

Noninvasively Collected Fecal Samples as Indicators of Multiple Pesticide Exposure in Wild Birds

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Abstract: Pesticide use poses a potential hazard to wild birds that use agricultural farmland as their foraging habitat. Whereas most current pesticide studies have found residues in liver samples and single active substances, noninvasive sampling methods and data on a wide variety of agrochemicals are needed to determine pesticide exposure of living wild birds for postregistration monitoring. We collected feces during autumn migration of Eurasian skylarks (*Alauda arvensis*), a species that commonly forages in winter cereal crops. Birds were kept in paper bags until we measured their body condition, individually marked and released them. We analyzed the feces dropped in paper bags for the presence of 80 pesticides including rodenticides and degradation products. Nine active substances from fungicides and herbicides commonly used in grain and maize fields were detected individually, or in combination, in 25% of the samples. We found no significant differences in body condition between exposed and unexposed birds, but Eurasian skylarks without pesticide residues had a better body condition score on average than birds with pesticide residues. Pesticide determination in noninvasively collected fecal samples allows a refined risk analysis, which takes pesticides used in the habitats of birds into account. It allows the search for the sources of pesticide contamination, but also enables research into potential deleterious effects on the fitness of farmland birds. *Environ Toxicol Chem* 2022;41:201–207. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Monitoring of pesticide residue levels has become an important tool to provide exposure data of wild animals and to allow an assessment of potential hazards in the postregistration period (see Badry et al., 2021; de Souza et al., 2020; Plaza et al., 2019). Such monitoring can help in deciding which substance has the least deleterious effect, and whether temporal and/or spatial restrictions for the use of pesticides are required. A prominent example is the anticoagulant rodenticides because they are commonly used worldwide for

commensal rodent management. Their residues have been found in many countries, and in a variety of nontarget species including birds (e.g., barn owl: Germany [Geduhn et al., 2016]; avian predators: United States [Murray, 2017], Australia [Lohr, 2018], Finland [Koivisto et al., 2018]; farmland birds: Germany [Walther et al., 2021]). The potential consequences of exposure for wildlife have been documented (Murphy, 2018; Rattner & Harvey, 2021), and there are restrictions on their use, such as only used in and around buildings (Buckle & Prescott, 2018).

Given that exposure of wildlife to active substances from a wide variety of pesticides is expected (see Tassin de Montaigu & Goulson, 2020), analytic methods for multiple-residue screening have recently been developed (Rial-Berriel et al., 2020; Badry et al., 2021). One such screening, for 30 pesticides, found residues of insecticides in addition to anticoagulant rodenticides in avian raptors (Badry et al., 2021). As usual, liver samples of carcasses and recently deceased individuals were examined (Valverde et al., 2021). A noninvasive method is needed to

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determine current pesticide exposure of living wild birds for postregistration monitoring.

Noninvasive monitoring is possible by sampling of serum (Murray, 2020), blood (Rial-Berriel et al., 2020; Valverde et al., 2021), and feces (Mateo et al., 2016). In particular, collecting birds' feces is relatively easy and can be done within currently running ringing programs by voluntary bird ringers. The potential of feces as an indicator has been shown for metals (Dauwe et al., 2004; Eeva et al., 2020) and fungicides (Mateo et al., 2016) and can reflect the pollution load in the environment and food items for bird species such as great tits (*Parus major*; Dauwe et al., 2004) and red-breasted goose (*Branta ruficollis*; Mateo et al., 2016). Gross et al. (2020) found up to 4.74% of orally single-dosed administered fungicides in fecal droppings of Japanese quail (*Coturnix japonica*) by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

To determine the potential of feces as an indicator for multiple pesticide exposure, we collected feces from Eurasian skylarks (*Alauda arvensis*) trapped for a bird-ringing program during autumn migration. Eurasian skylarks are known to be one of the focal species for assessing risks to birds from pesticides applied during drilling of winter cereals (Bonneris et al., 2019). We examined the samples for residues of approximately 80 different active substances of plant protection products, biocides, and metabolites to demonstrate the recent exposure of wild birds to current pesticide use. Poorer physical conditions of birds in intensively managed agricultural habitats have been reported for birds (Rioux Paquette et al., 2014) and have been associated with pesticide-induced food shortages (Boatman et al., 2004; Morris et al., 2005). Therefore, we further analyzed potential pesticide effects on birds' body condition, which was regularly monitored as part of the ringing program (e.g., body mass, fat score).

