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Demographic Determinants of Residue Profiles of Fungicidal Compounds in Common Voles (*Microtus arvalis*) under Semi-Natural and Natural Conditions

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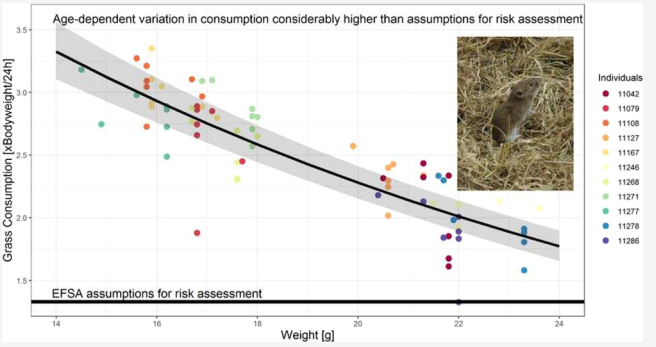
ABSTRACT: Environmental risks from plant protection products (PPPs) need to be assessed to ensure safe use. The risk assessments are generally carried out using the common vole as a focal species with conservative theoretical estimates of external exposure. These are then compared to dose-related toxicity endpoints established in toxicity studies, often with laboratory species. The aim of the present study was to determine the actual internal dosimetry of PPPs' active ingredients (AIs) in a population of common voles to provide the basis for informed higher tier risk assessment. As a proof of concept, two fungicidal AIs (fludioxonil and cyprodinil) were investigated using a range of application methodologies. Individuals were treated using oral gavage application (AI dose: 100/200 mg/kg) and fed treated grass (AI sprayed at 2 kg/ha) under laboratory, semi-natural, and natural conditions. Our results show that demographic factors play a significant role in the individual residue profile and that age structure is a key aspect that determines the overall exposure risk of a population. These results are consistent from laboratory to field conditions. Future approaches could establish dose−residue relationships that are reflective of natural food intake rates in wild common vole populations in the risk assessment of PPPs.



KEYWORDS: *generic focal species, registration, risk assessment, toxicokinetics*

1. INTRODUCTION

The potential environmental impact of compounds and products used in plant protection is assessed to make decisions on their safe use for the intended purpose and to regulate their use to ensure that the health and safety of humans, animals, and the environment is maintained. The risk assessments are mainly based on data that are derived from standardized test procedures outlined by organizations such as the FAO[1] and regulatory authorities like the EU.[2] However, for plant protection products (PPPs), risk assessments for terrestrial vertebrate wildlife are generally based on simple equations that estimate the total amount of a compound potentially ingested per day for a given model species. Acute dietary and reproductive risk assessments are carried out to calculate toxicological endpoints that are compared with the assumed external exposure (or dose) to be expected in the field when products are applied following good agricultural practice.

This approach simplifies decision-making but ignores complex species-specific traits like feeding behavior (frequency and size of meals) and toxicokinetic (TK) factors such as absorption and clearance, which affect the bioavailability and internal dosimetry of compounds. The TK of the compound determines the extent of actual internal exposure experienced by wildlife in nature as a key determinant of the safety margin maintained through the use of PPP and could provide a quantitative based refinement of the risk assessment. Purpose-bred, pathogen-free laboratory strains of rats and mice used to define no observed adverse effect levels (NOAELs) are unlikely to reflect the variability in dose response exhibited by genetically or behaviorally otherwise diverse wild populations of small mammals. For an approach utilizing TK, it is critical to estimate variability in at least the respective focal species to gain realistic insight into potential internal exposures experienced in a real wildlife scenario.

