Final Report

Sublethal Effects of the Insecticide Imidacloprid on Salamanders

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Project Summary

Imidacloprid is an insecticide that is currently being widely applied to hemlock (*Tsuga* spp.) stands in the eastern United States to reduce mortality caused by the exotic invasive insect hemlock woolly adelgid (*Adelges tsugae*). While this management strategy is effective for hemlock conservation, few studies have examined non-target effects of applying imidacloprid in hemlock systems. Our project investigated potential sublethal effects on stream salamanders of a real world imidacloprid application program in the National Parks of Southern West Virginia, USA. Specifically, we sought to understand if stress hormone levels (i.e., corticosterone level) for Northern Dusky Salamanders (*Desmognathus fuscus*) and Seal Salamanders (*Desmognathus monticola*) were correlated with presence and concentration of imidacloprid. Corticosterone (CORT) is a hormone released in response to unexpected stimuli to maintain homeostasis. The CORT-fitness hypothesis predicts that lower baseline levels of CORT indicate a healthier animal. Thus, we would expect that if exposure to imidacloprid in real world concentrations negatively affects salamander health, this would be detectable as higher baseline levels of CORT.

All CORT sampling field work was completed in 2017. We collected CORT samples from 121 Northern Dusky Salamanders and Seal Salamanders, sampling treated and untreated sections of 7 streams. Corticosterone levels were quantified by the Endocrine Technologies Core Team at the Oregon National Primate Research Center, Beaverton, Oregon, USA. Imidacloprid concentration at sampling sites was quantified using ultra-performance liquid chromatography-tandem mass spectrometry ([UP] LC-MS/MS).

Concurrent research by our team investigated additional potential sublethal effects of imidacloprid exposure on aquatic organisms, including bioaccumulation of the pesticide in *Desmognathus* spp. and benthic macroinvertebrates, and relationships between imidacloprid exposure and salamander body condition. For this report, we integrated our stress hormone investigation with these additional project components. This report represents Chapter 2 from *Crayton, S. M. 2019. Stream salamander and benthic macroinvertebrate community responses to imidacloprid exposure. Thesis, West Virginia University, Morgantown, West Virginia, USA.*

BIOACCUMULATION OF THE PESTICIDE IMIDACLOPRID IN STREAM ORGANISMS AND SUBLETHAL EFFECTS ON SALAMANDERS

ABSTRACT

The insecticide imidacloprid is widely used to mitigate hemlock (*Tsuga* spp.) mortality resulting from the invasive hemlock woolly adelgid (HWA; *Adelges tsugae*), but evidence suggests that imidacloprid can have negative impacts on adjacent stream systems. Laboratory studies have shown that imidacloprid bioaccumulates in anurans and spotted salamanders and causes sublethal effects, but no studies have investigated whether salamanders or insects in streams adjacent to HWA treatments can bioaccumulate imidacloprid. We collected *Desmognathus* spp. from seven streams directly adjacent to HWA treatments and four streams not adjacent to HWA treatments in West Virginia. We also collected benthic invertebrates from 15 streams adjacent to HWA treatments. We assessed the effect of imidacloprid exposure and imidacloprid bioaccumulation on levels of the stress hormone, corticosterone, and body condition indices (BCI). Of 107 tested salamanders, we detected imidacloprid bioaccumulation in the tissues of 14 *Desmognathus* spp. and the metabolite imidacloprid-olefin in the tissues of 19 *Desmognathus* spp. for a total of 29 individuals with one or both chemicals. Of 15 tested benthic invertebrate samples, we detected imidacloprid bioaccumulation in 15 samples and imidacloprid-urea in 13 samples. The top model for corticosterone included additive effects of species, sex, and number of imidacloprid applications in adjacent treated stands, and corticosterone levels increased with an increasing number of imidacloprid applications adjacent to the stream. The top model for BCI contained concentration of imidacloprid in stream water as a predictor, and BCI decreased with increasing imidacloprid concentration. This study provides strong evidence that salamanders and stream invertebrates bioaccumulate imidacloprid which leaches from HWA treatments and that imidacloprid is associated with sublethal effects in salamanders.

1. INTRODUCTION

Eastern hemlock (*Tsuga canadensis*) and Carolina hemlock (*T. caroliniana*) are ecologically important tree species in eastern North America that provide unique microhabitat conditions used by diverse invertebrate and vertebrate taxa (Becker et al. 2008, Ellison 2014, Snyder et al. 2002, Tingley et al. 2002). Hemlock trees exert a strong influence on the abiotic

environment by creating deep shade that reduces ground and stream temperatures, and by producing litter with a slow rate of decomposition, which stabilizes soil moisture levels (Daley et al. 2007, Hadley and Schedlbauer 2002, Mathewson 2009, Snyder et al. 2002). Hemlock populations are currently being impacted by the non-native insect hemlock woolly adelgid (HWA; *Adelges tsugae*), which has spread to ca. 50% of the geographic distribution of Eastern hemlock (Havill et al. 2014). In response to substantial mortality observed in infested hemlock stands (e.g., Krapfl et al. 2011), HWA control programs have been widely implemented on public lands in the United States (Vose et al. 2013). The most common and effective method of preventing HWA-induced tree death is application of the neonicotinic insecticide imidacloprid (Webb et al. 2003). Although neonicotinoids are highly selective for insect nicotinic acetylcholine receptors (nAChR), many studies have documented effects to health and survival of vertebrates exposed to this class of insecticides (Gibbons et al. 2015, Hallman et al. 2014, Matsuda et al. 2001, Yamamoto 1999). Imidacloprid is generally not lethal to adult vertebrates at concentrations typically found in the environment, but studies have found a variety of sublethal effects to mammals, birds, fish, and frogs, including effects on reproduction and growth (reviewed by Gibbons et al. 2015).

North America is a global hotspot for salamander diversity (Yap et al. 2015), particularly the Appalachian Mountains in the eastern United States (Petranka and Murray 2001). In headwater streams, salamanders are often the dominant vertebrates in terms of abundance and biomass (Burton and Likens 1975, Davic and Welsh 2004). Several studies have shown that imidacloprid can leach from HWA treatments into adjacent streams (Benton et al. 2017, Churchel et al. 2011, Wiggins et al. 2018), thus potentially exposing stream salamanders to the insecticide. Research investigating the physiological and ecological consequences of imidacloprid on salamanders is lacking, but in anurans, exposure to imidacloprid can cause DNA damage and increased mortality rates (Ade et al. 2010, Feng et al. 2004, Pérez-Iglesias et al. 2014). Additionally, laboratory studies have documented sublethal and lethal effects of imidacloprid exposure on benthic macroinvertebrates (Alexander et al. 2007, Columbo et al. 2013, Kreutzweiser et al. 2008), which are a major food resource for stream salamanders (Petranka 1998).

One potential result of exposure to imidacloprid is bioaccumulation of the pesticide. Measurable levels of imidacloprid were detected in northern cricket frog (*Acris crepitans*),

eastern narrowmouth toad (*Gastrophryne carolinensis*), barking tree frog (*Hyla gratiosa*), and southern leopard frog (*Lithobates sphenocephala*) tissues after 8 hours of exposure to imidacloprid in a laboratory (Glinski et al. 2018, Van Meter et al. 2014, Van Meter et al. 2015). To our knowledge, no studies have investigated whether HWA control programs are resulting in imidacloprid bioaccumulation in stream salamanders. Additionally, stream salamanders prey on crayfish and benthic macroinvertebrates (reviewed by Petranka 1998), and thus bioaccumulation of imidacloprid in stream invertebrates is a potential route of pesticide exposure for salamanders and other vertebrates.

