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Abstract

For terrestrial amphibians, accumulation of pesticides through dermal contact is a primary route of exposure in agricultural landscapes and may be contributing to widespread amphibian declines. To show pesticide transfer across the amphibian dermis at permitted label application rates, our study was designed to measure pesticide body burdens after two simulated exposure scenarios. We compared direct exposures, where amphibians were present when spraying occurred, and to indirect exposures, where amphibians were exposed to soils after pesticide application. During summer 2012, we reared barking (*Hyla gratiosa*) and green tree frogs (*H. cinerea*) through 60–90 days post-metamorphosis at a United States Environmental Protection Agency research laboratory. We tested exposure for 8 h to five pesticide active ingredients (imidacloprid, atrazine, triadimefon, fipronil, or pendimethalin) in glass aquaria lined with soil in the laboratory. We quantified total pesticide body burden and soil concentrations using liquid chromatography −mass spectrometry. All individuals in both treatments had measurable body burdens at the end of the study. A randomized block design analysis of variance ($n = 18$) showed that body burdens ($p = 0.03$) and bioconcentration factors (BCFs) ($p = 0.01$) were significantly greater in the direct overspray treatment relative to the indirect soil spray treatment for both species and tested pesticides. BCFs ranged from 0.1 to 1.16 and from 0.013 to 0.78 in the direct and indirect treatments, respectively. Our study shows dermal uptake for multiple pesticides from both direct spray and indirect soil exposures and provides empirical support for the degree to which terrestrial phase amphibians have higher body burdens after overspray pesticide exposure.

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Pesticide Uptake Across the Amphibian Dermis Through Soil and Overspray Exposures

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Abstract For terrestrial amphibians, accumulation of pesticides through dermal contact is a primary route of exposure in agricultural landscapes and may be contributing to widespread amphibian declines. To show pesticide transfer across the amphibian dermis at permitted label application rates, our study was designed to measure pesticide body burdens after two simulated exposure scenarios. We compared direct exposures, where amphibians were present when spraying occurred, and to indirect exposures, where amphibians were exposed to soils after pesticide application. During summer 2012, we reared barking (*Hyla gratiosa*) and green tree frogs (*H. cinerea*) through 60–90 days post-metamorphosis at a United States Environmental Protection Agency research laboratory. We tested exposure for 8 h to five pesticide active ingredients (imidacloprid, atrazine, triadimefon, fipronil, or pendimethalin) in glass aquaria lined with soil in the laboratory. We quantified total pesticide body burden and soil concentrations using liquid chromatography−mass spectrometry. All individuals in both treatments had measurable body burdens at the end of the study. A randomized block design analysis of variance ($n = 18$) showed that body burdens ($p = 0.03$) and bioconcentration factors (BCFs) ($p = 0.01$) were significantly greater in the direct overspray treatment relative to the indirect soil spray treatment for both species and tested pesticides. BCFs ranged from 0.1 to 1.16 and from 0.013 to 0.78 in the direct and indirect treatments, respectively. Our study shows dermal uptake for multiple pesticides from both direct spray and indirect soil exposures and provides empirical support for the degree to which terrestrial phase amphibians have higher body burdens after overspray pesticide exposure.

Direct dermal contact with pesticides presents a potentially significant but understudied route of exposure in terrestrial amphibians (Smith et al. 2007; Brühl et al. 2011). Unlike amniotes, amphibian skin is used for both gas and water exchange. Unique dermal properties associated with the biphasic life history of amphibians, including a ventral seat patch and aquaporins, which assist with water movement across the skin, may contribute to their increased susceptibility to pesticides and other contaminants (Smith et al. 2007; Quaranta et al. 2009; Ogushi et al. 2010; Brühl et al. 2011). To facilitate water uptake, many amphibians actively place the seat patch, a highly vascularized region of ventral skin, in direct contact with a moist substrate (McClanahan and Baldwin 1969). For amphibians living in arid climates or during periods of minimal precipitation, irrigated agricultural landscapes may be a preferred habitat in which individuals might come into direct contact with aerial overspray of pesticides or indirect contact with residually sprayed pesticides (Mann et al. 2009; Fryday and Thompson 2012). These pesticides may be accumulated across the amphibian dermis leading to measurable body burdens, physiological impairments, and mortality

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(Henson-Ramsey et al. 2008; Mendez et al. 2009; Brühl et al. 2013). Given that the dermal contact pathway in amphibians is not considered explicitly in ecological risk assessments, improving our understanding of this potentially significant route of pesticide exposure and accumulation may be critical to conserving many imperiled amphibian species.

Widespread use of pesticides in agricultural landscapes has been implicated as a major contributor to amphibian declines worldwide, but there are lacks of data on impacts to terrestrial, post-metamorphic life stages. With respect to aerially sprayed pesticides, many factors contribute to risks posed to amphibians such as distance from the site of application, changes to pH in soil and water, and vegetative buffers and vegetative cover (Thompson et al. 2004; Bernal et al. 2009; Hewitt et al. 2009). Although estimates indicate that as little as 12 % of the sprayed pesticide may actually reach the ground due to drift and interception by vegetation depending on crop stage during application (Thompson et al. 2004), applications of pre-emergent pesticides, which are applied to fields early in the growing season with newly germinated plants, can put amphibians in direct contact with much higher amounts. A comprehensive review of amphibian movements for 14 species associated with agricultural habitats throughout Europe recently confirmed that these animals often move across agricultural fields at times that coincide with large-scale pesticide applications (Fryday and Thompson 2012), and there is variability in the number and types of pesticides that amphibians come into contact with based on breeding phenology (Lenhardt et al. 2014). Studies in the United States (e.g., Bulger et al. 2003; Fellers and Kleeman 2007) have documented the willingness of the threatened California red-legged frog (*Rana draytonii)* to disperse through agricultural landscapes. The United States Geological Survey (USGS) recently reported detectable concentrations of 24 common-use pesticides in water and sediment samples from known amphibian breeding habitats, both agricultural and urban, that are monitored through the USGS Amphibian Research and Monitoring Initiative (Smalling et al. 2012). Furthermore, atmospheric transport of pesticides from agricultural areas has been linked to pesticide loads in sediments throughout habitats in California where tadpoles have measureable pesticide body burdens (Bradford et al. 2010), and several amphibian populations have suffered major losses (Davidson 2004; Davidson and Knapp 2007). Considering that >40 % of amphibian species are estimated to be experiencing some level of population decline (Stuart et al. 2004; Davidson et al. 2013; Christin et al. 2013), understanding the significance of pesticides in widespread amphibian losses is essential from both regulatory and conservation perspectives.

