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RESEARCH ARTICLE

Analysis of insecticide exposure in California hummingbirds using liquid chromatography-mass spectrometry

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Abstract

External feather rinses and homogenized whole-carcass tissue matrix from two hummingbird species found in California (*Calypte anna* and *Archilochus alexandri*) were analyzed for the presence of nine insecticides commonly used in urban settings. Using a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analytical method, samples were quantitatively tested for the following neonicotinoids: dinotefuran, nitenpyram, thiamethoxam, acetamiprid, thiacloprid, clothianidin, imidacloprid, and sulfoxaflor. This analytical method was also used to qualitatively screen for the presence of approximately 150 other pesticides, drugs, and natural products. Feather rinsates from both hummingbird species had detectable concentrations of carbamate and neonicotinoid classes of insecticides. Combined results of the rinsate and homogenized samples (n = 64 individual hummingbirds) showed that 44 individuals (68.75%) were positive for one to four target compounds. This study documented that hummingbirds found in California are exposed to insecticides. Furthermore, feather rinsates and carcass homogenates are matrices that can be used for assessing pesticide exposure in small bird species. The small body size of hummingbirds limits traditional sampling methods for tissues and whole blood to evaluate for pesticide exposure. Thus, utilization of this analytical method may facilitate future research on small-sized avian species, provide insight into pesticide exposure, and ultimately lead to improved conservation of hummingbirds.

Keywords Hummingbirds · Urban · Neonicotinoids · Insecticides · Pesticides · Birds · Non-target species

Introduction

The use of pesticides and other synthetic chemicals across the globe has increased rapidly over the last several decades and is a wide research topic across many disciplines (Bernhardt et al. 2017). Neonicotinoid insecticides were developed in the 1980s and currently includes the most widely used class of insecticides in the world (Goulson 2013). As of 2013, there were over 300 neonicotinoid products registered in California alone (Mineau and Palmer 2013). As systemic pesticides (meaning that the chemical is integrated in tissue throughout the entire plant), neonicotinoid pesticides offer long-lasting protection against insect herbivory (Tomizawa and Casida 2005). Compared to some other insecticides that neonicotinoids have replaced, such as organophosphates or carbamates, the acute toxicity of neonicotinoids to birds is relatively low (Mineau and Palmer 2013). Neonicotinoids act on insects by irreversibly binding to nicotinic acetylcholine receptors (nAChRs) located on the post-synaptic site of neuronal cells (Matsuda et al. 2001). Nicotinic acetylcholine

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receptors are activated by the endogenous neurotransmitter acetylcholine whereby low to moderate activation of these receptors cause nervous stimulation. Neonicotinoids, however, act as agonists and irreversibly bind at the receptor site causing overstimulation resulting in paralysis and death. Acetylcholinesterase, the enzyme which breaks down the endogenous ligand acetylcholine, cannot break down neonicotinoids.

Neonicotinoids can have effects on non-target species including bees and other invertebrates (Pisa et al. 2015) and vertebrates (Gibbons et al. 2015) in part because these chemicals are systemic and incorporated into the pollen and nectar of treated plants, where they come into contact with or are ingested by pollinating species. In neonicotinoid-treated plants, 11–24% of pollen and 17–65% of nectar is contaminated with the pesticide compound (Sanchez-Bayo and Goka 2014). For these reasons, pollinators are particularly vulnerable to neonicotinoid exposure (Hladik et al. 2018). Significant losses of bee colonies have been documented by beekeepers in many areas of the world and are an area of intense research due to the economic importance of pollination services by honeybees (Farooqui 2013). Experimental evidence suggests that exposure to neurotoxic pesticides can reduce survival and reproduction in honeybees (Farooqui 2013), induce immunosuppression in bees (Pamminger et al. 2018), and these sublethal effects can contribute to colony collapse disorder (Lu et al. 2014). While honeybee decline is a multi-faceted and synergistic issue involving habitat loss, pathogens, and agricultural pesticides (Goulson et al. 2015), three neonicotinoids (thiamethoxam, imidacloprid, and clothianidin) and two organophosphates (phosmet and chlorpyrifos) pose the biggest risk to honey bees overall (Sanchez-Bayo and Goka 2014).