Studies of efficacy and environmental risk are based on scientifically recognized test procedures (e.g., OECD, EC, and ISO), and resulting data are often applied to parameterize risk assessment models. This requires realistic settings for obtaining information about exposure, residue profiles, persistence, and effects of compounds. However, most studies of the

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toxicokinetics of compounds are based on findings in highly unnatural settings in the laboratory and on laboratory-reared surrogate species. This is of concern because there may be a higher variability among individuals in the wild versus in the laboratory and species tested in the laboratory may react differently to species relevant for field conditions.[3,4]

Routinely, generic model species of terrestrial vertebrates, which are of particular exposure risk, are used for assessing risk from PPP in Europe.[5] Such a species is the common vole (*Microtus arvalis*) because this rodent species has a high food intake relative to its body weight and should be especially prone to risk.[6] Common voles are the most abundant mammal species in Europe[7] and inhabit Eurasia from Northern Spain to the Middle East and Central Russia.[8,9] Their multi-annual population dynamics are characterized by outbreaks every 2−5 years[10] when considerable damage can be caused to crops,[11] but they also provide a number of vital ecosystem services.[6]

As a highly generalist species, the common vole is adapted to a wide range of habitats within its distributional range. It is highly socially flexible depending on population dynamics and environmental conditions[12] and very resilient to human disturbances.[13] Consequently, common voles exhibit marked genetic diversity, even at small scales.[14] In addition, the common vole is host to a variety of parasites and pathogens[15−17] that can affect host fitness. These characteristics of wild populations might culminate in a variable dose response when confronted with active ingredients (AIs) in a natural setting that might not be observed in laboratory trials. It is therefore important to include knowledge of dose response in wild population in the process of risk assessment.

Enclosure studies are used in basic and applied scientific studies to strike a reasonable balance between controlling experimental conditions and providing a natural setting. This is particularly the case for studying small mammals such as voles,[18] rats,[19] and mice.[20] However, surprisingly, few studies have used this approach for the determination of residue profiles in small mammals. In the context of risk assessment, field studies are rare as they inevitably incorporate multiple uncertainties like an open population with individual dispersal or the potential presence of predators. These factors can contribute to inter-individual variability in dose response, but they are nevertheless indicative of wild populations. Only a few published studies have considered the widely distributed and abundant common vole for screening for pesticide residues. As a result, only little data are available in the context of risk assessment. Some of the exceptions are studies of rodenticide residues,[21] heavy metal residues,[22] and the potential use of the species as a sentinel for a multitude of environmental pollutants.[23]

The aim of this study was to establish residue profiles of two fungicidal compounds under varying scenarios, ranging from laboratory exposure to semi-natural enclosures as well as field studies with the generic model species of terrestrial vertebrates used in risk assessment (common vole). We measured dissipation rates of both compounds in a variety of conditions and tested for consistency in the effect of individual covariates on the blood residues. If data are consistent and meaningful, the use of semi-natural and natural conditions for risk assessment is warranted and data can be used to parameterize TK models in the future.

2. MATERIALS AND METHODS

All trials were conducted at the, or close to the, premises of the Julius Kühn-Institute (JKI), Federal Research Centre for Cultivated Plants, Münster, Germany (51°58′29″N, 7°33′58″E), and procedures involving animals were conducted according to relevant legislation and by permission of the authorities of the German Federal State of North Rhine-Westphalia (permit number 84-02.04.2014.A273). Trials were conducted between 2015 and 2016 but never in parallel, so there would be no interaction between them.

2.1. Animals, Husbandry, and Enclosures. For laboratory trials, the experimentation room was fully climate controlled with a temperature of 22 °C ± 2 °C and an artificial day/night cycle of 12 h. Animals were wild caught in Münster's surroundings or their F_1 offspring bred at the facilities of the JKI. Voles were held individually in standard rodent cages (42 × 26 × 15 cm). Wood wool was provided as bedding, hay for nesting, and there were tap water and standard rodent chow pellets (Altromin 1324, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) available *ad libitum*. Health status was visually assessed daily, and bedding was changed at least once a week. Individuals were marked with individual Passive Integrated Transponders (PIT; Lux-IDent, Lanškroun, Czech Republic). The bodyweight (BW) of voles was measured at the start and at the end of each trial with a spring scale (Pesola Präzisionswaagen AG, Feusisberg, Switzerland) to the nearest gram. The enclosure trial was done in four rectangular enclosures with a base area of around 12 m^2. Metal mesh casing prevented voles from escaping and predators from entering, while concrete walls around the base of the enclosures (up to 1 m above ground) prevented voles from climbing up fences (for further details, see ref 24). A layer of about 40 cm of soil was freshly planted with a grass mix to mimic perennial grassland and to minimize the effects of previous experiments, and no other experiments were conducted in these enclosures during this study that might have influenced vole behavior.