MATERIALS AND METHODS

Ethical note

All required permits for the capture and ringing of birds were issued by the Ministerium für Umwelt, Landwirtschaft, Natur- und Verbraucherschutz of North Rhine–Westphalia (Germany) and the Heligoland bird ringing center (Germany). No animals were harmed during the present study.

Sampling

From October 5 to 7, 2019, we captured Eurasian skylarks during their active migration in Münster, North Rhine–Westphalia as part of the International Skylark Ringing Project. The captured birds possibly originated from breeding populations in Northern Germany or Northern Europe, and most likely migrated to Southern France during the non-breeding season (Bairlein et al., 2014). Three parallel rows of mist nets (each row consisting of two nets, each 18 m long) were placed on a harvested maize field, with approximately 3 m

between the rows of nets. One loudspeaker playing calls and songs of the Eurasian skylark was placed in the center of the mist nets to attract nocturnal migrants. Mist nets were open from sunset to 1 h after sunrise and were checked for birds every hour. All captured birds were put into paper bags (one bird/bag) before we ringed them. We measured wing length (mm) and body mass (g) and calculated relative body weight (in g/mm) by dividing individual body mass by wing length. We also estimated visible fat deposits ("fat score") and the condition of the flight muscle ("muscle score"; Eck et al., 2012). All birds were released within 1 h. Paper bags containing feces were stored at -20°C and at -80°C before analysis (Supporting Information, Table S1).

Chemical analysis of active ingredient residues

The paper bags were thawed before analysis. The solid components of the fecal samples that had adhered slightly to the paper were poured into 50-ml polypropylene Falcon tubes. Liquid components of the excreta had been absorbed by the paper bag during excretion. The soiled parts of the bags were cut out (in a circle of average size 5 cm) and also placed in the Falcon tubes (Supporting Information, Table S1). The determination of a sample weight was not feasible, because fecal droppings and paper could not be separated from each other. The selection of the analyzed active substances of the pesticides was based on a proposal for representative monitoring within the framework of the German Implementation of the National Action Plan for the Sustainable Use of Pesticides (Umweltbundesamt, 2019). At the beginning of the sample processing (Supporting Information, Table S2), each sample was spiked with the surrogates acenocoumarol, atrazin- d_5 , diphacinone- d_4 , and pirimicarb- d_6 for quality assurance. The mixture of fecal sample and paper was homogenized in methanol and water (2:1, v/v) with an Ultra-Turrax T25 device (IKA). Cleanup of the sample extract was carried out by solid-phase supported liquid–liquid extraction (ChemElut; Agilent) after the addition of a sodium chloride solution. The resulting dichloromethane extract was evaporated to dryness. The residue was reconstituted with an internal standard solution and filtered into an autosampler vial for analysis. The fecal sample was then analyzed using LC (Agilent 1290 Infinity II) coupled with MS/MS (QTRAP 6500+ [SCIEX]). Five different methods were employed, using two different reversed phase columns in positive and negative electrospray ionization modes (Supporting Information, Table S3). Identification of analytes was achieved with precursor/production transitions (Supporting Information, Table S4). Substance identity was confirmed with enhanced product ion spectra, using the QTRAP 6500+ linear ion trap mode with dynamic fill time. A substance was considered as accurately identified when its enhanced product ion spectra in the sample (with intensity greater than 500 cps) matched more than 80% of those in the reference spectra in the same sequence. Quantification of the substances was carried out using the internal standards. The calibration curves were linear with $r^2 > 0.99$. The reporting limit refers to the

lowest calibration level with a signal-to-noise ratio greater than 6:1 and a relative standard deviation less than 20% in the sequence. The analytical method was verified with a recovery test. Paper circles of 5 cm were cut out from unused paper bags of the same type and spiked before sample preparation with a mixture of 2.5 ng/analyte and surrogate. The results for the recovery of analytes and surrogates in the quality control samples, and for the quality assurance of all the fecal sample procedures, are shown in the Supporting Information, Table S5. No interference was observed in the blank samples. The concentrations of the analytes and surrogates were calculated using the Peak Area function in Analyst 1.7.1. We give residue concentrations as content of active ingredients/sample (ng/sample).