Much research has been done on the exposure and effects of neonicotinoids on bees (Fairbrother et al. 2014) but less work has evaluated the exposure and risks of these compounds on other pollinating species overall (Hladik et al. 2018), especially vertebrate pollinators. However, there has been a distinct increase in papers published on the ecological and environmental effects of neonicotinoids overall (Giorio et al. 2017). Pollination is a valuable ecosystem service that is in decline on a global scale (Potts et al. 2010). In bats (ecologically important both as pollinators and insectivores), pesticide exposure is a possible contributor to population declines and susceptibility to white-nose syndrome, but long-term data is lacking (Bayat et al. 2014). Recent analyses suggest that the use of neonicotinoid pesticides is associated with declines in bird populations on a continental scale (Goulson 2014), and insectivorous birds are thought to be impacted the most through a pesticide-mediated reduction in food supply (Hallmann et al. 2014). Thus, neonicotinoid pesticides appear to impact non-target species at exposure concentrations well below the amount that would induce acute toxic effects such as death (Goulson 2014).

In addition to the effects of direct mortality, sub-lethal toxic effects of pesticides can affect the survival and reproduction of birds (Fry 1995; Stanton et al. 2018). Attempts have been made to include pesticide exposure into models of avian survival and reproduction, but are often limited by the availability of direct controlled toxicological studies (Bennett et al. 2007; Etterson and Bennett 2013). Compared to some other insecticides that neonicotinoids have replaced, such as organophosphates or carbamates, the acute toxicity of neonicotinoids to birds is relatively low (Mineau et al. 2001). However, sublethal effects documented in birds include debilitation such as ataxia which can be induced in birds given imidacloprid orally at an order of magnitude below the lethal dose (Callahan and Mineau 2008). Migratory ability was shown to be impaired in white-crowned sparrows given 10% of the LD50 for imidacloprid (Eng et al. 2017). Thus, establishing if birds are exposed is a crucial first step in evaluating the impacts of these chemicals on the survival and reproduction of wild birds.

Small birds are generally more sensitive to toxic chemicals than larger birds (Mineau et al. 1996), thus hummingbirds may offer great potential for monitoring bird exposure to pollutants. Since the diet of hummingbirds includes both insects and nectar from many sources, hummingbirds could be used as sentinel species for assessing environmental exposure to pollutants, including pesticides (Godoy et al. 2014). Given that bees and hummingbirds often forage in the same areas (Pritchard et al. 2017), it makes sense that these two ecologically similar groups of species may be exposed to the same suites of pollutants. However, testing for pesticide exposure is a challenge for individual small-bodied animals due to the constraints of the minimum sample mass required for many existing analytical methods. One gram or more of a single tissue type (i.e., liver) is commonly necessary for toxicological assays (Berny et al. 1999), which makes it difficult to use currently established analytical methods on samples from very small-bodied individual animals. North American hummingbirds generally weigh less than 4–5 g, and thus require alternative methods to evaluate individual chemical exposure.

The objective of this study was to evaluate external carcass feather rinsates and composite tissue samples from hummingbirds found in California for exposure to nine insecticides commonly used in urban environments using a previously established liquid chromatography-high-resolution mass spectrometry (LC-HRMS) assay method (Filigenzi et al. 2019).

Methods

Carcass sampling

Anna's (ANHU; n = 21 Northern California; n = 22 Southern California) and Black-chinned (BCHU; n = 21) Hummingbirds that did not survive rehabilitation

efforts were used in this study. Bird carcasses were obtained from wildlife care and rehabilitation centers in California. No birds were killed for this study. We investigated these two hummingbird species found in California because one species is migratory (Black-chinned Hummingbird, *Archilochus alexandri*) and the other species has both migratory and resident populations (Anna's Hummingbird, *Calypte anna*). Black-chinned and some Anna's Hummingbirds are known to migrate outside the USA (Phillips 1975) where pesticide regulations are different, so they may be exposed to a different suite of chemicals than are resident Anna's Hummingbirds during their annual cycle.

Feather rinsate sample preparation

For the Northern California ANHU and BCHU, the intact carcasses were placed in a 50-mL plastic tube and enough acetonitrile was added to cover (approximately 40 mL) the sample. The tube was shaken for 10 min. The acetonitrile was then transferred to a glass tube, evaporated dry under nitrogen, and reconstituted for analysis in 200 μL of 20% methanol in water. For the Southern California ANHU, the carcasses were externally washed with a dilute solution of Alconox® detergent. The detergent solution used for the rinsates was not compatible with the existing liquid chromatography-high resolution mass spectrometry (LC-HRMS) analytical method and therefore these rinsates were not analyzed.