2.2. Blood Sampling and Analysis. In all trials, blood samples were obtained under isoflurane anesthesia by puncture of the lateral tail vein with a blood lancet as described by ref 25 and collected into a plastic 10 μL end-to-end microcapillary tube coated with EDTA. The blood sample was immediately transferred onto a dry blood spot (DBS) card (Whatman, FTA DMPK-B card), which was labeled and left to dry at room temperature for at least 2 h. DBS cards were then stored at room temperature in the dark until analysis. Sample analysis for both AIs is described in Imholt et al.[25] Briefly, the analysis was performed by liquid chromatography with tandem mass spectrometry (LC−MS/MS) following extraction from the DBS cards.

2.3. Oral Gavage Administration. Cyprodinil and fludioxonil were co-formulated in 0.5% (w/v) carboxymethylcellulose in Milli-Q water to obtain target concentrations of each compound. Individual BW was used to accurately determine the volume required for dosing, and the formulation was administered via oral gavage under general isoflurane anesthesia at a dose volume of 10 mL/kg to achieve target doses of 100 and 200 mg/kg.

Blood concentration−time profiles following a single oral dose administration for each dose group were constructed using a composite design (n = 12/dose group) where two groups of 6 voles (each containing 3 males and 3 females) were

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Table 1. Overview of Application Methodologies

application					
conditions	methodology	dose/spray	*N* individuals	trial duration	blood sampling regime
laboratory	oral gavage	100, 200 mg/kg	12 (2 groups, 6 males, 6 females)	48 h	group1: 0.25, 1, 4, 12, 32 [h] group2: 0.5, 2, 8, 24, 48 [h]
laboratory	feeding trial	2 kg/ha	12 (6 males, 6 females)	5 days	1× per day
semi-field	natural feeding	2 kg/ha	16 (4 enclosures, 8 males, 8 females)	7 days	as often as possible (min. every 2 h)
field	natural feeding	2 kg/ha	24 (179 captures)	7 days	as often as possible (min. every 2 h)

bled alternately ensuring that each vole was only bled on the minimum number of occasions to characterize the blood concentration−time profile (Table 1).

2.4. Dietary (Grass) Administration. Vegetation was prepared by spraying one of the aforementioned enclosures with the fungicidal product SWITCH (Syngenta UK Limited, Fulbourn, United Kingdom; Batch Code: SWO0L138) according to label instructions using a backpack pressurized hand sprayer at 5 bar to achieve an application rate of 2 kg/ha. Fresh grass was cut every 12 h from 6 to 8 different spots within the enclosure for the chemical residue analysis and as food for the voles. Spots were marked with a wooden marker stick, and grass was cut only in a 20 cm radius around the marker to ensure that sites were not sampled repeatedly.

To account for dissipation of compounds from the grass and for natural grass weight change over the 12 h, two 100 g "treatment control" samples were taken per 12 h period. A 100 g sample was immediately frozen at the start of each 12 h period, while a second 100 g control sample was subjected to the same experimental conditions as the grass provided to voles, weighed after the 12 h period and frozen. After each 12 h period, residual (uneaten) grass was collected and weighed to the nearest tenth of a gram with a laboratory scale (Sartorius U6100, Sartorius GmbH Göttingen, Germany) to get an accurate assessment of the amount of grass consumed. The relative weight change of the "treatment control" was used to correct the weight of residual grass. The trial was conducted for 4 days, which resulted in a total of 16 grass samples (including a 900 g baseline sample taken before the application of SWITCH).

