1).

Average total length at the start of the experiment ranged from 57 to 63.1 mm and final total length ranged from 64.7 to 69.9 mm. Average growth among treatment groups ranged from 5.7 to 12.3 mm, with the most growth occurring in the 0.1 μg/L group and the least amount of growth occurring in the 10 μg/L treatment (Fig. B2). On average, the 0.1 μg/L treatment grew 5.7 mm more than the control group, with a >98% probability that the difference was greater than zero (±2.69 SD;

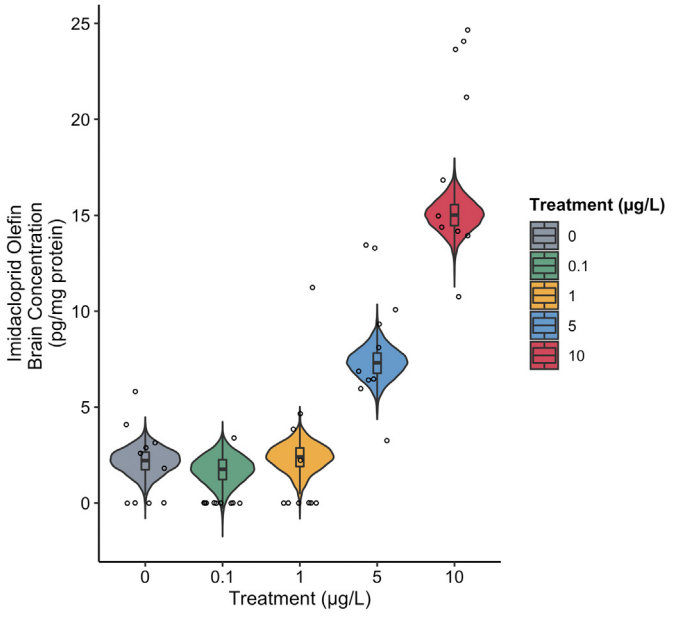

Fig. 2. Comparison of imidacloprid-olefin brain concentrations (pg/mg protein) in Northern Leopard frogs by treatment (n = 10 per treatment group). Points represent raw imidacloprid-olefin brain concentrations per individual. Points along the x-axis represent non-detections. Results are averages and 95% credible intervals from the posterior distribution of a Bayesian generalized linear mixed model.

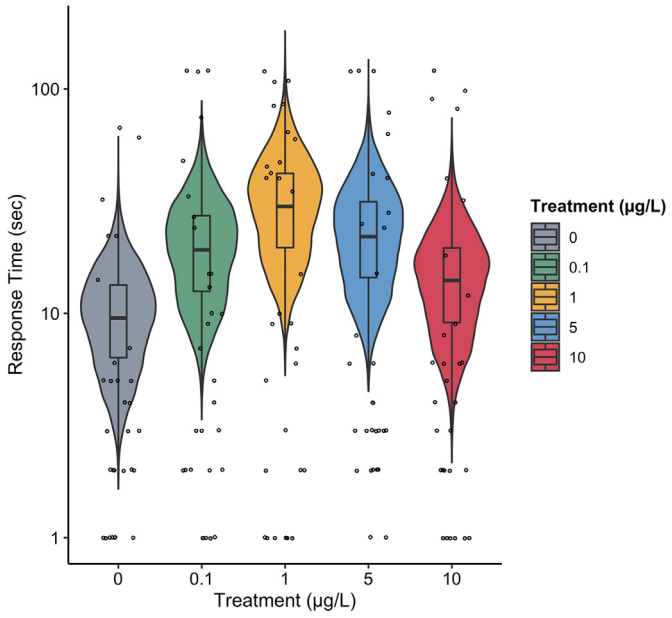

Fig. 3. Comparison of feeding response times (sec) between treatments and trial dates (n = 30 per treatment). Results are averages from the posterior distribution of a Bayesian generalized linear mixed model with 95% credible intervals. Y-axis is on the log scale.

Table 3
Average differences in response times between treatment groups. Averages were extracted from the posterior distribution of a Bayesian generalized mixed model with 89% credible intervals.

Treatment-pair	Average difference in response time (sec)	89% credible interval of difference	Probability difference is greater than zero
0–0.1	10.62	(0.35, 26.56)	95.18%
0–1	22.44	(5.78, 48.77)	99.53%
0–5	13.9	(1.94, 33.12)	97.47%
0–10	4.84	(−3.50, 16.18)	81.52%

89% CrI [1.46, 9.97]). All other treatments were not considered different from the control in terms of growth (Table B3).

4. Discussion

The present study represents the first detection of imidacloprid and imidacloprid-olefin in amphibian brain tissue, indicating the ability of imidacloprid to cross the BBB in adult amphibians. Furthermore, these results add to the growing body of evidence that neonicotinoids can alter body morphology, feeding behavior, and response times in amphibians at ecologically relevant concentrations.