Homogenized tissue sample preparation

After the feather rinsate process, the same hummingbird carcasses were analyzed using a previously described LC-HRMS method (Filigenzi et al. 2019). After rinsing, carcasses were air dried. Individual carcasses were frozen in liquid nitrogen and ground in a Stein mill. One gram of the ground carcass was transferred to a 50-mL plastic tube and a mixture of isotopically labelled neonicotinoid pesticide internal standards was added. Then, 5 mL of water was added and the tube was vortexed to mix the contents. Acetonitrile (15 mL) was added and the tube was shaken for 5 min. The tube was centrifuged for 5 min and the supernatant decanted into a 50-mL tube containing magnesium sulfate and ammonium acetate. The tube was shaken by hand and then centrifuged. The acetonitrile layer was transferred to a tube containing magnesium sulfate and primary-secondary amine, shaken, and centrifuged. The solvent was then decanted into a glass tube, evaporated dry, and reconstituted for analysis in 200 μL of 20% methanol in water.

Analysis of feather rinsates and homogenized tissue samples

Feather rinsate and homogenized tissue extracts were analyzed by LC-HRMS using a Dionex Ultimate 3000 ultrahigh pressure liquid chromatograph interfaced with a Thermo Q-Exactive high-resolution mass spectrometer. The column used was a 100 × 2.1 mm and 1.7 μm Eclipse Plus C-18 from Agilent Corporation. The mass spectrometer was run in positive ion mode using electrospray ionization. The resolution was set at 70,000 ($M/\Delta M$ at m/z 200). Neonicotinoid insecticides (dinotefuran, nitenpyram, thiamethoxam, acetamiprid, thiacloprid, clothianidin, imidacloprid, and sulfoxaflor) were analyzed in high-resolution MS/MS mode to provide enhanced specificity and quantitative data in carcass samples. While sulfoxaflor contains a sulfoximine group to make it chemically distinguishable from neonicotinoids (Zhu et al. 2011), sulfoxaflor acts on the same nicotinic acetylcholine receptors as neonicotinoids (Cutler et al. 2012). Thus, we included sulfoxaflor in our list of target neonicotinoids. Quantitation was performed on carcass samples using the isotopically labelled internal standards added prior to extraction (note that rinsate analysis was performed on a qualitative basis only). Five-point calibration curves ranging in concentration from 0.50 to 100 ng/g were used to establish instrument response and consistently showed r^2 values > 0.99. The limits of detection and quantification for each analyte are listed in Supplemental Table 1.

Full-scan high-resolution mass spectrometry (MS) data from these analyses was used to screen for a list of approximately 150 other pesticides, drugs, and natural products including some carbamate and organophosphate insecticides (Supplemental Table 2). Laboratory-raised 1–2-day-old chicken carcasses were used for quality control analyses, including negative control samples and fortified control samples.

Data analysis

Due to the limited number of samples with quantifiable neonicotinoid concentrations, any sample with pesticide concentrations greater than the limit of detection (LOD) was deemed positive and those with concentrations below the LOD were deemed negative. An individual bird was considered positive for compound exposure if either the feather rinsate, homogenized tissue sample, or both were positive. Data were analyzed to determine exposure prevalence using generalized linear mixed models (GLMM) in R (version 3.4.3, R Core Team 2017) and odds ratios in Microsoft Excel (version 15.32, 2017). A p value of < 0.05 was considered significant. Kappa coefficients (Cohen 1960) were calculated in R (KappaGUI package) to determine the level of agreement between rinsate and homogenized samples.

Results

A total of 42 ANHU and 42 BCHU samples (homogenized carcass or rinsate) from 64 individual hummingbirds were successfully analyzed for the following neonicotinoids: dinotefuran, nitenpyram, thiamethoxam, acetamiprid, thiacloprid, clothianidin, imidacloprid, and sulfoxaflor. In addition, all samples were successfully screened for a variety of other compounds (Supplemental Table 2). Out of 84 total samples, only 3 had quantifiable concentrations above the LOD. One adult male ANHU carcass from Southern California contained a quantifiable level of imidacloprid (3.2 μg/kg). Two BCHU carcasses contained quantifiable levels of acetamiprid (3.1 and 3.4 μg/kg; adult male from Northern California and juvenile male from an unknown region, respectively). Of the 64 individual hummingbirds, combined results of the rinsate and homogenized samples showed that 44 individuals (68.75%) were positive for between one and four target compounds, while 20 individuals (31.25%) were negative for any target compounds.