Sublethal effects of environmental stressors have been associated with hormone level changes in amphibians, particularly the hormone corticosterone. Corticosterone is a glucocorticoid hormone produced by the hypothalamus-pituitary-interrenal (HPI) axis that is associated with reproduction, development, growth, and stress in amphibians (Romero et al. 2004). Corticosterone enables an animal to maintain allostasis when exposed to a stressor by increasing available energy or causing behavioral changes (McEwen and Wingfield 2003). Long-term elevation of corticosterone levels induced by chronic stressors can have negative effects, including suppression of the immune system and growth (Romero et al. 2004). Multiple studies have documented increases in corticosterone levels in salamanders due to environmental stressors such as competition for habitat (Cooperman et al. 2004), increased temperature (Novarro et al. 2018), low moisture (Charbonnier et al. 2018), higher acidity (Chambers et al. 2013), and vernal pool size (Millikin et al. 2019). Similar associations were found in anurans where corticosterone was elevated in environments with limited food (Glennemeier and Denver 2002), higher anthropogenic disturbance like traffic noise (Troïanowski et al 2017), and presence of environmental contaminants (Hopkins et al. 1997).

In addition to influencing hormone levels, exposure to contaminants can negatively impact the size, growth rate, and body condition of individuals. For example, exposure to the herbicide atrazine was associated with smaller sizes and lower weights in Tiger Salamander (*Ambystoma tigrinum*) larvae (Larson et al. 1998), reduced Cuban tree frog (*Osteopilus septentrionalis*) tadpole snout-vent length (SVL) and mass (Gabor et al. 2018), and decreased growth rate in Southern Leopard Frogs (*Lithobates sphenocephala*; Adelizzi 2019). Negative effects on the health of individuals can ultimately result in population-level declines if they affect growth, reproduction, or survival rates (Hayes et al. 2010, Willson et al. 2012).

The purpose of this study was to determine if salamanders inhabiting streams adjacent to HWA treatments are bioaccumulating imidacloprid, and if there are detectable sublethal effects on individuals. In addition, we assessed bioaccumulation of imidacloprid and its metabolites in benthic macroinvertebrates at a subset of the study sites. We used corticosterone levels and body condition indices (BCI) to assess sublethal effects and tested whether they are correlated with imidacloprid concentration in salamander tissues and stream water at the time of sampling. We hypothesized that salamanders in streams with imidacloprid would have the chemical in their body, and that salamander imidacloprid concentration would be positively correlated with environmental concentration. We also hypothesized that salamanders with higher levels of imidacloprid in their tissues and salamanders collected from streams with higher imidacloprid concentrations would have higher levels of corticosterone and lower BCI scores, indicative of sublethal effects from imidacloprid exposure.

2. METHODS

2.1. Study Sites

This study was conducted in the Monongahela National Forest (MNF) and two units of the National Park Service (NPS): Gauley River National Recreational Area (GARI) and New River Gorge National River (NERI) in West Virginia, USA (Fig. 1). Hemlock stands in the MNF were treated with a single application of imidacloprid in 2014 or 2015. Hemlock stand treatments in NPS units began in 2006 and have continued annually, including repeated applications at 10 of the sampling locations. We did not select NPS treatment sites if the last treatment occurred prior to 2011. We used ArcGIS 10.4 to identify candidate streams based on proximity to HWA treatments. Candidate streams were visited to determine whether the stream depth, stream substrate, and water flow speeds were suitable for sampling salamanders and stream invertebrates. Final study sites were selected based on the suitability of the streams for sampling, the proximity of treated trees, or the absence of known treatments for non-impacted sites. Sites adjacent to HWA treatments had on average 306 ± 158.7 occurrences of imidacloprid applications (range = 5–3993 applications), with applications representing individual tree treatments.

We sampled headwater streams for stream salamanders in 24 sites in MNF, 14 sites in NERI, and ten sites in GARI. Of these 48 sites, 27 were directly adjacent to HWA treatments and

21 were not adjacent to HWA treatments. Sites that were not adjacent to HWA treatments were either a minimum of 100 m upstream of imidacloprid application or were in a watershed without known HWA treatments. We selected 100 m as a minimum distance because Benton et al. (2015) did not detect imidacloprid in stream sites that were 10–100 m upstream of treated trees, and because *Desmognathus* spp. typically have home ranges smaller than 100 m (reviewed by Petranka 1998).

We collected and euthanized adult individuals of the salamander genus *Desmognathus* to quantify imidacloprid bioaccumulation and corticosterone levels from 11 of the 48 sampled sites, including seven sites in GARI and four sites in NERI. For salamander collection, we selected sites that had high densities of large adult seal salamanders (*D. monticola*) and northern dusky salamanders (*D. fuscus*). Seven sites that were sampled for stream salamanders were directly adjacent to HWA treatments and four were not. For this subset of sites, sites adjacent to HWA treatments had an average of 244.3 ± 93.8 occurrences of imidacloprid application (range = 17–675 applications).

We also sampled benthic macroinvertebrates from 15 of the 48 sites used for salamander sampling. This subset of sites was comprised of five sites in NERI, two sites in GARI, and eight sites in MNF. For benthic macroinvertebrate collection, we selected sites with abundant riffles. All sites that were sampled for benthic macroinvertebrates were adjacent to HWA treatments.

2.2 Water and Sediment Sampling

We collected two liters of water from each stream in 1 L plastic bottles (Thermo Scientific Nalgene™ labware, Rochester, NY) without disturbing the stream sediment. Stream sediment was collected from the bottom of the stream using a trowel and enough sediment was collected to fill one quart-sized plastic bag. If the stream bottom did not have any sediment, we collected sediment from the sides of the stream bank. The trowel was wiped with 70% ethanol between each use to prevent contamination. The bottles of water and bags of sediment were placed in black bags and a backpack to prevent light exposure from metabolizing imidacloprid until the samples could be placed in a cooler with ice. The samples were stored at 4°C from the day of collection until extraction procedures began.

2.3 Water and Sediment Imidacloprid Extraction and Quantification

We adapted water and sediment extraction procedures from Baskaran et al. (1997). We filtered 1 L of water from each site through 0.22-µm filters. We then filtered the water samples through pre-conditioned C18 solid-phase extraction (SPE) cartridges on a vacuum manifold. We eluted the imidacloprid from the cartridges with 5 mL acetone into 15-mL glass test tubes and dried the eluent under nitrogen at 100°C and reconstituted the residue in 0.5 mL of acetonitrile. We then filtered the reconstituted samples through 0.20-µm filters into liquid chromatography (LC) vials.

We dried sediment samples at 100°C for 3 days in an oven before crushing and sieving the samples. We then weighed the sediment samples to 30 g, added 100 mL of deionized (DI) water, and stirred for 1 min. We sonicated the samples for 15 min at room temperature and filtered the samples through cheese cloth and a 0.22-µm filter. We completed the process of adding DI water and filtering twice before adjusting the final sample volume to 250 mL with DI water. We transferred the extract to a separatory funnel and added 25 mL of chloroform. We mixed the solution vigorously and extracted the chloroform layer through anhydrous sodium sulfate. We repeated this process twice before drying the solution under nitrogen at 100°C and reconstituting the residue in 0.5 mL of acetonitrile. We filtered the reconstituted samples through pre-conditioned florisil cartridges, eluted the imidacloprid using 5 mL of acetonitrile, and dried the eluent under nitrogen at 100°C. We reconstituted the residue in 0.5 mL of acetonitrile and filtered the reconstituted samples through 0.20 µm filters into LC vials

We quantified the concentration of imidacloprid in the stream sediment and water using ultra-performance liquid chromatography-tandem mass spectrometry ([UP] LC-MS/MS). We adapted the chromatographic and mass spectrometry conditions from Galeano et al. (2013). We used the Exion LC AD UHPLC system coupled with AB Sciex Qtrap 5500 triple quadrupole AcQuRate CEM detector. We separated the compounds imidacloprid, imidacloprid-urea, and imidacloprid-olefin and external standards on a Kromasil C_{-18} (M05CLD05) column (2.1 x 50 mm) maintained at an oven temperature of 40°C using a mobile phase gradient of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. We programmed the elution gradient as follows: 0–1.0 min, isocratic A to B (80:20, v/v); 1.0–1.3 min, from A to B (80:20, v/v) to A-B (0:100, v/v); 1.3–2.3 min, isocratic A to B (0:100, v/v); at 2.3 min, from A-B (0:100, v/v) to A to B (80:20, v/v); 2.3–6.0 min, isocratic A to B (80:20, v/v). We maintained the autosampler temperature at 10°C and the injection volume was 2 µL. The MS/MS detection of

the compounds was performed by electrospray ionization (ESI) source operated in positive ion mode.