The potential for pesticide uptake through dermal exposure in terrestrial-phase amphibians is supported by a small, but growing, number of studies (Henson-Ramsey et al. 2008; Mendez et al. 2009; Bernal et al. 2009; Dinehart et al. 2009; Belden et al. 2010; Edge et al. 2011; Brühl et al. 2013; Van Meter et al. 2014) and was recently reviewed by Brühl et al. (2011). The majority of these studies report mortality among post-metamorphic amphibians directly exposed to pesticide formulations through a simulated overspray exposure scenario (Bernal et al. 2009; Dinehart et al. 2009; Belden et al. 2010; Edge et al. 2011; Brühl et al. 2013). Mortality rates among these studies range from 0 to 100 %. Three controlled laboratory experiments document dermal uptake of pesticides in amphibians through indirect soil exposure scenarios using pesticide active ingredients (Henson-Ramsey et al. 2008; Mendez et al. 2009; Van Meter et al. 2014). Differences across these studies in measured endpoints, study designs, as well as active ingredients and formulations used, limit our deductive ability about pesticide risk in terrestrial habitats. Nonetheless, the significance of dermal routes of exposure in amphibians and the potential for sublethal and lethal outcomes has been shown.

In an effort to expand on the limited data currently available on dermal pesticide exposure and accumulation in amphibians, our study was designed to make inferences regarding pesticide body burden in amphibians after direct dermal contact with a simulated overspray compared with indirect contact with contaminated soil. In doing so, our goal was to provide body burden data that, when paired with sublethal and lethal effects data from current and future literature, will provide the data needed to better support regulatory models that estimate exposure, accumulation, and effects of pesticides to post-metamorphic amphibians. We chose five current-use pesticide active ingredients for this study that span a range of logK_{OW} values from 0.57 to 5.18: imidacloprid, atrazine, triadimefon, fipronil, and pendimethalin. LogK_{OW} is considered an important predictor in dermal contact models for mammals (United States Environmental Protection Agency 2007) because higher K_{OW} (lipophilic) and lower K_{OW} (hydrophilic) chemicals have separate pathways for dermal exposure (Michaels et al. 1975). Lipophilic molecules have received considerable emphasis in these models along with a subsequent focus on nonionic, neutral forms of contaminants (Flynn 1989) in dermal-exposure assessment. Although we recognize the importance of potentially mitigating factors in field conditions (e.g., interception by vegetation, soil organic carbon content, soil water content, amphibian dehydration state), our laboratory study reflects worst-case exposure scenarios of pesticides applied to bare soils at permitted label application rates. By simulating direct and indirect exposures to frogs simultaneously within an experimental chamber, we aimed to reproduce realistic pre-emergent agricultural field exposures to examine variability in dermal pesticide accumulation

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within an exposed frog population. Our hypotheses were as follows: (1) both direct and indirect pesticide exposure scenarios would lead to detectable and quantifiable body burdens; and (2) direct contact with a simulated overspray of pesticides would significantly increase total body burden relative to indirect contact with recently sprayed soil.

Experimental Section

Pesticides and Soils

All pesticide exposures were performed with analytic-grade pesticide active ingredients (Table 1). The pesticide active ingredients used in our study were imidacloprid (logK_{OW} 0.57), atrazine (logK_{OW} 2.5), triadimefon (logK_{OW} 3.11), fipronil (logK_{OW} 4.0), and pendimethalin (logK_{OW} 5.18) (Milne 1995). Each pesticide was obtained from the USEPA National Pesticide Standard Repository in Fort Meade, MD, with a purity ≥98 %. Pesticide active ingredients were applied at maximum legally allowable application rates (USEPA 2013), with the exception of pendimethalin, which was applied at 30 % of the permitted label rate due to its insolubility in the limited solvent and the water volumes used in this study. All application rates were scaled to the size of a 10-gallon aquarium (area 1225 cm^2) and confirmed from soil samples after application using liquid chromatography−mass spectrometry (LC–MS). Application rates were as follows: atrazine 22.9 μg cm^{-2}, fipronil 1.1 μg cm^{-2}, imidacloprid 5.7 μg cm^{-2}, pendimethalin 19.8 μg cm^{-2}, and triadimefon 2.7 μg cm^{-2}. All solvents were obtained from Fisher Scientific (Pittsburgh, Pennsylvania, USA).

Soil was collected from the Coweeta Long-Term Ecological Research (LTER) site in Otto, NC, in July and August 2012. With the exception of designated experimental watersheds within the Coweeta basin, there has been no history of pesticide applications within the past 30-40 years [Jennifer Knoepp (personal communication)]. Soil testing was completed by the University of Georgia's Soil, Plant and Water Laboratory through the Cooperative Extension Office, Athens, GA. The soil used in this study was a Plott series soil classified as sandy−clay−loam with an average of 14 % organic matter. In addition to being pesticide free, this soil type provided the highest recovery rates of soils spiked with pesticides during preliminary experimentation. Before use, roots and larger pieces of debris were removed from the soil by passing it through a 2-mm sieve. The soil was stored in a 4 °C walk-in cooler at the USEPA's Ecosystems Research Division (ERD) in Athens, GA, from the time of collection through experimentation.