We detected imidacloprid and imidacloprid-olefin concentrations up to 1365 and 15 pg/mg protein respectively in amphibian whole brain samples. Interestingly, previous studies have reported the potential low neurotoxicity and even the inability of imidacloprid to cross the BBB in vertebrates due to the varying number, subtypes, and electrical charges of vertebrate nAChRs (Tomizawa and Casida, 2003; Tomizawa, 2004; Sheets, 2010; Berheim et al., 2019; Thompson et al., 2020). However, our results show clear evidence of dose-dependent bioaccumulation in amphibian neural tissue, in which imidacloprid and imidacloprid-olefin brain concentrations increased monotonically. This dose-response relationship is characteristic of many other contaminants and is not particularly surprising, however, imidacloprid brain concentrations increased by a factor of 313, suggest imidacloprid may have stronger binding affinity that previously thought or has the ability to bind at multiple locations. Moreover, there was 9.75% protein in the brain (±6.48% SD, $n = 80$). Assuming 1 g/mL brain specific gravity (Larramendi et al., 2021), the highest exposure group had 140 μg/L of brain tissue or 14 times higher imidacloprid brain levels compared to exposure concentration. These results further support the notion that imidacloprid can bioaccumulate in the amphibian brain despite low exposure concentrations.

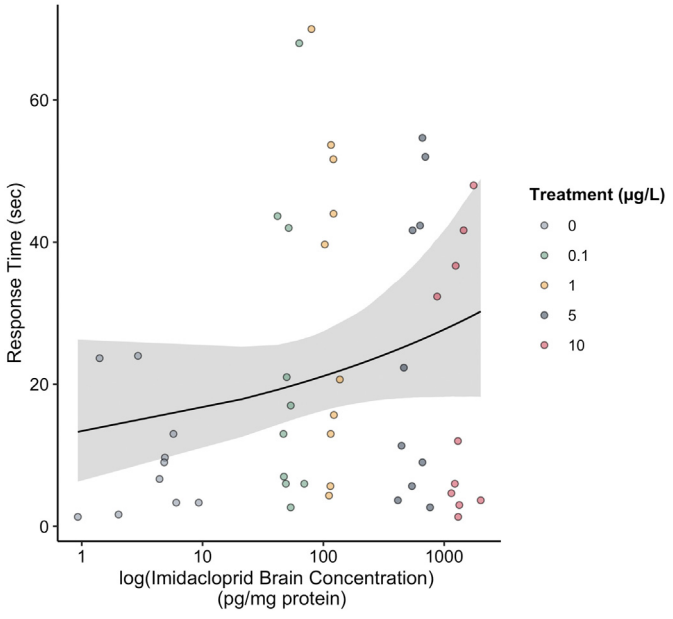

Fig. 4. Relationship between log-transformed imidacloprid brain concentrations in Northern Leopard frogs and average feeding response by treatment. Points represent raw mean response times per individual ($n = 10$ per treatment group). Regression line represents the average slope and 95% credible intervals from the posterior distribution of a Bayesian generalized linear model. X-axis is on the log-scale.

Mean imidacloprid brain concentrations in the control group were 4.36 pg/mg protein, despite never being exposed to imidacloprid during the course of the experiment. This is likely due to pre-exposure in the field prior to collection for this study. Based on a previous field study (Campbell et al. unpublished), baseline imidacloprid brain concentrations in juvenile Northern Leopard frogs collected from the same Reference Wetland were 2.25 pg/mg protein, which was similar to the amount of imidacloprid in control group brains in the present study (variation in brain concentration may be due to sampling in different months and age class). Since control animals were not exposed to imidacloprid during the 21 days of the experiment, the presence of imidacloprid in control brains approximately 1 month (1 week of depuration +3 weeks of experiment) following potential field exposure likely suggests poor neuronal detoxification in adult Northern Leopard frogs.

Similarly, average imidacloprid-olefin brain concentrations in the control group were 2.18 pg/mg protein, owning to metabolism of imidacloprid in the brain. Neonicotinoids often induce delayed toxicity (van Lexmond et al., 2015), therefore response times in the control group may represent the effects of low imidacloprid and imidacloprid-olefin brain concentrations on foraging behavior instead of the behavior of naïve, un-exposed amphibians. To fully understand the behavioral effects of imidacloprid on adult Northern Leopard frogs, future studies should induce mating of unexposed adults and use the reared offspring for experimental exposures and behavioral trials.

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 (Hussain et al., 2016; Thompson et al., 2020). The three main imidacloprid metabolites include imidacloprid-olefin, 5-hydroxy-imidacloprid (5-OH-imidacloprid), and 4,5-dihydroxy-imidacloprid, two of which can be directly converted to imidacloprid-olefin. Additionally, imidacloprid-olefin has been reported to be 10 times more toxic to insects and mammals and shows a higher affinity for mammalian nAChRs (Honda et al., 2006; Hussain et al., 2016). Recent studies have reported imidacloprid and imidacloprid-olefin residues in the brain tissue of lizards (Wang et al., 2019), rock pigeons (Abu Zeid, 2018), and chameleon cichlids (Iturburu et al., 2017); suggesting that imidacloprid can cross the BBB of a wide range of taxonomic groups and could have broad negative impacts on biodiversity and human communities.