We used multiple reaction monitoring (MRM) for the detection and quantification of imidacloprid and metabolites. MRM parameters were as follows: imidacloprid, Q1 mass 256.000 Da, Q3 mass 209.000 Da, 50.0 msec; imidacloprid urea, Q1 mass 213.200 Da, Q3 mass 129.000, 50.0 msec; imidacloprid olefin, Q1 mass 254.100 Da, Q3 mass 171.000 Da, 50 msec. We maintained the IonSpray voltage and source temperature at 4.50 kV and 450°C, respectively. We used the LC-MS/MS software Analyst (Sciex, Version 1.6.3) for data acquisition and processing. Due to project constraints, we were unable to quantify recovery success of imidacloprid from sediment, and thus we treated sediment data as presence-absence only for this study. We note that estimated imidacloprid concentrations in sediment were minor compared to estimated concentrations in stream water (i.e., typically <15% of the concentration in stream water). We were not able to test for presence/absence of metabolites in water for sites at NERI and GARI.

2.4 Salamander Sampling

Within each of the 48 sites, we established three 3.3 x 2 meter subplots for a total plot area of 10 x 2 meter. One meter of the subplot width was on the bank and one meter was within the wetted stream channel. We primarily placed subplots in riffles, but occasionally placed subplots in runs or pools if the site did not have riffle habitat. We chose subplots which were similar in terms of stream depth, substrate, canopy cover, vegetative community, and flow regime. We completed salamander sampling in the NPS sites between April and July of 2017 and in the MNF sites between April and July of 2018, sampling each site 6–7 times during the year. We conducted surveys during baseflow conditions. While moving upstream to prevent stream sediment from flowing downstream and obscuring our view, we flipped every cover object greater than 50 mm in diameter and searched through leaf packs.

We removed all captured salamanders and placed them in plastic bags. We identified all salamanders to species, or genus when identification to species was not possible. We weighed all captured salamanders to the nearest 0.1 g with a spring scale (Pesola Precision Scales, Schindellegi, Switzerland) and measured snout-vent length (SVL) and total length to the nearest 0.1 mm with dial calipers (Wiha Tools, Monticello, Minnesota, USA). We measured salamanders using a salamander stick to maximize accuracy (Margenau et al. 2018). We took

note of any missing limbs or tails and if salamanders were gravid. After processing, we returned salamanders to their point of capture.

2.5 Salamander Sampling for Corticosterone Concentration and Imidacloprid Bioaccumulation

We hand captured 175 *D. monticola* and *D. fuscus* salamanders by flipping rocks and other cover objects in the stream between 8 July and 24 November 2017. We selectively collected large salamanders because of a minimum tissue sample requirement for imidacloprid extraction and quantification. We measured and weighed the salamanders, placed them in individual plastic bags and transferred them to the laboratory, and humanely euthanized them through exposure to carbon dioxide followed by decapitation. Salamanders were then frozen until processing for imidacloprid extraction.

We quantified plasma levels of corticosterone for a subset of the salamanders (n = 126). For these samples, we decapitated salamanders in the field and collected a blood sample within three minutes of initial disturbance of the salamander to minimize the influence of capture stress on corticosterone level (Romero and Reed 2005). We then transported salamanders to the laboratory, measured and weighed them, and froze them until processing for imidacloprid extraction. We centrifuged blood samples for 5 min and plasma was collected and stored at -20°C until analysis. Plasma samples were packed in a cooler on dry ice and sent to the Endocrine Technology Laboratory at the Oregon National Primate Research Center (ONPRC) and assayed for corticosterone using radioimmunoassay (RIA; Thomas and Woodley 2017). Recovery was 98.8% and intra-assay coefficient of variation (CV) was 8.1%.

2.6 Benthic Macroinvertebrate and Crayfish Sampling

We collected benthic macroinvertebrates and crayfish between 17 September and 20 November 2018. We collected individuals by placing a D-net flush with the stream bottom and disturbing the substrate upstream or by sweeping the D-net under stream overhangs. Only crayfish smaller than 2.5 cm were collected to ensure that they were of a size that could be consumed by a salamander. Benthic macroinvertebrates and crayfish from each site were stored together in a tube containing 75% ethanol and covered with foil to prevent light exposure until imidacloprid extraction.

2.7 Salamander and Invertebrate Imidacloprid Extraction and Quantification

We quantified imidacloprid concentrations in 164 *Desmognathus* salamanders (62 *D. fuscus* and 102 *D. monticola*). We adapted pesticide extraction and chromatographic and mass spectrometry conditions from procedures developed by Lehotay (2006) and Galeano et al. (2013). We placed individual salamanders and the composite invertebrate samples from each site into 50-mL tubes. We flash froze samples in liquid nitrogen and placed them in a freeze dryer for 3 days. We placed 3 5-mm steel beads into each 50-mL tube and ground the salamanders and invertebrates in a Retsch MixerMill (MM 400, Haan, Germany) for 3 mins at 30 rep/min. We then removed the steel beads and added 10 mL of deionized water and 10 mL of acetonitrile. Samples were briefly vortexed then sonicated for 20 min at room temperature in a sonication bath. We then added Quick Easy Cheap Effective Rugged Safe (QuEChERS) Mylar salt pouches (UCT, ECQUEU7-MP) to each sample, and the samples were vortexed for 10 sec and shaken by hand for 1 min. We centrifuged the samples at 2,200 relative centrifugal force for 5 min. We assembled a high-throughput vacuum apparatus with clean-up cartridges (UCT, ECPSAC1856) and conditioned with 5 mL of acetonitrile. Eight mL of the organic layer (acetonitrile) of each sample was collected and cleaned through the cartridges, and deposited in 15-mL glass test tubes. We then dried the test tubes under nitrogen at 50°C, reconstituted them in 0.5 mL of acetonitrile, and filtered the samples through PTFE Whatman Mini-UniPrep Syringeless Filter vials. We followed the same procedure to quantify imidacloprid concentration as described above for water and sediment.

We calculated limit of detection (LOD) values from an external standard solution containing imidacloprid, imidacloprid-urea, and imidacloprid-olefin ranging from 5–300 ng/mL in LC-MS grade acetonitrile. We performed a linear regression on the data points in the concentration range (n = 7) and used the formula $3*[(SE)/(R^2)]$ (SE = standard error, R^2 = coefficient of determination) to calculate the LOD. In all sample types, the LOD values were 5.98 ng/mL, 33.7 ng/mL, and 4.15 ng/mL for imidacloprid, imidacloprid-urea, and imidacloprid-olefin, respectively.

2.8 Statistical Analyses

We assessed relationships between exposure to imidacloprid and the following response variables: bioaccumulation in salamanders, bioaccumulation in invertebrates (benthic

macroinvertebrates and crayfish combined), salamander corticosterone concentration, and salamander BCI score. For bioaccumulation, we assessed the influence of imidacloprid concentration in stream water on imidacloprid concentration in the salamanders and invertebrates. For salamanders, we only included individuals with detectable levels of imidacloprid or one of its metabolites. For both salamanders and invertebrates, we used the total estimated concentration of imidacloprid and its metabolites as the response variable. For sublethal effects, we assessed the influence of imidacloprid concentration in stream water and three additional predictors of imidacloprid exposure, including whether the site was adjacent to treated trees, number of applications in adjacent treated stands (a measure of treatment intensity), and whether imidacloprid was detected in the environment. We considered a site to be present for imidacloprid if imidacloprid, imidacloprid-urea, or imidacloprid-olefin was detected in either stream water or sediment, or the sampling site was located adjacent to imidacloprid-treated trees.