Tree Frog Collection and Rearing

To minimize the potential for previous pesticide exposure, barking tree frogs (*Hyla gratiosa*) and green tree frogs (*H. cinerea*) were collected from an isolated ephemeral pond in the University of Georgia's Whitehall Forest research facility in Athens, GA. Tree frogs were chosen for experimentation because their arboreal habitat preferences may make them more susceptible to aerial pesticide deposition in addition to some soil exposure during breeding events through movement to and from ponds. Three mating pairs of both barking tree frogs and green tree frogs were obtained on June 12, 2012, and transported to the ERD laboratory. After oviposition, adult tree frogs were returned to Whitehall Forest and the embryos reared through metamorphosis in outdoor wading pools at the ERD. All tree frog larvae were fed Tetra Fin fish food ad libitum. As metamorphs emerged from the wading pools, they were transferred to 600-L polyethylene tanks lined with sphagnum moss and leaf litter to simulate a terrestrial habitat. All juvenile tree frogs were fed cultured fruit flies and purchased crickets for 50–60 days post-metamorphosis.

Table 1 Pesticide active ingredient, pesticide class, logK_{OW}, application rate, and composite sample soil concentrations for direct overspray and indirect soil exposure treatments by tree frog species

Pesticide a.i.	Class	logK_{OW}	Application rate (μg cm^{-2})	Barking tree frog		Green tree frog	
				Direct exposure composite soil concentration (ppm)	Indirect exposure composite soil concentration (ppm)	Direct exposure composite soil concentration (ppm)	Indirect exposure composite soil concentration (ppm)
Imidacloprid	Insecticide	0.6	5.7	2.5	0.5	NA	NA
Atrazine	Herbicide	2.5	22.9	14.4	15.1	17.6	19.1
Triadimefon	Fungicide	3.1	2.7	6.0	8.4	5.2	8.1
Fipronil	Insecticide	4.0	1.1	2.5	3.4	2.7	3.8
Pendimethalin	Herbicide	5.2	19.8	13.6	12.1	12.4	12.5

NA not applicable; green tree frogs were not exposed to imidacloprid

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Tree Frog Pesticide Exposure Treatments

This study is considered a worst-case scenario exposure that was designed to maximize water uptake across the dermis on contaminated soils. All juvenile tree frogs were dehydrated overnight for 12 h in clean, unlined 10-gallon glass aquaria before pesticide exposure. Experimental units were 10-gallon glass aquaria lined with 750 g of soil (depth approximately 1 cm), and all exposures were initiated between 7:00 and 9:00 am. To simulate direct contact with an overspray of pesticides, individual pesticide active ingredients were dissolved in 300 mL of 5 % methanol (MeOH) in deionized water. In pilot studies, 5 % MeOH in deionized water had no apparent sublethal or lethal affects on control tree frogs when sprayed directly over the surface of the aquaria and frogs. The control treatment for the direct overspray consisted of 300 mL of 5 % MeOH. The testing sequence for species was based on their morphological development, but within each species the pesticide/control sequence for the experimental units was randomized. After lining the 10-gallon aquaria with 750 g of soil, five dehydrated conspecifics were added to each aquarium. The simulated overspray was initiated by spraying pesticides in random order using compressed air propellant Preval Spray Gun canisters attached to gradated, clean glass jars. In the direct pesticide-exposure treatment, amphibians were exposed to pesticides directly during the overspray and subsequently through soil exposure for the remainder of the 8-h study.

Five individuals of each tree frog species were exposed to each pesticide with the exception that green tree frogs were not exposed to imidacloprid due to the low availability of metamorphs at the time of testing. In total, 90 tree frogs were used in this study across 18 experimental units (aquaria)—50 barking tree frogs and 40 gray tree frogs—divided evenly among the direct and indirect pesticide treatments. In addition, 16 frogs were not exposed to an active ingredient for control purposes and analyzed for any pesticide background contamination. Body burdens were estimated for individual frogs within each aquarium; therefore, means (with $n = 5$) from each of the 18 experimental units served as the basis for hypothesis testing. Direct overspray experiments were performed with a randomization procedure and initiated within 1 week after completing the indirect exposure treatments for all treatment combinations. To keep tree frogs in contact with pesticide-contaminated soil, a 0.5 cm−diameter hardware mesh screen insert was placed inside each aquarium at a height of 2.5 cm above the soil surface. This insert allowed tree frogs to explore the soil surface fully, but it prevented their climbing on the glass walls of the aquaria. Only 1 pesticide active ingredient was applied to each aquarium to avoid ameliorative or synergistic effects of exposure to numerous pesticide stressors. Including the initial 10 min required to release the 300-mL mixture of pesticide and solvent through the Prevel Spray Gun, the exposure duration for the simulated overspray was 8 h.

An 8-h exposure duration was chosen because it has been documented as a time point at which dehydrated toads lose significant amounts of water through elimination and evaporative cooling after being rehydrated in atrazin-treated water (Mendez et al. 2009). This time frame also minimizes the likelihood of pesticide loss through the excretion of waste products. Separate laboratory studies AQ3 have confirmed 8 h as a time point for maximal loading of the parent pesticide in dermally exposed amphibians [Glinski et al. (in preparation)]. Post-exposure effects can continue to occur after 8 h of exposure, but the loss of pesticide mass through elimination, excretion, metabolism, and evaporative processes compromise the exposure end points and render uptake estimates less conservative for longer study durations.

For the indirect soil-spray treatment, a randomized approach for spraying experimental units with single pesticide active ingredients was used. The pesticide treatments were dissolved in 150 mL of 100 % MeOH over the soil surface and applied with a Spray Gun canister. A control treatment with 150 mL of 100 % MeOH was also applied. Dissolution of active ingredients in 100 % MeOH was more readily achieved for hydrophobic compounds. After pesticide application, aquaria were placed in a fume hood overnight to allow the MeOH to evaporate off the soil surface. The next morning, the aquaria were removed from the fume hood and the soil rehydrated with 300 mL of deionized water using a hand-held spray bottle. Two soil samples from each experimental unit were composited for analysis. Five dehydrated conspecifics were added immediately after soil rehydration to each aquarium for the entire duration of the 8-h pesticide exposure. As in the direct treatment, each indirect experimental unit yielded a mean estimate of five body burdens per each combination of pesticide and tree frog. All experimentation was performed at room temperature (approximately 20 °C). At the termination of each experiment, individual tree frogs were placed in prelabeled scintillation vials and killed in an −80 °C freezer.