A total of 12 individuals (6 males, 6 females) were fed grass treated with SWITCH (Table 1). Each vole was provided with 30 g of treated freshly cut grass every 12 h at 07:00 and 19:00 h, which was deemed sufficient to provide for feeding *ad libitum*.[5,26] Blood sampling took place once a day at 07:00, resulting in 4 blood samples per individual.

2.5. Experimental Design Enclosure Study. *2.5.1. Grass Spraying.* The fungicidal product combining the compounds cyprodinil (C; 37.5% w/w) and fludioxonil (F; 25% w/w) (SWITCH; Batch Code: SWO0L138) was sprayed in accordance with label instructions inside the enclosures using a backpack pressurized hand sprayer. A solution of SWITCH was prepared and sprayed at 5 bar pressure to give a nominal application rate of 2 kg/ha (600 L/ha). The mixture was also sprayed in two smaller (5 m^2) adjacent enclosures that were similar in structure to the four experimental enclosures to obtain grass samples for monitoring dissipation rates over the course of the study period without vole grazing.

2.5.2. Vole Trapping and Measuring Vole Activity. When trapping voles for blood sampling, the ERMINEA permanent monitoring system[27] was used for rodent detection based on infrared sensors. The sensor detects both body heat and movement. A signal indicating capture or movement of trap or a sensor was sent to handheld pagers allowing for immediate extraction of the captured vole and subsequent blood sampling.

Sensors were combined with Ugglan multiple-capture live traps (Grahnab, Hillerstorp, Sweden) that are frequently used in studies of small mammals.[28] Ugglan traps (24 cm × 9 cm × 6 cm) are made of galvanized metal mesh with a plastic base. The trap consists of two compartments, namely, an unbaited entrance compartment and a baited capture compartment connected through a trap door. Animals heavier than ∼4 g trigger the trap door and are caught in the capture compartment. The trap was equipped with an aluminum roof, pierced with a hole of ∼1.5 cm diameter to mount the sensor on the roof, positioned over the center of the capture compartment. Each of the seven traps per enclosure was baited with a slice of apple that was placed in a metal tea ball to ensure the baiting effect of the apple while preventing the voles from consuming it.

For monitoring the above-ground activity, assumed to represent foraging behavior, seven additional trap lids with mounted sensors were placed into each enclosure. Voles passing under those lids were registered by the internal storage system of the ERMINA permanent monitoring system for rodent detection. Traps and lids were placed near centers of activity. Traps were set from 7 am until 7 pm each day. After 7 pm, the trap doors were opened, while the respective sensors remained active during the night to continue recording activity without capturing rodents. In the morning, the traps were baited and set again.

2.6. Experimental Design Field Study. *2.6.1. Field Location.* The field site was located in the North-East of Münster (51°59′20″N, 7°31′55″E) in close vicinity to the grounds of the Julius Kühn-Institut. The grassland was used to harvest forage for cattle of a nearby farm. The site was chosen as it showed adequate vole activity that indicated adequate numbers of individuals for the field study. To estimate vole activity, all burrow entrances were closed, and 24 h later, the number of reopened burrows was counted. Of a total of 348 closed burrows, 144 burrows were reopened, which indicated suitable conditions for the trial.

2.6.2. Study Design. A 2.25 ha grid (150 × 150 m) was established on the field. The grid consisted of a 120 × 120 m trapping grid and a 30 m similarly sprayed buffer strip where no traps were set. This buffer was established to ensure that voles caught at the edge of the core grid most likely fed on treated grass, even if they foraged within the buffer strip. As burrows were not evenly distributed in the field, because voles rather live in kinship groups (so-called nests), the grid was established at a site that maximized trapping success and incorporated several activity clusters. At each of these clusters, live traps were set near burrow entrances and on above-ground runways. In total, 110 traps were set simultaneously where activity was observed.