Statistical analysis

All statistics were carried out using R Ver 3.6.1 (R Core Team, 2011). Birds were divided into two groups—those with and those without evidence of pesticide residues in their fecal samples. We tested for differences in the distribution of sexes between groups using a chi-square test. We built a linear model with relative weight (body mass/wing length) as the dependent variable and generalized linear models with fat score and muscle score as dependent variables, to test for differences in body condition between groups. Pesticide (yes/no) was implemented as the only explanatory variable in all models. We used the analysis of variance (ANOVA) function to compare the models with a null model, and to calculate a *p* value for the explanatory variable in all models. We modeled the probabilities of a sample containing pesticide residue (yes/no) as a function of relative weight, fat, and muscle score using logistic regressions using the R package *popbio* (Stubben et al., 2020).

RESULTS

Residues of nine active pesticide ingredients were detected in 12 (25%) of the 48 feces samples of Eurasian skylarks (Supporting Information, Table S6). We found pesticides in 4 out of 12 males (33%), and 8 out of 33 females (24%). There was no evidence for significant differences between sexes ($\chi^2 = 0.05$, $p = 0.81$). No pesticides were found in individuals of undetermined sex ($n = 3$).

We found diflufenican in six samples, with contents between 0.3 and 6.3 ng/sample (Supporting Information, Table S6). Cyprodinil and epoxiconazole were found in three individuals, with contents between 2.8 and 6.1 ng/sample and 0.4 and 1.7 ng/sample, respectively. Flufenacet, metribuzin, pro-sulfocarb, S-metolachlor, terbuthylazine-2-hydroxy, and difenoc-nazole were found in one or two individuals with contents between 0.1 and 1.8 ng/sample. Mostly single, but up to four, pesticides were found in one individual. Six individuals had residues of one, three individuals had residues of two, two individuals had residues of three, and one individual had residues of four active pesticide substances in their fecal samples

(Figure 1). We found no significant differences in body condition between exposed and unexposed Eurasian skylarks (relative weight: mean 0.32 vs. 0.33 g/mm, $F = 0.41$, $df = 46$, $p = 0.525$; fat score: mean 2.75 vs. 2.97, $df = 46$, $p = 0.696$; muscle score: mean 1.50 vs. 1.86, $df = 46$, $p = 0.416$), although mean values for all three parameters were higher in birds without pesticide residues.

DISCUSSION

Pesticide occurrence

Overall, we found residues of eight active substances from herbicides and fungicides and one herbicide metabolite individually, or in combination, in 25% of the 48 Eurasian skylark fecal samples. We can therefore confirm the suitability of this species as a bioindicator for pesticide exposure, as suggested by Bonneris et al. (2019). The highest concentrations of residues were found for diflufenican and cyprodinil, at 6.3 and 6.1 ng/sample, respectively. Cyprodinil is considered to be nontoxic to mallard ducks (safety data sheet from Syngenta, 2014). The same applies for diflufenican for bobwhite quails (safety data sheet from Adama Agricultural Solutions, 2014), as well as the other active substances we found (Supporting Information, Table S7).

Our results show the contact foraging behavior of the migrating skylarks in autumn and the use of pesticides. To interpret the concentrations in the feces samples, it would be crucial to investigate the links among pesticide specific uptake, bio-accumulation, metabolism, and excretion. Future studies should measure the mass of the collected feces to calculate concentrations of residues for interpretations.