Soil and Tree Frog Extraction

Extraction methods for both whole-body amphibians and soils are detailed in Van Meter et al. (2014). Briefly, 5 mL of methanol (MeOH) was added to each sample followed by sonication, vortexing, and centrifugation. The supernatant was collected, and each sample was extracted again for a total of two extractions per sample. The resulting supernatant from the second extraction was combined with

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the first extract supernatant and evaporated to 1 mL under nitrogen gas. For final pesticide extraction, 10 mL of Milli-Q water, 3 mL of methyl-*tert*-butyl ether (MTBE), and sodium sulfate were added to each sample. The MTBE layer was transferred off the top of the final sample and centrifuged, and 1 mL of the final extract was analyzed using LC–MS after being blown down with nitrogen and reconstituted with 30 % methanol.

In addition to the five pesticide active ingredients, soil and frog tissue samples were also scanned for primary metabolites. When metabolites were detected, their concentrations were summed with that of the associated parent compound as follows: desethyl-atrazine (DEA) and deisopropyl atrazine (DIA) with atrazine, triadimenol with triadimefon, and fipronil sulfone with fipronil. We did not detect quantifiable metabolites of either pendimethalin or imidacloprid. After analysis, bioconcentration factors (BCFs) were determined for each species and pesticide:

$$\mathrm{BCF} = C_f/C_s$$

where C_f is the frog whole-body tissue concentration, and C_s is the average composite soil concentration within an experimental unit, both at the end of the 8-h exposure. Although BCFs typically refer to the accumulation of contaminants from an aquatic medium at steady state, they have also been used to describe dietary and dermal accumulation in terrestrial environments as presented in our study (Kenaga 1980; Henson-Ramsey et al. 2008).

LC–MS Instrumentation

A 1-mL aliquot of each soil and frog sample extract was analyzed on an Agilent 1100 Series high-performance liquid chromatograph coupled to a 6120 mass spectrometer. Chromatographic separation was achieved on an Eclipse XDB-C18 (3.5-µm particle size, 3.0 × 150 mm^2; Agilent Technologies). Initial mobile phase was 70 % water with 0.1 % formic acid (A) and 30 % acetonitrile with 0.1 % formic acid (B). Starting conditions were held for 2 min and ramped to 90 % B over 16 min and held for 4 min before returning to initial conditions of 30 % B and re-equilibrated for 5 min (total run time 30 min). Samples were analyzed in positive electrospray ionization (ESI) from 0 to 19 min, then switched to negative ESI from 19 to 23 min, and back to positive ESI from 23 to 30 min; both used selected ion monitoring (SIM) mode. Switching between positive and negative ESI was due to the respective elution times of the pesticide active ingredients. Active ingredients were identified based on the following SIM ions: atrazine 216 m/z and 174 m/z, triadimefon 294 m/z and 225 m/z, fipronil 435 m/z and 330 m/z, imidacloprid 256 m/z and 175 m/z, pendimethalin 212 m/z and 282 m/z, and tetraconazole 372 m/z. Pesticide metabolites were identified as follows: DIA 174 m/z and 176 m/z, DEA 188 m/z and 146 m/z, triadimenol 296 m/z and 227 m/z, and fipronil sulfone 451 m/z and 415 m/z.

Statistical Analysis

All analyses were performed in R version 3.0.1 [R Core Team 2013, Vienna, Austria (http://www.R-project.org/)]. To verify that the measured pesticide concentration in soil samples was directly and positively related to the amount of pesticide applied, we used Kendall's Tau correlation coefficient, a nonparametric statistic that is less sensitive to outliers in data that are not normally distributed. Hypothesis tests of whole-body pesticide tissue concentrations and BCF within species and by pesticide were compared for direct overspray and indirect soil exposure. A paired comparison randomized block design analysis of variance (ANOVA) (Wu and Hamada 2009) that treats species and pesticides as factorial nuisance variables was run to test the hypothesis concerning differences in pesticide residues and BCFs between direct and indirect applications. ANOVA was also performed for the composite soil samples to ensure that there were no significant treatment effects between direct and indirect application methods. It contains $n-1$ degrees of freedom (d*f*) associated with the sum of squares among the paired comparisons. In addition to ANOVA, a nonparametric sign test was performed for the nine paired comparisons of direct overspray and indirect soil exposure to test whether tissue residues and BCFs were higher for the direct overspray. The sign test does not require any normality assumptions about the data and simply involves counting the number of positive differences between the matched pairs and relating these to the binomial distribution.

Results

Pesticide Concentrations in Soil

Mean composite soil pesticide concentrations correlated positively with application amounts (Kendall's Tau correlation coefficient = 0.91, p = 7.7E−7; Table 1). Pesticide metabolites were detected in soils sprayed with atrazine, triadimefon, and fipronil. On average, DEA and DIA in combination constituted <1.0 % of total recoverable atrazine in both the direct and indirect exposure treatments. Triadimenol accounted for 35.3 % (direct) and 3.5 % (indirect) of total triadimefon whereas fipronil sulfone constituted 1.7 % (direct) and 1.3 % (indirect) of total fipronil exposures. ANOVA comparison of soil concentrations between direct and indirect application methods indicated no significant difference in soil concentrations

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between application methods across the treatment units ($p = 0.33$) as did the sign test comparison ($p = 0.18$).