We created a BCI by regressing (log) SVL on (log) weight (Schulte-Hostedde et al. 2005). Positive residuals indicate a higher-than-average weight for a given SVL, and vice versa. We did not include salamanders missing portions of their tails or legs in BCI analyses. We created separate BCIs for each salamander species, and for gravid females within-species, including *D. fuscus* (n = 207), *D. monticola* (n = 274), *D. ochrophaeus* (Allegheny Mountain dusky salamander; n = 141), *Eurycea* spp. (northern and southern two-lined salamanders; n = 59), and *Gyrinophilus porphyriticus* (spring salamander; n = 121). We did not include larval salamanders that weighed ≤0.1 g, larval *Eurycea* spp. because SVL was not a strong predictor of weight, or additional salamander species captured because sample sizes were small (< 20 captures). For each BCI, we z-score transformed the data so that standard deviations were equal across species (Legendre and Legendre 2012).

We used linear regressions and a model selection approach using Akaike's Information Criterion corrected for small sample size (AIC_c) to assess the influence of imidacloprid predictors on our response variables (Burnham et al. 2011, Zuur et al. 2009). For all analyses, we assessed assumptions of normality using quantile-quantile plots and homoscedasticity using residual plots (Zuur et al. 2009, 2010). For the invertebrate bioaccumulation data set, we removed the two highest water concentration samples to satisfy the assumption of homoscedasticity. For the corticosterone data set, we removed the four highest corticosterone concentration samples to satisfy the assumption of normality. For the corticosterone model

selection, we accounted for inherent differences in corticosterone concentration among species and sex (i.e., males, females, gravid females; Dickens and Romero 2013, Gormally et al. 2019, Scott and Ellis 2007) by including these factors in all imidacloprid models. For the BCI model selection, we did not include sex as a predictor because we did not identify sex for many of the captures, but we did include life stage (i.e., larva or adult/sub-adult). We created linear regressions using the GLS function with a constant variance structure in the package nlme (Pinheiro et al. 2016; version 3.1-137) in program R (R Core Team 2019; version 3.4.1). We gauged model support based on ΔAIC_c and Akaike weight (w_i), and considered candidate models to have support when $\Delta AIC_c < 7$ (Burnham et al. 2011).

3. RESULTS

3.1 Water and Sediment Imidacloprid Concentration

Of the 48 sampled sites, 27 were directly adjacent to HWA treatments. Imidacloprid was detected in the stream water at 24 sites, with a mean concentration of 49.83 ± 20.22 ng/mL (range = 6.52–489.56 ng/mL). Imidacloprid-urea was not detected in the stream water at any site. We detected imidacloprid-olefin in the water at two sites, both of which had detectable levels of imidacloprid. Imidacloprid was detected in sediment at eight sites, five of which were adjacent to HWA treatments. We did not detect imidacloprid-olefin or imidacloprid-urea in the sediment at any site.

3.2 Salamander Imidacloprid Bioaccumulation

Of the 107 salamanders tested for bioaccumulation, 29 had detectable levels of imidacloprid or imidacloprid-olefin. We detected imidacloprid in the tissues of 10 *D. monticola* and 4 *D. fuscus*, with a mean concentration of 24.63 ± 3.55 ng/mL (range = 6.36–51.27 ng/mL). We detected imidacloprid-olefin in the tissues of 13 *D. monticola* and 6 *D. fuscus*, with a mean concentration of 8.33 ± 0.80 ng/mL (range = 4.16–19.78 ng/mL). Three *D. monticola* and one *D. fuscus* had detectable levels of both imidacloprid and imidacloprid-olefin. We did not detect imidacloprid-urea in the tissues of any salamanders. We detected imidacloprid or imidacloprid-olefin in the tissue of three salamanders collected from sites that were not adjacent to treated trees. The model containing concentration of imidacloprid in stream water received higher support than the null model ($w_i = 0.60$). Imidacloprid concentration in salamanders increased

with concentration in stream water, but the 95% confidence interval (CI) overlapped 0 (β= 0.093, 95% CI: -0.013–0.199).

3.3 Benthic Macroinvertebrate and Crayfish Imidacloprid Bioaccumulation

We detected imidacloprid in all 15 benthic macroinvertebrate/crayfish samples, with a mean concentration of 26.36 ± 3.76 ng/mL (range = 12.05–68.38 ng/mL). We also detected imidacloprid-urea in 13 of these samples. We did not detect imidacloprid-olefin in any samples. For three sites adjacent to HWA treatments, imidacloprid was not detected in the water or sediment, but was detected in the invertebrate samples (mean concentration of these samples = 26.08 ± 4.94 ng/mL). The null model received higher support than the model containing concentration of imidacloprid in stream water (w_i = 0.71).

3.4 Sublethal Effects of Imidacloprid on Salamanders

For the corticosterone model selection, the model with the strongest support contained additive effects of species, sex, and number of imidacloprid applications in adjacent treated stands (w_i = 0.39; Table 1). Corticosterone concentration increased with increasing number of applications (β = 0.0010, 95% CI: 0.0003–0.0017). The second most supported model contained an interaction effect between sex and number of applications (w_i = 0.28), and indicated that effects were stronger for non-gravid females than for males and gravid females (Fig. 2a). Concentration of imidacloprid in stream water (ΔAIC_c = 2.77) and presence of treated trees (ΔAIC_c = 6.68) also had some support as predictors of corticosterone concentration (Table 1), and both estimated positive relationships.

For the BCI model selection, the model with the strongest support contained concentration of imidacloprid in stream water as a predictor (w_i = 0.44; Table 2). BCI decreased as concentration of imidacloprid in stream water increased (β = -0.0009, 95% CI: -0.0017 – -0.0001; Fig. 2b). The null model also had some support (ΔAIC_c = 2.62, w_i = 0.12). There was no support for an interaction between species and concentration of imidacloprid in stream water (ΔAIC_c = 12.35). For all 5 species, mean BCI score was lower at sites with presence of imidacloprid in the environment, and median BCI score was lower for 4 of the species (Fig. 3).

4. DISCUSSION

This study provides strong evidence that salamanders and stream invertebrates uptake imidacloprid that leaches into their environment from treated hemlock stands. In this study, we found that benthic macroinvertebrates and crayfish, which are both important prey sources of stream salamanders, can also bioaccumulate imidacloprid. Thus, prey consumption may represent an additional route of imidacloprid exposure for salamanders. However, additional research is needed to confirm if salamanders bioaccumulate imidacloprid after consuming contaminated prey.

Corticosterone in *D. monticola* and *D. fuscus* was positively associated with number of imidacloprid applications, and this effect was the strongest for non-gravid females. Conclusions from previous studies investigating pesticide-associated changes in corticosterone levels are conflicted. For example, larval western tiger salamanders (*Ambystoma mavortium*) had higher corticosterone levels in agricultural wetlands with elevated levels of neonicotinoid insecticides, compared to reference wetlands (Davis et al. In Press). In contrast, wood frog (*Lithobates sylvaticus*) tadpoles experimentally exposed to environmentally relevant concentrations of the neonicotinoid thiamethoxam had lower corticosterone concentrations, and there was no difference in corticosterone concentrations in juveniles (Gavel et al. 2019). Male African clawed frogs (*Xenopus laevis*) had elevated corticosterone concentrations when exposed to the pesticide concoction (Hayes et al. 2006). Additional studies are needed to determine if corticosterone responses to imidacloprid exposure are predictable based on species, sex, and life stage.

We found that salamander BCI was negatively associated with imidacloprid concentration in stream water, and BCI was lower in streams with environmental imidacloprid for all five species. Body condition is an important indicator of amphibian health and correlates with survival, productivity, and movement dynamics (e.g., Lowe et al. 2006, Reading 2007, Roznik et al. 2015). For example, body condition is correlated to territory size and number of prey within territories (Gabor 1995) and larger body size is advantageous in mate competition (Howard et al. 1996). Our results suggest that HWA treatments can negatively affect the health of individual salamanders.