Source of pesticides

All the active substances we found are used for protection of grain and maize crops in the European Union. Substance combinations found in single individuals might allow for a determination of which specific plant protection products have been used. For example, the combinations of diflufenican and pro-sulfocarb, or diflufenican and flufenacet, found in individual T115681 (Figure 1 and Supporting Information, Table S3), are contained in several spray herbicides used during autumn on winter crops (Supporting Information, Table S7). Sprayed winter crops are the most likely origin for most of the herbicide and fungicide residues, because Eurasian skylarks are known to use autumn-sown cereals as habitat during autumn, winter, and spring (Bonneris et al., 2019; Donald et al., 2001; Hiron et al., 2012). Maize stubble is also commonly used as habitat by Eurasian skylarks during autumn and winter (Geiger et al., 2014), which might explain the presence of residues of two spray herbicides used on maize as well as lupin and millet fields (S-metolachlor, terbuthylazine-2-hydroxy) in three of our sampled individuals. Our results demonstrate that Eurasian skylarks face exposure risks from herbicides during autumn migration.

Some combinations can only be traced back to more than one registered product (Supporting Information, Table S7),

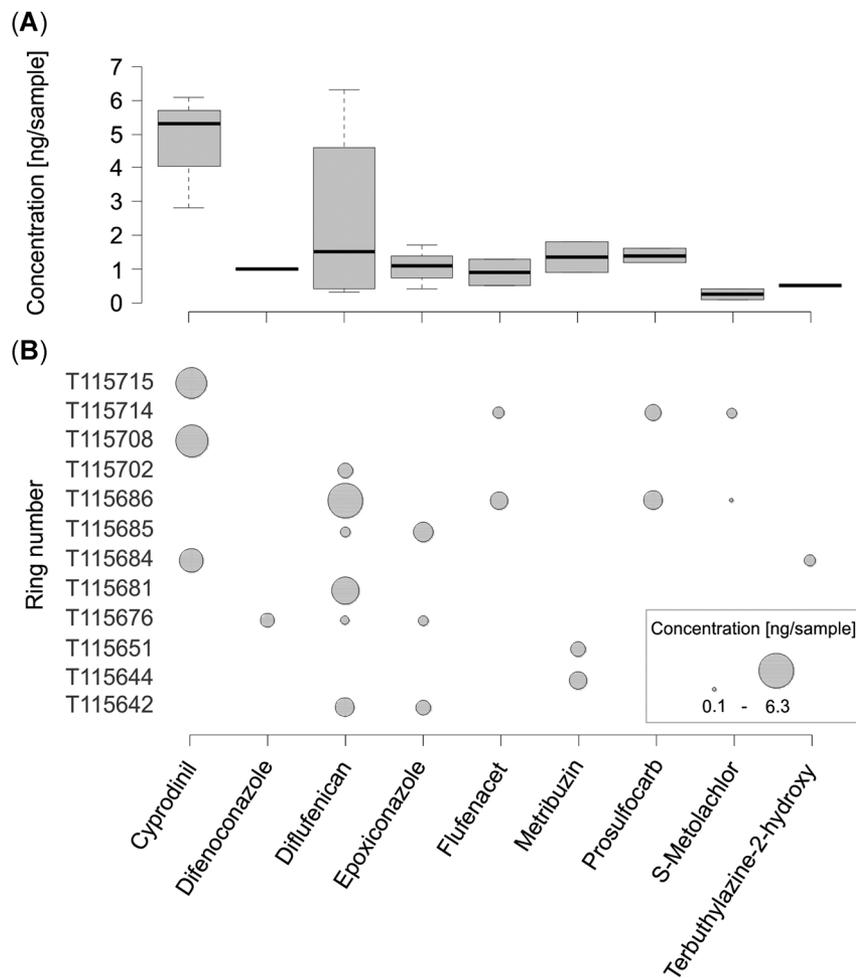


FIGURE 1: Concentrations of active ingredients found in the fecal samples (A) of each of the 12 exposed Eurasian skylarks (B). Individuals are marked by ring number.

which means that those individuals must have had contact with multiple pesticide products. For example, we found residues of the fungicide difenoconazole and the herbicide diflufenican in one individual (T115702; Figure 1). The fungicide epoxiconazole, found in three individuals, is known to present a high long-term risk to herbivorous birds in terms of its use on cereals (European Food Safety Authority, 2015). European Union approval for epoxiconazole as an active ingredient in plant protection products according to Implementing Regulation (EU) No. 2019/168 ended on April 30, 2020 (German Federal Office of Consumer Protection and Food Safety, 2020). In Germany, the authorizations for disposal, storage, and use ended on October 31, 2021.