Across all 64 individual hummingbirds screened, 42.2% (n = 27) tested positive for imidacloprid, 32.8% (n = 22) tested positive for carbaryl, 17.2% (n = 11) tested positive for acetamiprid, 4.7% (n = 3) tested positive for dinotefuran, 3.1% (n = 2) tested positive for thiacloprid, 3.1% (n = 2) tested positive for caffeine, 1.6% (n = 1) tested positive for thiamethoxam, and 1.6% (n = 1) tested positive for malathion (Tables 1 and 2). Imidacloprid, thiacloprid, acetamiprid, carbaryl, and caffeine were detected in the homogenized tissue samples. Imidacloprid, thiamethoxam, acetamiprid, carbaryl, malathion, and dinotefuran were detected in the feather rinsate samples. Nitenpyram, sulfoxaflor, and clothianidin were not detected in any hummingbird samples.

Agreement between homogenized carcass and rinsate samples in compound detections was found to have a kappa value of 0.38 for all compounds combined. Rinsate samples had more detections (n = 49) overall than did homogenized carcass samples (n = 30), although we were unable to screen for metabolites of target compounds which may have affected this observed pattern.

For compound prevalence and odds ratio calculations, ANHU were used as the referent category for species, females were used as the referent category for sex, hatch-year birds were used as the referent category for age, and northern California was used as the referent category for region.

Results of GLMMs are summarized in Table 3, where most odds ratio calculations did not reach the $p < 0.05$ threshold of statistical significance. However, some significant patterns were found for carbaryl, imidacloprid, and acetamiprid. BCHU were 0.23 times less likely to be positive for carbaryl (p = 0.036) and 0.20 times less likely to be positive for imidacloprid (p = 0.012) compared to ANHU. ANHU from the Southern California region were 0.26 times less likely to

Table 1 Number of feather rinsate or homogenized carcass samples from salvaged Anna's Hummingbird (*Calypte anna*; ANHU) carcasses from Northern (n = 42) and Southern California (n = 22) positive for target insecticides using a novel liquid chromatography high-resolution mass spectrometry analytical method for detecting pesticides and other compounds in small mass avian tissue samples (Filigenzi et al. 2019)

Insecticide (class)	Northern California ANHU feather rinsate samples positive for insecticides (n = 21 samples analyzed)	Northern California ANHU homogenized tissue samples positive for insecticides (n = 21 samples analyzed)	Southern California ANHU homogenized tissue samples positive for insecticides (n = 22 samples analyzed)
Carbaryl (carbamate)	19	0	0
Sulfoxaflor (neonicotinoid)	0	0	0
Dinotefuran (neonicotinoid)	2	0	0
Nitenpyram (neonicotinoid)	0	0	0
Thiamethoxam (neonicotinoid)	0	0	0
Acetamiprid (neonicotinoid)	0	0	0
Thiacloprid (neonicotinoid)	0	0	2
Clothianidin (neonicotinoid)	0	0	0
Imidacloprid (neonicotinoid)	15	2	8

Feather rinsate results are not available for the Southern California birds as they were rinsed with detergent versus organic solvent. Samples were deemed positive if they were above the limit of detection and deemed negative if below the limit of detection for each compound

Table 2 Number of feather rinsate samples (n = 21) and homogenized tissue samples (n = 21) from salvaged Black-chinned Hummingbird (BCHU) carcasses positive for target insecticides using a novel liquid chromatography high-resolution mass spectrometry analytical method for detecting pesticides and other compounds in small mass avian tissue samples (Filigenzi et al. 2019)

Insecticide (class)	BCHU feather rinsate samples positive for insecticides (n = 21 samples analyzed)	BCHU homogenized tissue samples positive for insecticides (n = 21 samples analyzed)
Carbaryl (carbamate)	3	0
Malathion (organophosphate)	1	0
Sulfoxaflor (neonicotinoid)	0	0
Dinotefuran (neonicotinoid)	1	0
Nitenpyram (neonicotinoid)	0	0
Thiamethoxam (neonicotinoid)	1	0
Acetamiprid (neonicotinoid)	3	2
Thiacloprid (neonicotinoid)	0	0
Clothianidin (neonicotinoid)	0	0
Imidacloprid (neonicotinoid)	4	0

Samples were deemed positive if they were above the limit of detection and deemed negative if below the limit of detection for each compound

be exposed to imidacloprid (p = 0.018) compared to ANHU from the Northern California region. Adult birds were 0.14 times less likely to be exposed to acetamiprid (p = 0.006) compared to juvenile birds.