Regarding morphological changes in imidacloprid dosed groups, all groups experienced a decrease in body mass, however these differences were not considered different between treatments. Previous studies have reported a loss in body mass in rabbits (Memon et al., 2014), mice (Arfat et al., 2014), rats (Mohamed et al., 2016), and birds (Eng et al., 2017) following imidacloprid exposure. Alternatively, Robinson et al. (2017) did not detect any impacts of imidacloprid exposure on body mass in chronically exposed wood frog tadpoles. It is evident that imidacloprid can have variable responses on body mass among taxonomic groups and varying age-class, thus it is imperative for future studies to focus on the direct effects of imidacloprid on metabolic activity and how this corresponds to an increase or decrease in body mass.

Unexpectedly, all groups experienced an increase in body length, however, only the lowest exposure group (0.1 μg/L) experienced significant growth compared to the control. Additionally, the highest exposure group (10 μg/L) experienced the least amount of growth, however, it was not different from the control group. This is a prime example of hormesis, in which there is low dose stimulation and inhibition at higher doses. These findings are in opposition of previous studies that suggest imidacloprid exposure often results in decreased growth in terms of body length (Gibbons et al., 2015).

We found exposure to imidacloprid at ecologically relevant concentrations resulted in slower feeding response times in adult Northern Leopard frogs, however the 10 μg/L treatment was not considered different from the control (Table 3). Additionally, there was a positive relationship between log transformed imidacloprid brain concentrations and average feeding response time, suggesting imidacloprid brain levels may alter foraging ability in adult amphibians. Our findings are consistent with previous studies that have detected altered foraging behavior, vision loss, and impaired visual learning following imidacloprid exposure in honey bees and flies (*Drosophila melanogaster*) (Martelli et al., 2020). Recent research has also shown that wood frogs exposed to imidacloprid as tadpoles were less likely to escape simulated predator attacks, suggesting imidacloprid exposure at 10 and 100 μg/L may negatively impact perception and cognitive function in tadpoles (Lee-Jenkins and Robinson, 2018; Sweeney et al., 2021).

Behavior is considered an obvious and environmentally relevant measure of neurotoxicity because behavioral alterations often correlate with nervous system functionality (Fitzgerald et al., 2021). However, neurotoxic substances can interact with binding sites in different, and potentially multiple, regions of the brain, resulting in a wide variety of behavioral phenotypes. During the present study, all amphibians were collected from the same Reference Wetland site to minimize variation; nevertheless there was a clear distinction in response time between individuals from the same treatment group (Fig. 4). This suggests that some individuals were more "sensitive" while others were "resistant" to the behavioral effects of imidacloprid exposure.

Adult Northern Leopard frogs can have home ranges that span several hectares and are known to travel between adjacent wetlands (Knutson, 2018); therefore it's likely these individuals had different life histories and may have been previously exposed to variable durations and concentrations of imidacloprid. Studies have shown that chronic exposure to nicotine, which neonicotinoids were modeled after, leads to upregulation, resulting in higher numbers of nAChRs, changes in stoichiometry and trafficking, and an increase in the number of high-affinity binding sites (Henderson and Lester, 2015). Furthermore, previous research with bumblebees has shown that chronic exposure to imidacloprid enhances nAChR sensitivity (Moffat et al., 2016). Based on this information, pre-exposure to imidacloprid during other amphibian life stages could result in changes to nicotinic receptor conformations, which in turn, could alter the sensitivity of these receptors to imidacloprid, resulting in "sensitive" and "resistant" individuals.

These results have widespread implications for aquatic and semi-aquatic organisms and will serve as the basis for neonicotinoid neurotoxicity in amphibians. It is evident that exposure to low concentrations of imidacloprid can induce variable morphological and behavioral responses in adult amphibians; therefore future research should focus on whether conformational changes occur in amphibian brains and to pinpoint the affected receptors. Additionally, amphibians are often exposed to a wide range of environmental contaminants in the wild; therefore future studies should also investigate the impacts of combinations of various neonicotinoids and other classes of pesticides.

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CRediT authorship contribution statement

K.S. Campbell: Conceptualization, Data curation, Methodology, Software, Formal analysis, Funding acquisition, Investigation, Visualization, Writing – original draft, Writing – review & editing, Project administration. **P.G. Keller:** Investigation, Writing – review & editing. **L.M. Heinzel:** Investigation, Writing – review & editing. **S.A. Golovko:** Methodology, Resources, Validation, Writing – review & editing. **D.R. Seeger:** Methodology, Resources, Validation, Writing – review & editing. **M.Y. Golovko:** Methodology, Resources, Validation, Writing – review & editing. **J.L. Kerby:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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