Interestingly, we found imidacloprid in the tissues of invertebrates from three sites where we did not detect imidacloprid in the stream water or sediment. Presence of imidacloprid in stream water varies temporally and increases after rain events (Churchel et al. 2011, Cowles et al. 2009, McGrath et al. 2010). Grab sampling of stream water for pesticide runoff research does

not account for spatial and temporal variation and can lead to underestimates of pesticide residues (Xing et al. 2013). Our results suggest that sampling stream invertebrates may be more reliable than sampling stream water to confirm presence of imidacloprid in streams. However, we recognize that our sample size was small, and we were unable to assess temporal dynamics in our data sets. Additional research on this topic is warranted.

Three sites that were not directly adjacent to HWA treatments contained salamanders with detectable levels of imidacloprid or imidacloprid-olefin. One of these sites was located downstream of farmland and we did detect imidacloprid in the stream sediment. Imidacloprid is commonly used in agriculture (Elbert et al. 2008), and thus leaching from upstream farmland may explain presence of imidacloprid at this site. Similarly, another site was near private homes, and imidacloprid is used in residential areas for treating ornamental trees and for termite control (McCullough et al. 2005, Parman and Vargo 2010). However, we did not detect imidacloprid in the stream water or sediment at this site. The third site was located 100 m upstream of a known treatment site, and we speculate that the salamander traveled upstream following exposure to imidacloprid.

In summary, this research indicates that treating hemlock stands adjacent to streams with imidacloprid can result in the pesticide entering aquatic food webs, and can result in sublethal effects on salamanders. Imidacloprid is one of the world's most widely used pesticides, with heavy application in agricultural, urban, and forest systems. Given the widespread use of imidacloprid and documented bioaccumulation in invertebrate and vertebrate organisms, additional studies investigating potential biomagnification in food webs are warranted.

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LITERATURE CITED

Ade, C.M., Boone, M.D., Puglis, H.J. 2010. Effects of an insecticide and potential predators on green frogs and northern cricket frogs. Journal of Herpetology, 44(4): 591–601.

Adelizzi, R., Portmann, J., Van Meter, R. 2019. Effect of individual and combined treatments of pesticide, fertilizer, and salt on growth and corticosterone levels of larval southern leopard frogs (*Lithobates sphenocephala*). Archives of Environmental Contamination and Toxicology, 2019: 1–11.

Alexander, A.C., Culp, J.M., Liber, K., Cessna, A.J. 2007. Effects of insecticide exposure on feeding inhibition in mayflies and oligochaetes. Environmental Toxicology and Chemistry, 26(8): 1726–1732.

Baskaran, S., Kookana, R.S., Naidu, R. 1997. Determination of the insecticide imidacloprid in water and soil using high-performance liquid chromatography. Journal of Chromatography A, 787(1–2): 271–275.

Becker, D.A., Brittingham, M.C., Goguen, C.B. 2008. Effects of hemlock woolly adelgid on breeding birds at Fort Indiantown Gap, Pennsylvania. Northeastern Naturalist, 15(2): 227–240.

Benton, E.P., Grant, J.F., Mueller, T.C., Webster, R.J., Nichols, R.J. 2016. Consequences of imidacloprid treatments for hemlock woolly adelgid on stream water quality in the southern Appalachians. Forest Ecology and Management, 360(2016): 152–158.

Benton, E.P., Grant, J.F., Nichols, R.J., Webster, R.J., Schwartz, J.S., Bailey, J.K. 2017. Risk assessment of imidacloprid use in forest settings on the aquatic macroinvertebrate community. Environmental Toxicology and Chemistry, 36(11): 3108–3119.

Burnham, K.P., Anderson, D.R., Huyvaert, K.P. 2011. AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. Behavioral Ecology and Sociobiology 65: 23–35.

Burton, T.M., Likens, G.E. 1975. Salamander populations and biomass in the Hubbard Brook Experimental Forest, New Hampshire. Copeia, 1975(3): 541−546.

Chambers, D.L., Wojdak, J.M., Du, P., Belden, L.K. 2013. Pond acidification may explain differences in corticosterone among salamander populations. Physiological and Biochemical Zoology, 86(2): 224–232.

Charbonnier, J.F., Pearlmutter, J., Vonesh, J.R., Gabor, C.R, Forsburg, Z.R., Grayson, K.L. 2018. Cross-life stage effects of aquatic larval density and terrestrial moisture on growth and corticosterone in the spotted salamander. Diversity, 10(3): 68.

Churchel, M.A., Hanula, J.L., Berisford, C.W., Vose, J.M., Dalusky, M.J. 2011. Impact of imidacloprid for control of hemlock woolly adelgid on nearby aquatic macroinvertebrate assemblages. Southern Journal of Applied Forestry, 35(1): 26–32.

Cohen, J. 1992. A power primer. Psychological Bulletin, 112(1): 155–159.

Colombo, V., Mohr, S., Berghahn, R., Pettigrove, V.J. 2013. Structural changes in a macrozoobenthos assemblage after imidacloprid pulses in aquatic field-based microcosms. Archives of Environmental Contamination and Toxicology, 65(4): 683–692.

Cook, R.D. 1977. Detection of influential observation in linear regression. Technometrics, 19(1): 15–18.

Cooperman, M.D., Reed, J.M., Romero, L.M. 2004. The effects of terrestrial and breeding densities on corticosterone and testosterone levels in spotted salamanders, *Ambystoma maculatum*. Canadian Journal of Zoology, 82(11): 1795–1803.

Cowles, R.S. 2009. Optimizing dosage and preventing leaching of imidacloprid for management of hemlock woolly adelgid in forests. Forest Ecology and Management, 257(3): 1026–1033.

Daley, M.J., Phillips, N.G., Pettijohn, C., Hadley, J.L. 2007. Water use by eastern hemlock (*Tsuga canadensis*) and black birch (*Betula lenta*): implications of effects of the hemlock woolly adelgid. Canadian Journal of Forest Research, 37(10): 2031–2040.

Davic, R.D., Welsh, H.H. 2004. On the ecological roles of salamanders. Annual Review of Ecology, Evolution, and Systematics, 35(2004): 405–434.

Davis, D.R., Ferguson, K.J., Schwarz, M.S., Kerby, J.L. In press. Effects of agricultural pollutants on stress hormones and viral infection in larval salamanders. Wetlands.

Dickens, M.J., Romero, L.M. 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. General and Comparative Endocrinology, 191(2013): 177–189.

Elbert, A., Nauen, R., Leicht, W. 1998. Imidacloprid, a novel chloronicotinyl insecticide: biological activity and agricultural importance. In Insecticides with novel modes of action (pp. 50–73). Springer, Berlin, Heidelberg.

Ellison, A.M. 2014. Experiments are revealing a foundation species: a case study of eastern hemlock (*Tsuga canadensis*). Advances in Ecology, 2014: article 456904.

Ertl, H.M., Mora, M.A., Brightsmith, D.J., Navarro-Alberto, J.A. 2018. Potential impact of neonicotinoid use on northern bobwhite (*Colinus virginianus*) in Texas: a historical analysis. PLoS ONE, 13(1): e0191100.

Feng, S., Kong, Z., Wang, X., Zhao, L., Peng, P. 2004. Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell*. Chemosphere, 56(5): 457–463.

Gabor, C.R. 1995. Correlational test of Mathis' hypothesis that bigger salamanders have better territories. Copeia, 729-729.

Gabor, C.R., Knutie, S.A., Roznik, E.A., Rohr, J.R. 2018. Are the adverse effects of stressors on amphibians mediated by their effects on stress hormones? Oecologia, 186(2): 393-404.

Galeano, M., Scordino, M., Sabatino, L., Pantò, V., Morabito, G., Chiappara, E., Traulo, P., Gagliano, G. 2013. UHPLC/MS-MS analysis of six neonicotinoids in honey by modified QuEChERS: method development, validation, and uncertainty measurement. International Journal of Food Science, 2013: 1–7.

Gavel, M.J., Richardson, S.D., Dalton, R.L., Soos, C.E., Ashby, B., McPhee, L., Forbes, M.R. and Robinson, S.A. 2019. Effects of two neonicotinoid insecticides on blood cell profiles and corticosterone concentrations of wood frogs (*Lithobates sylvaticus*). Environmental toxicology and chemistry, 2019(38): 1273–1284.

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.