Discussion

This study showed that a sensitive analytical method can be used for evaluating feather rinsate and homogenized tissue samples for presence of insecticides in free-ranging hummingbirds. Imidacloprid, acetamiprid, and thiacloprid insecticides were detected in homogenized carcass samples while carbaryl, malathion, dinotefuran, imidacloprid, thiamethoxam, and acetamiprid were detected in feather rinsates from Anna's and Black-chinned Hummingbirds found in California. This analytical method will also be useful for future studies as it provides a means for detecting carbamates, organophosphates, and neonicotinoids in animals with low body mass. Given the small sample mass and low pesticide concentrations, qualitative data predominated our current findings although three samples did contain quantifiable concentrations of target compounds (imidacloprid and acetamiprid).

To date, most studies evaluating insecticide exposure of pollinators have focused on bees. While direct insecticide residue data for free-ranging honeybees are sparse, compounds have been detected in honeybee tissue samples (and other bee products such as honey and pollen). A study in France found that 44.3% of honeybee samples contained between one and five insecticide compounds (of 42 screened for, including carbaryl) with concentrations ranging from 0.4 to 1545.6 μg/kg (Chauzat et al. 2011). Another study of declining bee populations in Greece detected imidacloprid in 60% of bee samples at concentrations of 14–39 ng/g of tissue, while other target insecticides were not detected (Bacandritsos et al. 2010).

Our results show that two avian pollinator species found in California (ANHU and BCHU), are exposed to a suite of insecticides. Moreover, greater numbers of insecticide compounds were detected in the external feather rinsate samples (n = 6 compounds) per bird than in the homogenized carcass samples (n = 5 compounds). Different compounds were also detected in different sample types; thiacloprid and caffeine were only detected in homogenized carcass samples, while thiamethoxam, malathion, and dinotefuran were only detected in feather rinsate samples, and imidacloprid, acetamiprid, and carbaryl were detected in both sample types. It is intriguing that more insecticides were detected externally, given that it has been previously reported that the greatest pesticide residue concentrations are generally found in the digestive tract contents of birds, at least in cases of acute poisoning (Mineau and Tucker 2002). The high number of external pesticide detections relative to internal detections overall raises the possibility that hummingbirds are exposed to pesticides through a mechanism other than dietary intake. However, due to a lack of commercial standards for the metabolites, our analytical method only evaluated for parent compounds. If analytical standards for the metabolites were available, we may have detected a greater number of positive samples and/or quantifiable concentrations of target compounds.

One challenge with this study was the external rinsate method for feather samples. After multiple rinses, it was unclear if the rinsate represented surface contamination or insecticides that had been incorporated into the feather during development and extracted during the feather rinsing process. Therefore, with the Southern California

Table 3 Summary of target insecticide prevalence by species, sex, age (hatch year [HY] and after hatch year [AHY]), and region in Anna's (ANHU; n = 42 samples) and Black-chinned (BCHU; n = 42 samples) Hummingbird feather rinsate and homogenized carcass samples using a novel liquid chromatography high-resolution mass spectrometry analytical method for detecting pesticides and other compounds in small mass avian tissue samples (Filigenzi et al. 2019)