Pesticide Concentrations in Amphibians

Barking tree frog average mass (±SE) was 2.41 g (±0.08) and 2.24 g (±0.12), whereas green tree frog average mass was 2.38 g (±0.23) and 1.99 g (±0.14) for the direct and indirect pesticide treatments, respectively. None of the pesticides tested in this study were detected in the tissue of the control frogs. Within the direct overspray treatment, mean pesticide tissue concentrations ranged from 0.63 to 12.59 ppm for barking tree frogs and from 1.7 to 20.46 ppm for green tree frogs. The indirect soil spray exposure resulted in mean pesticide concentrations in barking tree frog tissues ranging from 0.019 to 6.11 ppm and from 0.165 to 3.74 ppm in green tree frog tissues (Table 2). Atrazine produced the highest measurable body burdens in both species and across treatments (Fig. 1). The direct overspray treatment consistently resulted in significantly higher whole-body tissue concentrations in barking and green tree frogs across all active ingredients tested (Fig. 1). Exposure to pendimethalin resulted in the lowest measured body burdens among green and barking tree frogs in the indirect soil treatment, whereas triadimefon body burdens were lowest for both species in the direct overspray treatment (Fig. 1). Statistical hypothesis testing performed with a paired-comparison ANOVA rejected a null hypothesis of no difference between tissue concentrations from direct and indirect applications ($p = 0.03$; $df = 8$), which shows that the direct uptake was significantly higher than indirect uptake. For the nonparametric sign test comparison, all nine tissue concentrations were higher in the direct versus the indirect application, and therefore the sign test also rejected the no-difference null hypothesis with $p = 0.004$. Ratios of whole-body pesticide tissue concentrations for the direct to indirect treatments are given in Table 2.

BCFs

BCFs are often used in ecological risk assessments to estimate body burdens in exposed populations. Across all pesticides, BCFs for barking and green tree frogs were significantly higher for the direct overspray treatment relative to the indirect soil treatment (Fig. 2). The paired-comparison ANOVA rejected the null hypothesis of no difference between BCFs from direct and indirect applications ($p = 0.01$), again showing that the direct bioconcentration was higher than the indirect. For the nonparametric sign test comparison, eight of the nine BCFs were higher in the direct application; therefore, the sign test also rejected the null hypothesis ($p = 0.04$). Mean BCFs for barking tree frogs ranged from 0.1 to 0.87 for pesticides in the direct treatment and from 0.026 to 0.78 for the indirect treatment. BCFs and ratios of direct to indirect exposures for barking and green tree frogs are given in Table 2. On average, atrazine was the most bioconcentrated pesticide in the direct exposure treatment, whereas imidacloprid resulted in the highest BCF in the indirect exposure treatment (Fig. 2). The lowest average barking

Table 2 Mean treatment tissue concentrations ($n = 5$ for species × pesticide treatment combinations) and mean ratios of direct to indirect exposures for whole-body tissue concentrations and BCFs by pesticide

Species/ pesticide	Mean direct tissue concentration	Mean indirect tissue concentration	Tissue concentration ratio	Tissue concentration sign	Mean direct BCF	Mean indirect BCF	BCF ratio	BCF sign
Barking tree frog								
Imidacloprid	1.08	0.39	2.7	+	0.43	0.78	0.5	–
Atrazine	12.59	6.11	2.1	+	0.87	0.40	2.2	+
Triadimefon	0.63	0.21	2.9	+	0.10	0.03	4.1	+
Fipronil	1.94	0.57	3.4	+	0.78	0.17	4.6	+
Pendimethalin	2.09	0.19	11.1	+	0.15	0.02	9.9	+
Green tree frog								
Atrazine	20.46	3.74	5.5	+	1.16	0.20	5.9	+
Triadimefon	1.70	0.37	4.6	+	0.33	0.05	7.1	+
Fipronil	2.10	0.21	9.9	+	0.78	0.06	13.9	+
Pendimethalin	2.76	0.17	16.8	+	0.22	0.01	16.9	+

Significance testing (sign test) of the direct overspray tissue concentrations versus indirect soil exposure tissue concentrations showed that the direct oversprays were significantly greater for tissue concentrations ($p = 0.004$) and BCFs ($p = 0.04$)

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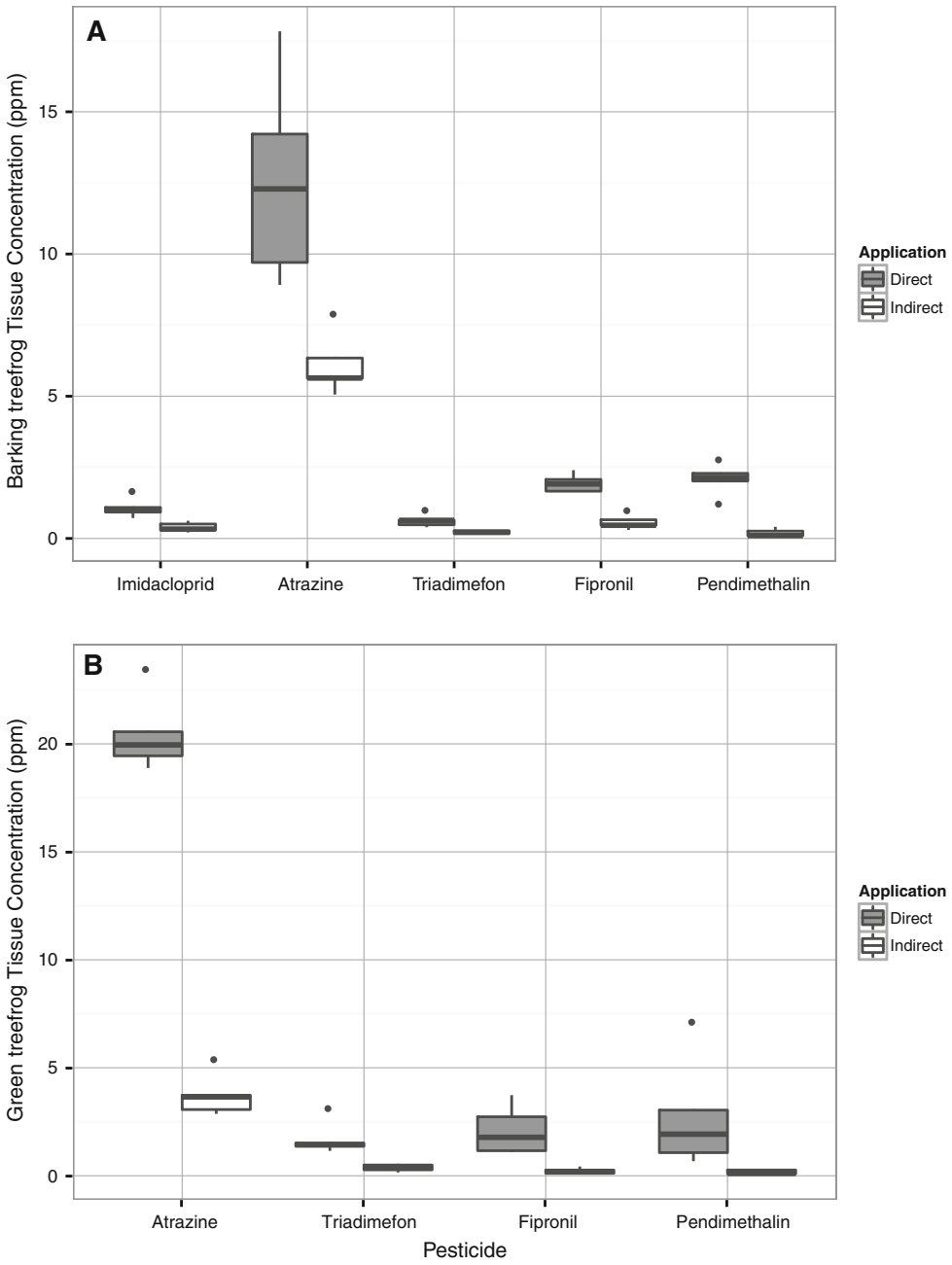