Similar to the enclosure trial, the ERMINEA permanent monitoring system in combination with Ugglan multiple-

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Table 2. Results of Generalized Linear Mixed Models for the Grass Consumption in the Dietary Trial[a]

					random effects			
					time		ID:time	
factor	estimate	std. error	*t*-value	*p*-value	variance	standard deviation	variance	standard deviation
intercept	1.270	0.110	11.920	<0.001	0.005	0.070	0.007	0.086
weight	−0.060	0.000	−15.960	<0.001				
sex [m]	0.000	0.020	0.000	0.998				
daytime [morning]	0.080	0.060	1.440	0.149				
time	0.020	0.010	1.320	0.188				

[a]*Sex* and *Daytime* are categorical variables with *female* as the reference category for *Sex* and *evening* as the reference category for *Daytime*.

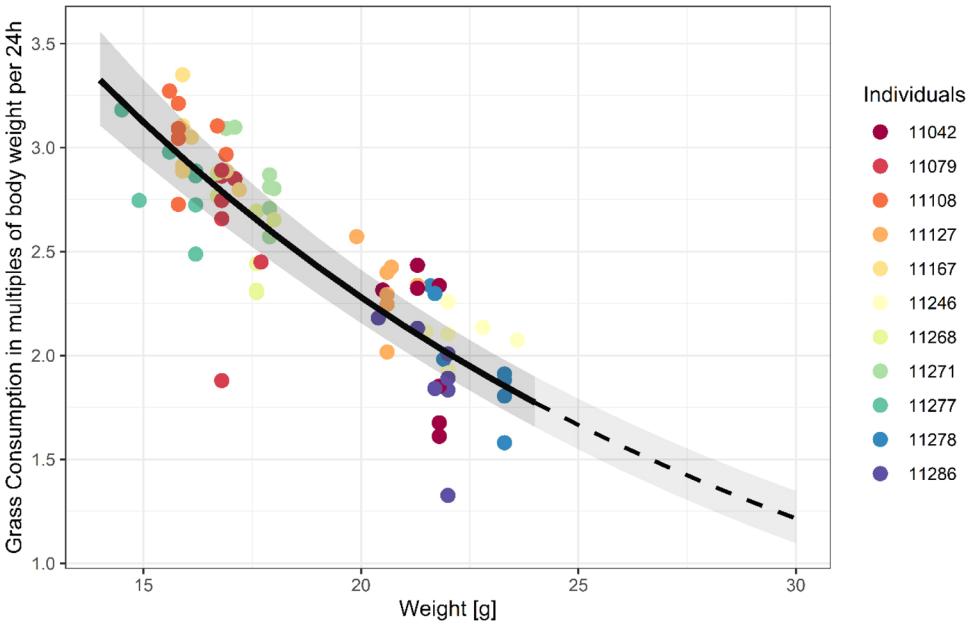

Figure 1. Graphical representation of the effect of individual body weight on grass consumption. The *y*-axis represents the multiples of individual body weight that were consumed per day. Study data means are presented by a solid line. Predicted values, not covered by original data set, for heavier individuals are presented by a dashed line.

capture live traps was used. The trial was performed from 12/09/16 until 18/09/16 and ran from 7 am until 9 pm each day. After 7 pm, the Ugglan traps were opened, allowing free access for voles to enter and leave, while the respective sensors remained active during the night. In the morning, the traps were baited and set again. All animals were marked with an RFID/PIT transponder tag at first capture and weighed once a day when recaptured. Additionally, sex and reproductive status of each individual were determined at first capture.

2.7. Application of SWITCH. SWITCH was applied to the entire 2.25 ha grid including the core grid and the 30 m buffer strip using a trailer boom sprayer with a 30 m beam. SWITCH was applied at a spray volume of 600 L/ha to give an application rate of 2 kg/ha. To determine the dissipation rates of the compounds within the sprayed grid, six different bulk grass samples were taken at days 0, 1, 2, 4, and 7 of the study. Each bulk sample consisted of six 100 g samples, taken each at separate 10 m^2 grids, randomly chosen across the site. A total of 30 grass samples were taken, frozen, and analyzed for residues of cyprodinil and fludioxonil.