Risk factor		Prevalence	Odds ratio	p value	Number
Prevalence of carbaryl					
Species	ANHU	41.9% (27.4–57.8%)	Ref.	–	43
	BCHU	14.3% (3.8–37.4%)	0.23 (0.06–0.93)	0.036*	21
Sex	F	32.4% (18.6–49.9%)	Ref.	–	37
	M	36% (18.7–57.4%)	0.85 (0.29–2.54)	0.771	25
Age	HY	35.3% (15.3–61.4%)	Ref.	–	17
	AHY	32.6% (20.0–48.1%)	0.89 (0.27–2.93)	0.841	46
Region	North	71.4% (51.1–86.0%)	Ref.	–	28
	South	0% (0–15.0%)	NA	0.992	28
Prevalence of dinotefuran					
Species	ANHU	4.7% (0.8–17.1%)	Ref.	–	43
	BCHU	4.8% (2.5–25.9%)	1.02 (0.08–12.60)	0.984	20
Sex	F	0% (0–16.6%)	Ref.	–	25
	M	8.1% (2.3–24.8%)	NA	0.996	37
Age	HY	5.9% (0.3–32.3%)	Ref.	–	17
	AHY	4.3% (0.8–16.7%)	0.73 (0.06–9.02)	0.800	46
Region	North	7.1% (1.3–26.6%)	Ref.	–	28
	South	3.6% (0.2–20.9%)	0.48 (0.04–5.93)	0.560	28
Prevalence of imidacloprid					
Species	ANHU	53.5% (37.8–68.5%)	Ref.	–	43
	BCHU	19% (6.3–42.6%)	0.20 (0.06–0.73)	0.012*	21
Sex	F	36% (18.7–57.4%)	Ref.	–	25
	M	48.6% (32.2–65.3%)	1.68 (0.58–4.87)	0.326	37
Age	HY	41.2% (19.4–66.5%)	Ref.	–	17
	AHY	43.5% (29.2–58.8%)	1.10 (0.35–3.47)	0.870	46
Region	North	60.7% (40.7–77.9%)	Ref.	–	28
	South	28.6% (14.0–48.9%)	0.26 (0.08–0.81)	0.018*	28
Prevalence of thiamethoxam					
Species	ANHU	0% (0–10.2%)	Ref.	–	43
	BCHU	4.8% (0.3–26.9%)	NA	0.997	21
Sex	F	0% (0–16.6%)	Ref.	–	25
	M	2.7% (0.1–16.2%)	NA	0.998	37
Age	HY	0% (0–22.9%)	Ref.	–	17
	AHY	2.2% (0.1–13.2%)	NA	0.997	46
Region	North	3.6% (0.2–20.9%)	Ref.	–	28
	South	0% (0–15.0%)	NA	0.998	28
Prevalence of thiacloprid					
Species	ANHU	4.7% (0.8–17.1%)	Ref.	–	43
	BCHU	0% (0–19.2%)	NA	0.996	21
Sex	F	4% (0.2–22.3%)	Ref.	–	25
	M	2.7% (0.1–15.8%)	0.67 (0.04–11.84)	0.778	37
Age	HY	0% (0–22.9%)	Ref.	0.997	17
	AHY	4.3% (0.8–16.0%)	NA	–	46
Region	North	0% (0–15.0%)	Ref.	–	28
	South	7.1% (1.2–25.0%)	NA	0.996	28

Table 3 (continued)

Risk factor		Prevalence	Odds ratio	*p* value	Number
Prevalence of caffeine					
Species	ANHU	4.7% (0.8–17.1%)	Ref.	–	43
	BCHU	0% (0–19.2%)	NA	–	21
Sex	F	4% (0.2–22.3%)	Ref.	–	25
	M	2.7% (0.1–15.8%)	0.67 (0.04–11.84)	0.778	37
Age	HY	5.9% (0.3–30.8%)	Ref.	–	17
	AHY	2.2% (0.1–13.0%)	0.35 (0.02–6.39)	0.474	46
Region	North	0% (0–15.0%)	Ref.	–	28
	South	7.1% (1.2–25.0%)	NA	0.996	28
Prevalence of malathion					
Species	ANHU	0% (0–10.2%)	Ref.	–	43
	BCHU	4.8% (0.2–25.9%)	NA	0.997	21
Sex	F	0% (0–16.6%)	Ref.	–	25
	M	2.7% (0.1–15.8%)	NA	0.998	37
Age	HY	0% (0–22.9%)	Ref.	–	17
	AHY	2.2% (0.1–13.0%)	NA	0.997	46
Region	North	3.6% (0.2–20.2%)	Ref.	–	28
	South	0% (0–15.0%)	NA	0.997	28
Prevalence of acetamiprid					
Species	ANHU	0% (0–10.2%)	Ref.	–	43
	BCHU	52.4% (30.4–73.6%)	NA	0.994	21
Sex	F	4% (0.2–22.3%)	Ref.	–	25
	M	24.3% (0.2–22.3%)	7.71 (0.87–68.24)	0.061	37
Age	HY	41.2% (19.4–66.5%)	Ref.	–	17
	AHY	8.7% (2.8–21.7%)	0.14 (0.03-0.57)	0.006*	46
Region	North	14.3% (4.7–33.6%)	Ref.	–	28
	South	7.1% (1.2–25.0%)	0.46 (0.07–2.85)	0.397	28