Fig. 1 Paired box plots for whole-body pesticide tissue concentration for barking tree frogs (**a**) and green tree frogs (**b**) to compare indirect soil spray and direct overspray treatments ($n = 5$/species and pesticide treatment). Box 25th, 50th, and 75th percentiles represent the middle three data points, and the line extends to the minimum and maximum data points (*points* indicate outliers)

tree frog BCF was for triadimefon in the direct exposure treatment and pendimethalin in the indirect exposure treatment (Fig. 2a). For green tree frogs, BCFs ranged from 0.22 to 1.16 in the direct overspray treatment and from 0.013 to 0.2 in the indirect soil treatment. Patterns in maximum and minimum BCF values for green tree frogs largely coincide with patterns in BCFs for barking tree frogs. The highest green tree frog average BCF values were associated with atrazine for both direct and indirect treatments (Fig. 2b). The lowest BCF values were related to pendimethalin in the direct overspray treatment and to triadimefon in the indirect soil spray treatment (Fig. 2b).

Discussion

Direct dermal contact with pesticides is a concern for post-metamorphic, terrestrial amphibians that reside in agricultural habitats or that move large distances through agricultural fields during breeding season (Smith et al. 2007; Brühl et al. 2011; Fryday and Thompson 2012; Smalling et al. 2012; Lenhardt et al. 2014). The same dermal characteristics that make amphibians ideally suited for both aquatic and terrestrial habitats also may leave them more susceptible to pesticides, which may be readily taken up across the skin and distributed to various organs in the

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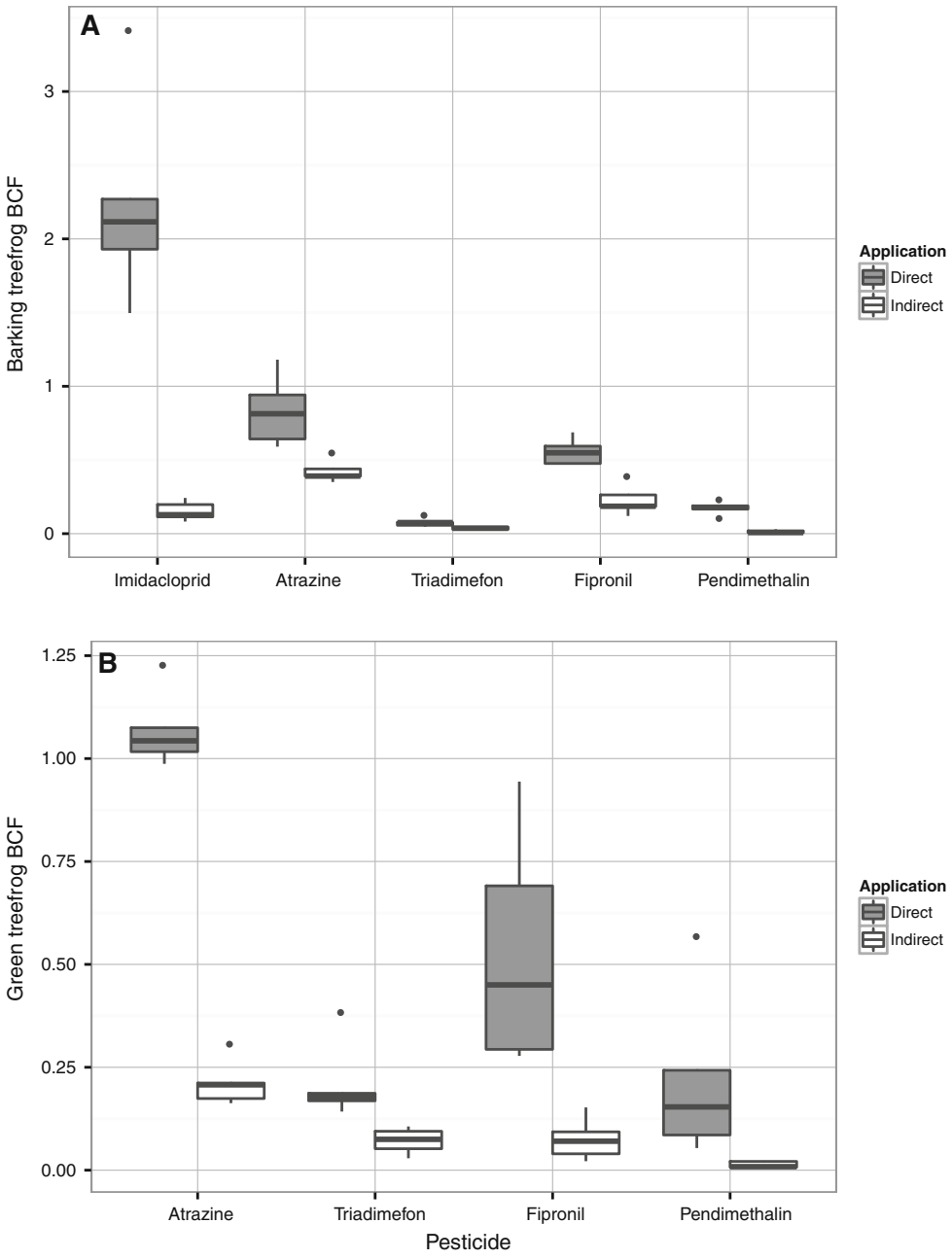