Finding sources of pesticide residues is a complex undertaking because many factors influence if and when a pesticide comes into contact with birds (Tassin de Montaigne & Goulson, 2020). Gross et al. (2020) analyzed fecal samples in the 24-h period following dosing and found residues of the parent active substance. In less than 24 h, Eurasian skylarks can fly more than 1200 km (Scebba et al., 2017), defining the potential distance to sources. Tracking by GPS, radiotelemetry,

and observations of ringed individuals could help to determine sources precisely in the future.

Potential of fecal sampling

Following application, pesticides may be absorbed by consumption. Analysis of fecal samples allows for an estimation of exposure risk. Mateo et al. (2016) found that geese were feeding on wheat seeds that had been treated with the four fungicides thiram, tebuconazole, difenoconazole, and fludioxonil and reported the presence of thiram and tebuconazole in fecal samples of the geese.

The active ingredients we detected are formulated together with other active ingredients in approved crop protection products. Most of the combination partners were part of the analytical mix. However, we do not claim to be able to detect all associated active ingredients because they are present in the products in different amounts and have different intrinsic properties that are expressed by different toxicokinetics. After all, not all relevant substances can be detected with sufficient sensitivity with just one analytical method. Similar observations were made in the

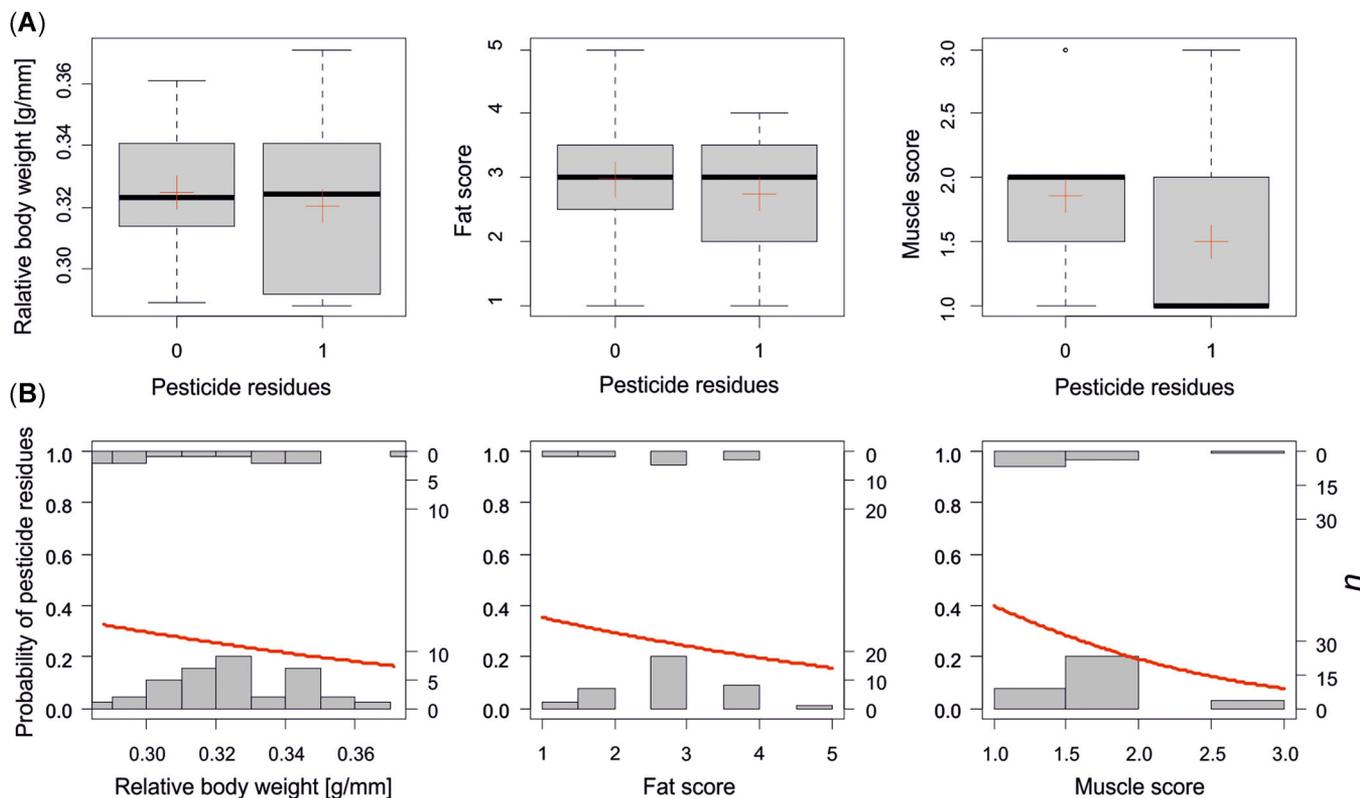


FIGURE 2: (A) Relative weight, fat, and muscle scores of exposed (pesticide residues=1) and unexposed (pesticide residues=0) individual Eurasian skylarks. Red crosses indicate mean values. (B) Logistic regression models (red line) showing a declining probability of a sample containing pesticide residues with increasing relative weight, fat, and muscle scores. Sample sizes (n) are shown as gray histograms.

analysis of skin swab samples of amphibians: although terbutylazine was detected, none of the other active substances (e.g., flufenacet) that were part of the approved terbutylazine-containing herbicides could be found (Schenke et al., 2021).

Potential effects of pesticides