All samples with concentrations above the limit of detection were deemed positive and all samples below the limit of detection were deemed negative for each target compound. Combined results of feather rinsate and homogenized carcass samples were used to determine the overall presence of a target compound for an individual hummingbird. Sulfoxaflor, nitenpyram, and clothianidin were not detected in any samples, and were thus excluded from this summary. Prevalence and odds ratios are reported with accompanying 95% confidence interval in parentheses (when applicable). ANHU, female, hatch-year, and North are used as the referent category for all comparisons. NA = not applicable

*p values that are significant (< 0.05)

ANHU, the feather rinsate method was switched to detergent washing. However, the detergent-based rinsate was incompatible with the organic solvent used for the extraction method. In the future, it would be advantageous to further develop and validate optimal methods for feather rinsates.

Since hummingbirds are a highly mobile group of species with varying migration strategies, we cannot know where or how this exposure is occurring. However, hummingbirds may be exposed to these chemicals through dietary intake of contaminated flower nectar (Bishop et al. 2018) and/or more general exposure through contaminated air or water sources (Giorio et al. 2017). Some pesticides that are not approved by the EPA for use in the USA are approved for legal use in Mexico (US General Accounting Office 1992), thus exposing migratory birds (i.e., BCHU) to a different suite of chemicals than USA resident populations of birds (i.e., some ANHU). Public records of pesticide usage show that malathion is used commercially in the county where the hummingbird positive for malathion was obtained (Contra Costa County 2015). In addition, as these insecticides may degrade over time (Rouchaud et al. 1994) combined with the lack of analytical standards for metabolite compounds, our results likely underestimate total exposure in California hummingbirds.

The qualitative detection of neonicotinoids and other pesticide classes from avian tissue is a necessary first step for improving wildlife toxicology assays for new anthropogenic chemicals, which are often lacking in the rapidly changing world of chemical registration (Mineau and Tucker 2002). Even though only three carcasses had

quantifiable concentrations of insecticide parent compounds (ranging from 3.1 to 3.4 μg/kg), our study establishes a method for future assessment of insecticide exposure in wild birds. For comparison of quantifiable concentrations of insecticides in free-ranging birds, a recent study of wild Eurasian Eagle Owls found one individual positive for imidacloprid at a concentration of 3.28 ng/mL in blood (Taliansky-Chamudis et al. 2017). A recent study of hummingbirds in an agricultural area in Canada found a combined concentration of imidacloprid, thiamethoxam, and clothianidin to be 3.63 ppb in pooled samples of cloacal fluid from many individuals (Bishop et al. 2018). Similar to our findings in hummingbirds, this demonstrates the relatively low concentrations that seem to dominate the results of wild bird exposure studies that are not cases of acute poisoning. Given the growing interest in the effects of pesticides on pollinators, which are declining on a global scale (Potts et al. 2010), toxicology studies on both pollinating and non-pollinating bird species are likely to be of increasing interest.

The analytical method applied here will be key to future work investigating the health and/or conservation impacts of chronic low dose pesticide exposure on wild birds. In addition, our studies provide first reported evidence that hummingbirds found in California are exposed to insecticides even though the location of exposure is unknown. This finding provides a glimpse into a necessary body of work that will examine the role hummingbirds play as a potential sentinel species for environmental contaminants. A similar sample preparation procedure combined with LC-low-resolution MS has been used for the analysis of pesticides in bees (Bargańska et al. 2014; García et al. 2018) so the method applied to the hummingbirds should also be applicable for LC-HRMS analysis of insects and other small bodied species.

Future research should attempt to resolve the observed differences in compound detection between homogenized carcass and feather rinsate samples. Assessing these differences of internal versus external insecticide exposure in wild hummingbirds in more detail will be important in determining the origin of exposure and to mitigate these practices. Future availability of metabolite compound analytical standards could also improve detection accuracy. Ultimately, the goal would be to understand the routes of exposure and establish best practices to protect hummingbird populations.

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