Glennemeier, K.A., Denver, R.J. 2002. Role for corticoids in mediating the response of *Rana pipiens* tadpoles in intraspecific competition. Journal of Experimental Zoology, 292(1): 32–40.

Glinski, D.A., Henderson, W.M., Van Meter, R.J., Purucker, S.T. 2018. Effect of hydration status on pesticide uptake in anurans following exposure to contaminated soils. Environmental Science and Pollution Research, 2018: 1–10.

Gormally, B.M., Fuller, R., McVey, M., Romero, L.M. 2019. DNA damage as an indicator of chronic stress: correlations with corticosterone and uric acid. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 227: 116–122.

Hadley, J.L., Schedlbauer, J.L. 2002. Carbon exchange of an old-growth eastern hemlock (*Tsuga canadensis*) forest in central New England. Tree Physiology, 22(15–16): 1079–1092.

Hallmann, C.A., Foppen, R.P., van Turnhout, C.A., de Kroon, H., Jongejans, E. 2014. Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature, 511 (7509): 341–343.

Harper, C.A., Guynn Jr, D.C. 1999. Factors affecting salamander density and distribution within four forest types in the Southern Appalachian Mountains. Forest Ecology and Management, 114(2–3): 245–252.

Havill, N.P., Vieira, L.C., Salom, S.M. 2014. Biology and control of hemlock woolly adelgid. USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown, West Virginia.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environmental Health Perspectives, 114(Suppl 1): 40–50.

Hayes, T.B., Falso, P., Gallipeau, S., Stice, M. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. Journal of Experimental Biology, 213(6): 921–933.

Hopkins, W.A., Mendonça, M.T., Congdon, J.D. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. General and Comparative Endocrinology, 108(2): 237–246.

Hothorn, T., Zeileis, A., Millo, G., Mitchell, D., 2010. Package 'lmtest'. Testing Linear Regression Models. Available from https://web.archive.org/web/20190706155233/https://cran.r-project.org/web/packages/lmtest/lmtest.pdf

Howard, R.D., Moorman, R.S., Whiteman, H.H. 1997. Differential effects of mate competition and mate choice on eastern tiger salamanders. Animal Behaviour, 53(6): 1345-1356.

Iturburu, F.G., Zömisch, M., Panzeri, A.M., Crupkin, A.C., Contardo-Jara, V., Pflugmacher, S., Menone, M.L. 2017. Uptake, distribution in different tissues, and genotoxicity of imidacloprid in the freshwater fish *Australoheros facetus*. Environmental Toxicology and Chemistry, 36(3): 699–708.

Krapfl, K.J., Holzmueller, E.J., Jenkins, M.A. 2011. Early impacts of hemlock woolly adelgid in *Tsuga canadensis* forest communities of the southern Appalachian Mountains. Journal of the Torrey Botanical Society 138(1): 93–106.

Kreutzweiser, D.P., Thompson, D.G., Scarr, T.A. 2009. Imidacloprid in leaves from systemically treated trees may inhibit litter breakdown by non-target invertebrates. Ecotoxicology and Environmental Safety, 72(4): 1053–1057.

Larson, D.L., McDonald, S., Hamilton, S.J., Fivizzani, A.J., Newton, W.E. 1998. Effects of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: duration, larval growth, and hormonal response. Physiological Zoology, 71(6): 671–679.

Legendre, P., Legendre, L. 2012. Numerical Ecology. Third edition. Elsevier, Amsterdam, The Netherlands.

Lehotay, S. 2006. Quick, easy, cheap, effective, rugged, and safe approach for determining pesticide residues. Pages 239–261 in J. L. Martínez Vidal and A. Garrido Frenich, editors. Pesticide protocols, Humana Press, New York, New York, USA.

Margenau, E.L., Crayton, S.M., Rucker, L.E., Jacobsen, C.D., Brown, D.J. 2018. Modified salamander stick to facilitate accurate measurement of small individuals. Herpetological Review, 49(2): 243–246.

Mathewson, B. 2009. The relative abundance of eastern red-backed salamanders in eastern hemlock-dominated and mixed deciduous forests at Harvard Forest. Northeastern Naturalist, 16(1): 1–12.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M., Sattelle, D.B. 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends in Pharmacological Sciences, 22(11): 573–580.

McCullough, D.G., Cappaert, D., Poland, T.M., Lewis, P., Molongoski, J. 2005. Long-term (three-year) results of truck injections for emerald ash borer control in landscape ash trees. Emerald Ash Borer Research and Technology Development, 2005: 31.

McEwen, B.S., Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. Hormones and Behavior, 43(1): 2–15.

McGrath, G., Hinz, C., Sivapalan, M. 2010. Assessing the impact of regional rainfall variability on rapid pesticide leaching potential. Journal of Contaminant Hydrology, 113(1–4): 56–65.

Millikin, A.R., Woodley, S.K., Davis, D.R., Anderson, J.T. 2019. Habitat characteristics in created vernal pools impact spotted salamander water-borne corticosterone levels. Wetlands, 2019: 1–12.

Novarro, A.J., Gabor, C.R., Goff, C.B., Mezebish, T.D., Thompson, L.M., Grayson, K.L. 2018. Physiological responses to elevated temperature across the geographic range of a terrestrial salamander. Journal of Experimental Biology, 221: jeb178236.

Parman, V., Vargo, E.L. 2010. Colony-level effects of imidacloprid in subterranean termites (Isoptera: Rhinotermitidae). Journal of Economic Entomology, 103(3): 791-798.

Pérez-Iglesias, J.M., de Arcaute, C.R., Nikoloff, N., Dury, L., Soloneski, S., Natale, G.S., Larramendy, M.L. 2014. The genotoxic effects of the imidacloprid-based insecticide formulation Glacoxan Imida on Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae). Ecotoxicology and Environmental Safety, 104(2014): 120–126.

Petranka, J.W. 1998. Salamanders of the United States and Canada. Smithsonian Institute Press, Washington, D.C., USA.

Petranka, J. W., Murray, S. S. 2001. Effectiveness of removal sampling for determining salamander density and biomass: a case study in an Appalachian streamside community. Journal of Herpetology, 35(1): 36–44.

Pinheiro, J., Bates, D., DebRoy, S., Heisterkamp, S., Van Willigen, B. 2016. Package "nlme." Fit and compare Gaussian linear and nonlinear mixed-effects models. Available at https://web.archive.org/web/20190706154601/https://cran.r-project.org/web/packages/nlme/nlme.pdf.

R Core Team. 2019. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://web.archive.org/web/20190706154601/https://cran.r-project.org/web/packages/nlme/nlme.pdf

Romero, L.M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology and Evolution, 19(5): 249–255.

Romero, L.M., Reed, J.M. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology, Part A 140(1): 73–79.

Sardo, A.M., Soares, A.M.V.M. 2010. Assessment of the effects of the pesticide imidacloprid on the behaviour of the aquatic oligochaete *Lumbriculus variegatus*. Archives of Environmental Contamination and Toxicology, 58(3): 648–656.

Schulte-Hostedde, A. I., Zinner, B., Millar, J.S., Hickling, G.J. 2005. Restitution of mass-size residuals: validating body condition indices. Ecology, 86(1): 155–163.

Scott A.P., Ellis T. 2007. Measurement of fish steroids in water—a review. General and Comparative Endocrinology, 153(1–3): 392–400.

Smitley, D.R., Herms, D.A, Davis, T.W. 2015. Efficacy of soil-applied neonicotinoid insecticides for long-term protection against emerald ash borer (Coleoptera: Buprestidae). Journal of Economic Entomology, 108(2015): 2344–2353.

Snyder, C.D., Young, J.A., Lemarié, D.P., Smith, D.R. 2002. Influence of eastern hemlock (*Tsuga canadensis*) forests on aquatic invertebrate assemblages in headwater streams. Canadian Journal of Fisheries and Aquatic Sciences, 59(2): 262–275.

Sokal, R.R., Rohlf, F.J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. 3rd Edition. Freeman, New York, New York, USA. 887 pp.