Fig. 2 Paired box plots for BCFs for barking tree frogs (**a**) and green tree frogs (**b**) to compare pesticide indirect soil spray and direct overspray treatments ($n = 5$/species and pesticide treatment)

body (e.g., Henson-Ramsey et al. 2008; Mendez et al. 2009). For amphibians occupying terrestrial habitats, dermal contact with pesticides may be the primary route for uptake and accumulation, especially during pre-emergent spray events and life history periods prone to dehydration such as drought-type conditions and long-distance dispersal events. Our study contributes to the limited published data for post-metamorphic terrestrial amphibians experiencing pesticide exposure by validating that amphibians readily absorb pesticides from soils and overspray through the skin. This experiment is unique in comparing pesticide accumulation through the dermis in amphibians after direct overspray and indirect soil exposure treatments that simulate pre-emergent pesticide applications. The amphibian BCFs we report here for the indirect treatment are consistent with those we reported for barking and green tree frogs in Van Meter et al. (2014) for atrazine, triadimefon, fipronil, and pendimethalin. Indirect exposure to imidacloprid resulted in a higher BCF in the current study for barking tree frogs, and the direct exposure treatment produced much higher BCFs than our previously reported data for both species (Van Meter et al. 2014) as expected.

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Regulatory Application and Exposure Study Design

Exposure studies carry unique restrictions on study design. Laws regulating pesticide registration require the estimation of exposure and effects as distinct steps. Understanding exposure dynamics within and across ecological taxa is a critical aspect of creating science-based regulatory approaches that are founded on sets of species/taxa. Although exposure studies are an integral component for human health toxicological studies, this area is little explored on the ecological side for many taxa (Lioy and Smith 2013). Exposure-based studies allow for inference across a taxa (e.g., amphibians) on hypotheses that may be unique to exposure studies. These research questions include pertinent questions as to whether terrestrial amphibians uptake pesticides at different rates than other vertebrate taxa more commonly studied and used as surrogates for regulatory purposes (e.g., birds, mammals). The unique dermal properties of amphibians may also affect exposure processes such as the relative uptake of direct and indirect pesticide exposure tested here. However, exposure-based hypotheses also present restrictions different than typical ecotoxicological effects studies. Specifically, there is the potential for the expression of effects to confound exposure estimation.

In vivo metabolism and uptake of chemicals may be significantly altered in an organism experiencing toxicological effects. This was a concern for our experimental design because we witnessed behavioral changes and some mortality in test runs with exposure durations typically used for effects studies (e.g., 48 h). Under these conditions, a significant proportion (if not all) of exposed organisms could have their physiology affected. Our stated observational end point (tissue residue, bioconcentration) would then be statistically compromised based on when exactly they died or how significantly their physiology was affected by the pesticide effects. In contrast, the objective in these experiments is to characterize the temporal dynamics of uptake and pesticide exposure in a relative manner (testing our hypothesis concerning direct and indirect uptake) while not statistically compromising the uptake estimates by way of expressed effects in the exposed amphibians.

Immediately on entering the amphibian body, pesticides begin to be metabolically transformed into different daughter compounds. Some, but not all, of these daughter compounds are known and can be analyzed for. This means that the ratio of the amount of pesticide active ingredient in tissue residue to the amount applied in the study becomes less meaningful over time. We collected metabolic data showing that 8 h is the peak concentration period observed for these parent analytes, species, and life stage [Glinski et al. (in preparation)].

Dermal Exposure

Tree frogs in our study accumulated greater concentrations of pesticides from the simulated overspray compared with the soil exposure, thus indicating that direct contact with pesticides over a larger area of the dermis leads to greater uptake. Several investigations of pesticide risk through aerial spraying in agricultural habitats have indicated minimal risk to terrestrial amphibians (Thompson et al. 2004; Dinehart et al. 2009; Brain and Solomon 2009), whereas a very recent study reported mortality rates as high as 100 % after 1 h of exposure to aerially sprayed pesticides (Brühl et al. 2013; Belden et al. 2010). Our data indicate that risk associated from the aerial spray of pesticides can be much greater than contact with contaminated soil alone. This may be particularly true for amphibians subject to aerial pesticide applications during pre-emergence when foliar cover is absent. Amphibian dispersal to breeding ponds in spring coincides temporally with application of many pre-emergent pesticides. Later in the growing season, vegetative canopy cover can reduce ground deposition and downwind exposure from airborne droplets associated with aerially sprayed pesticides, although organisms within 10 m of the spray zone can be subject to substantially higher exposure amounts (Hewitt et al. 2002). We used juvenile frogs in this study, which have a smaller surface area than adult frogs. Therefore, pesticide accumulation in adult frogs may be even greater given the larger surface area of their body in contact with contaminated soils and vegetation. Although amphibian dermis is susceptible to pesticide uptake from overspray and soil applications, we found that direct and full-body contact with an aerially sprayed pesticide is likely to lead to higher body burdens.