Over the past few decades, research has found a range of lethal and sublethal effects on birds caused by pesticides (Tassin de Montaigu & Goulson, 2020). Reduced survival and fertility, poisoning, abnormalities, and reduced mobility and navigation ability were found (Tassin de Montaigu & Goulson, 2020). We found no significant differences in relative weight, fat, or muscle score between exposed and unexposed Eurasian skylarks, but we did observe a possible decrease in the probability of a sample containing pesticide residues in individuals with better body condition (Figure 2). Poorer physical condition of other bird species has been reported in intensively managed agricultural habitats (Rioux Paquette et al., 2014), and could potentially be linked to pesticide exposure. Indirect effects of pesticides on birds are well known, for example, through a reduction in food abundance (Boatman et al., 2004; Morris et al., 2005). Herbicides can affect seed production (Heard et al., 2003) and potentially reduce the availability of seeds for granivorous bird species (Marschall et al., 2003). A large-scale monitoring of fecal samples is needed to validate whether pesticide exposure leads to poorer

physical condition. Fecal sampling and recording of physical condition in connection with ringing programs could be part of postregistration monitoring, to identify unexpected direct and indirect impacts on organisms by accounting for multiple propagation routes and exposures (Vijver et al., 2017). One of the greatest challenges will be the identification of the effects of pesticide mixtures (Panizzi et al., 2016).

CONCLUSIONS

We show that LC-MS/MS is an efficient method to detect a wide range of pesticide residues in fecal samples collected from Eurasian skylarks. Our method therefore enhances the ecotoxicological toolbox by providing a noninvasive method to monitor pesticide contamination of wildlife. It allows investigation of high numbers of samples with consideration of animal welfare. This is especially relevant for rare and protected species, for which tissue samples cannot be collected invasively in sufficient sample sizes. In collaboration with citizen scientists, such as bird ringers, a large-scale sampling scheme to monitor pesticide residues and potential effects on survival and fitness parameters could be established.

Supporting Information—The Supporting information are available on the Wiley Online Library at <https://doi.org/10.1002/etc.5260>.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (alexandra.esther@julius-kuehn.de).

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