Thomas, J.R., Woodley, S.K. 2017. Testing the immunocompetence handicap hypothesis: testosterone manipulation does not affect wound healing in male salamanders. General and Comparative Endocrinology, 247(2017): 8–15.

Tingley, M.W., Orwig, D.A., Field, R., Motzkin, G. 2002. Avian response to removal of a forest dominant: consequences of hemlock woolly adelgid infestations. Journal of Biogeography, 29(10–11): 1505–1516.

Troïanowski, M., Mondy, N., Dumet, A., Arcanjo, C., Lengagne, T. 2017. Effects of traffic noise on tree frog stress levels, immunity, and color signaling. Conservation Biology 31(5): 1132–1140.

Van Dijk, T.C., Van Staalduinen, M.A., Van der Sluijs, J.P. 2013. Macro-invertebrate decline in surface water polluted with imidacloprid. PLoS ONE, 8(5): e62374.

Van Meter, R.J., Adelizzi, R., Glinski, D.A. and Henderson, W.M., 2019. Agrochemical mixtures and amphibians: the combined effects of pesticides and fertilizer on stress, acetylcholinesterase activity and bioaccumulation in a terrestrial environment. Environmental Toxicology and Chemistry, 38(5): 1052–1061.

Van Meter, R.J., Glinski, D.A., Henderson, W.M., Garrison, A.W., Cyterski, M., Purucker, S.T. 2015. Pesticide uptake across the amphibian dermis through soil and overspray exposures. Archives of Environmental Contamination and Toxicology, 69(4): 545–556.

Van Meter, R.J., Glinski, D.A., Hong, T., Cyterski, M., Henderson, W.M., Purucker, S.T. 2014. Estimating terrestrial amphibian pesticide body burden through dermal exposure. Environmental Pollution, 193(2013): 262–268.

Vose, J.M., Wear, D.N., Mayfield III, A.E., Nelson. C.D. 2013. Hemlock woolly adelgid in the southern Appalachians: control strategies, ecological impacts, and potential management responses. Forest Ecology and Management, 291(2013): 209–219.

Webb, R.E., Frank, J.R., Raupp, M.J. 2003. Eastern hemlock recovery from hemlock woolly adelgid damage following imidacloprid therapy. Journal of Arboriculture, 29(5): 298–302.

Wiggins, G., Benton, E., Grant, J., Kerr, M., Lambdin, P. 2018. Short-term detection of imidacloprid in streams after applications in forests. Journal of Environmental Quality, 47(3): 571–578.

Willson, J.D., Hopkins, W.A., Bergeron, C.M., Todd, B.D. 2012. Making leaps in amphibian ecotoxicology: translating individual-level effects of contaminants to population viability. Ecological Applications, 22(2012): 1791–1802.

Xing, Z., Chow, L., Rees, H., Meng, F., Li, S., Ernst, B., Benoy, G., Zha, T., Hewitt, L.M. 2013. Influences of sampling methodologies on pesticide-residue detection in stream water. Archives of Environmental Contamination and Toxicology, 64(2): 208–218.

Yamamoto, I., Casida, J.E., eds. 1999. Nicotinoid insecticides and the nicotinic acetylcholine receptor. Springer Verlag, Tokyo, Japan.

Yap, T.A., Koo, M.S., Ambrose, R.F., Wake, D.B., Vredenburg, V.T. 2015. Averting a North American biodiversity crisis. Science, 349(6247): 481–482.

Zuur, A.F., Ieno, E.N., Elphick, C.S. 2010. A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution, 2010(1): 3–14.

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M. 2009. Mixed effects models and extensions in ecology with R. Springer, New York, New York, USA.

Table 1. Model selection results for the influence of species (*Desmognathus fuscus* [northern dusky salamander] and *D. monticola* [seal salamander]), sex (males, females, and gravid females), and imidacloprid exposure on salamander corticosterone concentration. We sampled 119 salamanders (115 included in the analysis) at Gauley River National Recreational Area (GARI) and New River Gorge National River (NERI) in West Virginia, USA. We tested four predictors of imidacloprid exposure, including whether the site was adjacent to treated trees (Trees), number of applications in adjacent treated stands (Number), whether imidacloprid was detected in the environment (Presence), and concentration in the stream water (ng/mL; Concentration). We used Akaike's Information Criterion corrected for small sample size (AIC_c) to rank candidate models. The null model is shown as (.) and includes only the intercept. Akaike weights are represented as w_i.

Model	**Parameters**	**AIC_c**	**ΔAIC_c**	**Adj-R^2**	**w_i**
Species + Sex + Number	6	311.55	0.00	0.15	0.39
Species + Sex × Number	8	312.20	0.65	0.16	0.28
Sex + Species × Number	7	313.82	2.27	0.14	0.13
Species + Sex + Concentration	6	314.32	2.77	0.13	0.10
Species × Sex	7	315.19	3.64	0.13	0.06
Species + Sex	5	317.93	6.38	0.09	0.02
Species + Sex + Trees	6	318.23	6.68	0.10	0.01
Species + Sex + Presence	6	319.94	8.39	0.09	0.01
Species	3	321.46	9.91	0.05	0.00
Sex	4	321.93	10.38	0.05	0.00
(.)	2	325.90	14.35	NA	0.00

Table 2. Model selection results for the influence of species, age (larva or adult/sub-adult), and imidacloprid exposure on salamander body condition index (BCI) score. We standardized BCI scores for each species and thus did not include species as an independent factor in the model selection. Species included *Desmognathus fuscus* (northern dusky salamander), *D. monticola* (seal salamander), *D. ochrophaeus* (Allegheny Mountain dusky salamander), *Eurycea* spp. (northern and southern two-lined salamanders), and *G. porphyriticus* (spring salamander). We tested four predictors of imidacloprid exposure, including whether the site was adjacent to treated trees (Trees), number of applications in adjacent treated stands (Number), whether imidacloprid was detected in the environment (Presence), and concentration (ng/mL) in the stream water (Concentration). We used Akaike's Information Criterion corrected for small sample size (AIC_c) to rank candidate models. The null model is shown as (.) and includes only the intercept. Akaike weights are represented as w_i.

Model	**Parameters**	**AIC_c**	**ΔAIC_c**	**Adj-R^2**	**w_i**
Concentration	3	2269.34	0.00	0.005	0.44
(.)	2	2271.95	2.62	NA	0.12
Presence	3	2272.00	2.67	0.001	0.12
Number	3	2272.18	2.84	0.001	0.11
Trees	3	2272.44	3.10	0.001	0.09
Life Stage × Concentration	5	2273.15	3.81	0.002	0.07
Life Stage	3	2273.77	4.43	-0.001	0.05
Species × Concentration	11	2281.69	12.35	-0.001	0.00