Individual pesticide properties—such as soil partition coefficient (K_{OC}), octanol−water partition coefficient (K_{OW}), inactive ingredients, and soil properties such as organic carbon content—may play a large role in the degree to which they permeate amphibian dermis. In particular, a pesticide's $\log K_{OW}$ indicates that compound's hydrophobicity and is considered an important predictor in pesticide accumulation. Hydrophobic pesticides (i.e., high $\log K_{OW}$) were found to permeate excised frog skin in a flow-through cell more readily than hydrophilic pesticides (Quaranta et al. 2009). Although the current study was not designed to investigate $\log K_{OW}$ exclusively as a factor in pesticide accumulation, the data corroborates the report of Van Meter et al. (2014) showing that $\log K_{OW}$ is not the best predictor of pesticide accumulation in living terrestrial amphibians. In both direct overspray and indirect soil exposure treatments, atrazine and imidacloprid led to higher body burdens and BCFs compared with pendimethalin, a much more hydrophobic pesticide. Among the

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pesticides studied by Quaranta et al. (2009), atrazine also produced the highest permeation rate. However, fipronil, another hydrophobic pesticide, resulted in higher tissue concentrations in barking tree frogs than imidacloprid in our study. To keep the active ingredients in this study in solution, we used methanol as a solvent to isolate the potential for accumulation of only active ingredients. The extent to which the inert ingredients in formulated products contribute to pesticide permeability and cause behavioural and physiological alterations in amphibians needs further study.

Exposure Versus Effects

Quantifying body burdens and BCFs from controlled laboratory exposures is a critical first step toward improving our understanding of the relationship between dermal exposure and pesticide accumulation in terrestrial amphibians. Adult female green frogs (*Lithobates clamitans*) exposed to 10 μg L^{-1} fipronil in water for 8 days reached a maximum BCF of 11.7 after 6 days, and, after only 1 day of exposure, had already equaled or exceeded the maximum BCF values we report (Reynaud et al. 2012). Although both datasets highlight the susceptibility of the amphibian dermis to pesticides, making direct comparisons is inappropriate given the variation in exposure mediums, fraction of body exposed, and exposure duration. Furthermore, there are lacks of data for terrestrial amphibian exposures needed to relate our pesticide body burdens to reported LC50 or LD50 values. Estimates of lethal concentrations or doses for larval amphibians have been published for several of the pesticide active ingredients we tested (e.g., Overmyer et al. 2007; Weir et al. 2012), but they are much lower than the body burdens we measured in our tree frogs, which indicates significant differences in pesticide tolerance between life stages. An extensive data set on dermal pesticide LD50 values among birds has been published (Mineau et al. 2001), but amphibian skin is much less keratinized and has adaptations to enhance water uptake, thus preventing a meaningful comparison.

Gaining a foundational understanding of pesticide accumulation, toxicokinetics, and associated sublethal or lethal effects in terrestrial amphibians is vital to conservation and policy efforts. In Mendez et al. (2009), dehydrated American toads (*Bufo americanus*) offered both ^{14}C-atrazine treated water and dry space in a controlled laboratory study actively used their seat patch to rehydrate and accumulated the associated atrazine. Although the highest levels of radioactivity were found in the gall bladder and intestines, atrazine was also found in other sensitive organs such as the liver, brain, and kidneys (Mendez et al. 2009). Similarly, Reynaud et al. (2012) report fipronil accumulation in the gall bladder, intestines, fat bodies, skin, and ovaries among green frogs in an 8-day aquatic exposure. Fipronil also reduced liver metabolism by as much as 80 % relative to control frogs, consequently limiting the ability of the body to detoxify contaminants (Reynaud et al. 2012). Given that we did not remove the dermis from our amphibians before homogenization, we cannot differentiate between pesticides that may have been residually on the skin versus those that had passed through the skin and into specific organ systems. Nonetheless, our data indicate the formation and presence of metabolites for three of the five pesticides tested, therefore further supporting the conclusion that pesticides are readily taken up across amphibian skin and distributed throughout the body where they are chemically transformed. Direct comparisons with other studies cannot be made given the different exposure media and whole-body versus organ-specific pesticide loads reported. Additional studies are needed to improve our understanding of distribution and the toxicokinetics associated with pesticide exposure and any potential sublethal effects that may be induced in terrestrial amphibians.

Conclusion

The amphibian dermis is a showed route of pesticide uptake and accumulation in terrestrial habitats creating the potential for lethal and sublethal impacts to this declining fauna in habitats where pesticides are commonly applied. Pesticide exposure among amphibians may not necessarily result in immediate direct mortality, but repeated exposure and accumulation of pesticides may induce subsequent mortality and sublethal impacts that limit population viability over time. Agricultural landscapes may be the greatest concern given the variety and timing of pesticide applications throughout the year and the likelihood that irrigated farmland may be a preferred habitat for terrestrial amphibians during times of low water availability. The vulnerability of early amphibian life stages to pesticide contamination has been well-documented, but juveniles and adults have been poorly represented in published datasets to date. This is particularly true for amphibians residing in or traversing agricultural landscapes during their breeding season when they may be subject to overspray pesticide applications or residual pesticides on soil and vegetation. In combination with dietary ingestion of contaminated food resources, dermal uptake presents an additional and significant threat to the long-term success of amphibian populations worldwide. Given their distinctive skin properties designed for bulk movement of water, amphibians may receive higher chemical doses, particularly from hydrophilic pesticides, compared with reptiles, birds, and mammals. Acknowledging the essential role of

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the dermis in pesticide uptake in amphibians, particularly in terrestrial habitats, is crucial to properly estimating amphibian exposures on agricultural landscapes and the resulting individual and population-level impacts.

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