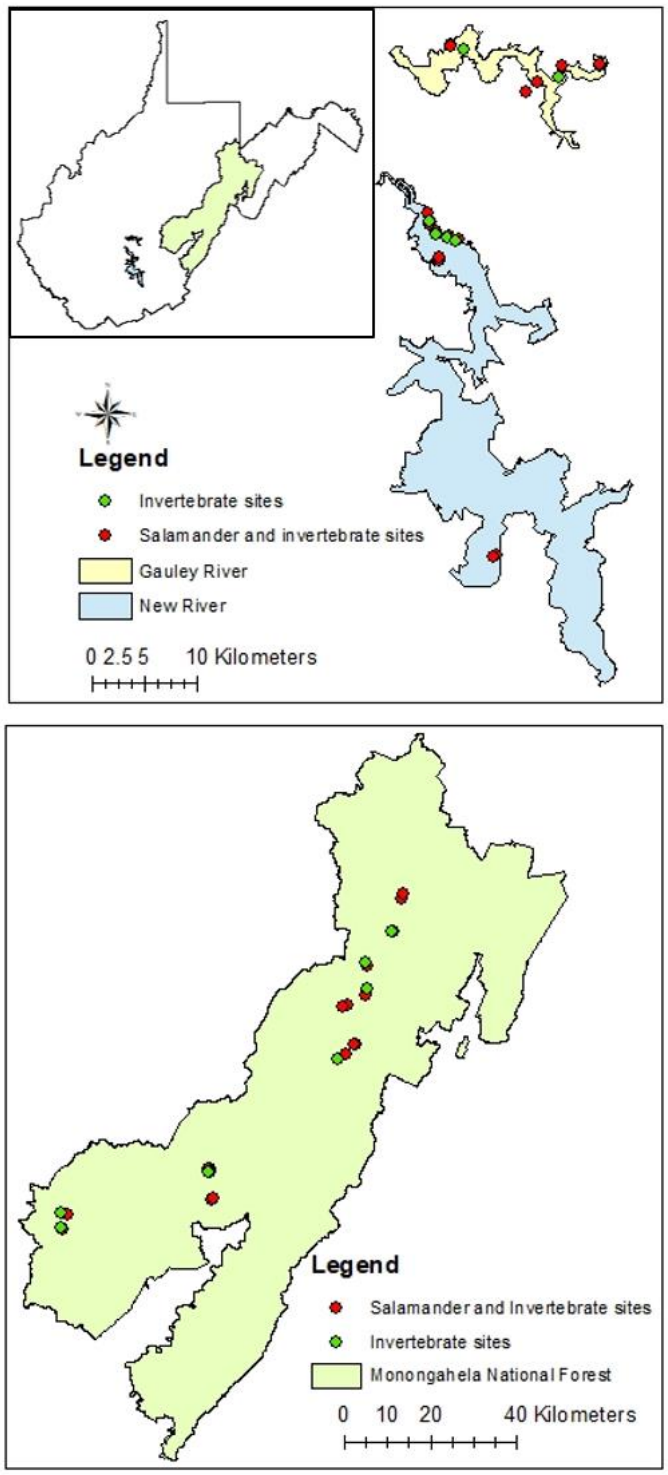

Figure 1. Map of study sites to investigate bioaccumulation of imidacloprid and its metabolites in stream salamanders and invertebrates, and sublethal impacts of imidacloprid on salamanders. West Virginia, USA (insert).

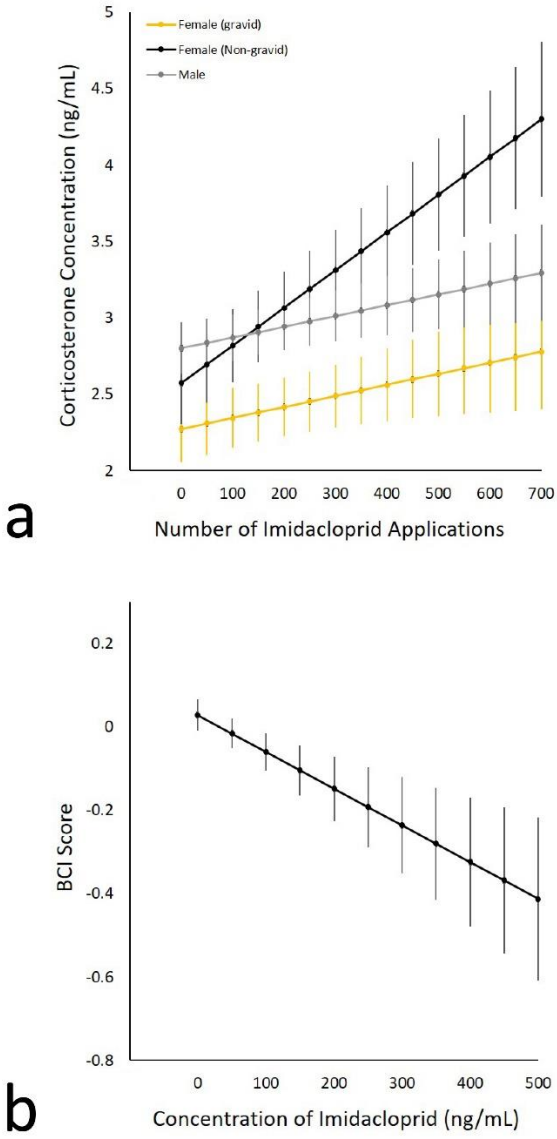

Figure 2. Potential sublethal effects of imidacloprid exposure on stream salamanders in West Virginia, USA. (a) Model-estimated relationship between concentration of the hormone corticosterone and total number of imidacloprid applications at the sampling site for male, non-gravid female, and gravid female *Desmognathus* spp. (n = 115). The intercepts represent *D. fuscus* (northern dusky salamander). (b) Model-estimated relationship between salamander body condition index (BCI) score and concentration of imidacloprid in stream water. BCI analyses included 802 individuals representing 5 species. Bars represent model standard errors.

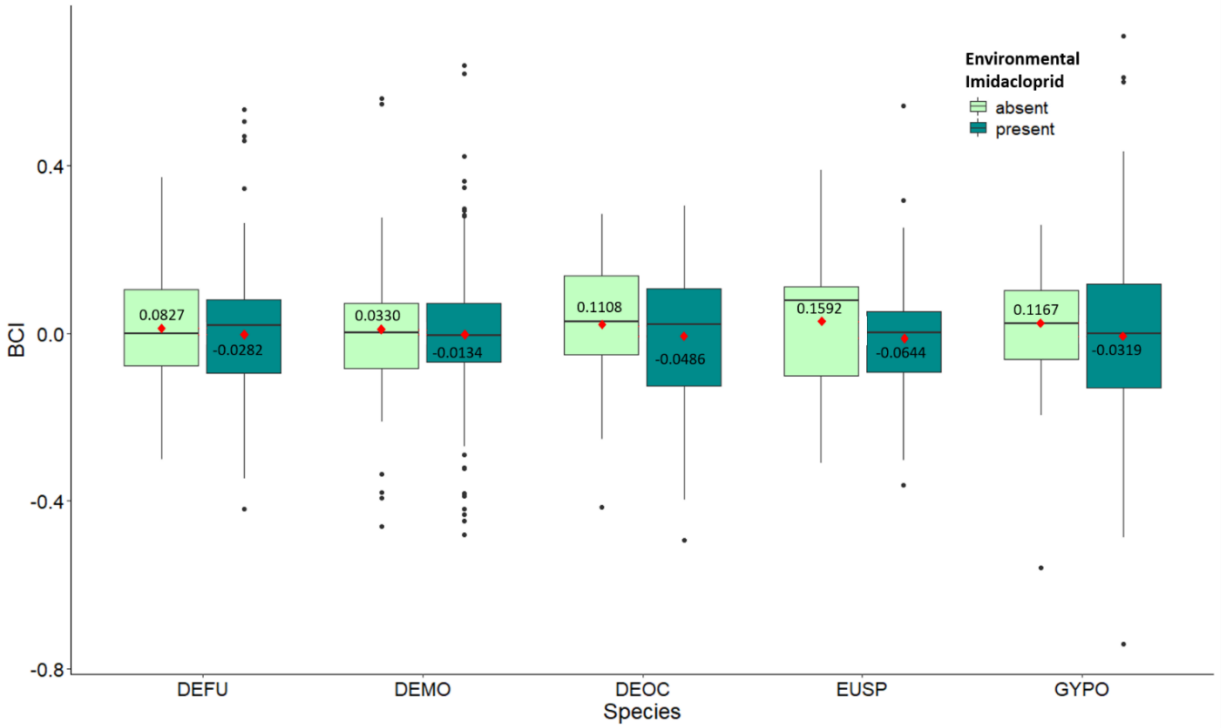

Figure 3. Boxplot summaries of body condition index (BCI) values used in this study assessing potential sublethal effects of imidacloprid exposure on *D. fuscus* (DEFU, absent, n = 54, present, n = 153), *D. monticola* (DEMO, absent, n = 79, present, n = 195), *D. ochrophaeus* (DEOC, absent, n = 43, present, n = 98), *Eurycea* spp. adults (EUSP, absent, n = 17, present, n = 42), and *G. porphyriticus* (GYPO, absent, n = 26, present, n = 95). Mean BCI is indicated with a red circle and generally was lower in streams with imidacloprid present in the environment for each species tested.