2.8. Grass Sample Analysis. The cyprodinil and fludioxonil residues in grass samples of the dietary, enclosure, and field trial were determined using a QuEChERS-Multi method. Samples were extracted using acetonitrile, followed by sample cleanup using dispersive solid-phase extraction. The solvent extract was analyzed by LC−MS/MS using an AB Sciex API 5500 run in both positive and negative ion mode.

2.9. Statistical Analysis. Grass consumption in the dietary trial was analyzed using generalized linear mixed models (GLMMs) with a gamma error structure (log link) where grass consumption per 24 h standardized to the respective BW was the dependent variables, while individual weight and sex as well as sampling time were fixed factors. For blood residues of both AIs of each trial, a separate GLMM was constructed using individual residues as the dependent variable and weight, sex, and sampling time as fixed factors. In all GLMMs, a random factor was incorporated where each individual was nested with the respective sampling time to account for the repeated measures design. Single factor effects were explored using the *ggeffects*-package (estimated marginal means). Frequencies of enclosure activity fluctuations were assessed using spectral densities on first-differenced log-transformed hourly detections.

To identify the dominant above-ground activity pattern in each enclosure, the data set of motion sensors was analyzed using spectral densities. To account for the observed decrease in above-ground activity in the first 2 days, the data set was detrended (first differenced) using the ln-transformed hourly rates of change in activity (as $r = \ln(N_t) - \ln(N_{t-1})$). Frequencies of activity fluctuation were assessed using spectral

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densities on first-differenced log-transformed activities. All analyses were performed using R.[29]

3. RESULTS AND DISCUSSION

3.1. Grass Residues and Consumption. AI residues on sprayed grass for the dietary study fluctuated during the study period, potentially reflecting wet conditions shortly after the spraying, which might affect the adhesion of compounds on the grass (Figure S1). Residues of cyprodinil and fludioxonil on grass fed to voles were highly correlated. The concentration of both compounds markedly decreased after the application for 24 h. Similarly, AI residues in the enclosure and field trial showed peak concentration directly after application, which then steadily decreased over the study period (Figure S2). No precipitation occurred during the field or enclosure trial. In the enclosure trial, grass showed a mean concentration of 103 mg/kg for cyprodinil and 61 mg/kg for fludioxonil. Both decreased over the study period to 14.9 mg/kg for cyprodinil and 23.6 mg/kg for fludioxonil. In the field trial, grass showed mean concentrations of 44 mg/kg for cyprodinil and 27 mg/kg for fludioxonil. Both decreased over the study period to 13 mg/kg for cyprodinil and 17 mg/kg for fludioxonil on day 7.

Individual voles consumed on average 23.3 g per 12 h (range 14.6 to 26.6 g) of fresh grass treated with SWITCH (Figure S3). Mixed modeling revealed no significant impact of time of day or individual sex on consumption, nor did consumption fluctuate during the experimental period (Table 2). However, lighter individuals consumed more grass per BW compared to heavier ones (Figure 1).

Voles consumed generally more fresh grass per gram BW than assumed by EFSA.[5] In our investigation, the EFSA assumption was accurate for a common vole of around 28 g, while it underestimated the consumption of juveniles about 2−3 fold. The amount of grass eaten by the experimental voles was also much higher than those reported for the uptake of 4.4 to 7.8 g of pelleted food per day[26] or of alfalfa (2.5 to 5 g).[30] Certainly, food quality and moisture content will be important drivers of food requirements. The nutritional content of grass is low, and processes related to the wilting of grass in the cages may have required voles to consume more than they normally do when fresh food is available continuously. There may also be consumption of wilted grass in nature when voles store grass in their burrows. However, it is unknown what the proportion of stored grass is at the time of application and whether storage conditions affect uptake. Given the comparable residue profiles in all dietary grass administrations, it is unlikely that, in our experimental conditions, stored food was consumed in large quantities.

3.2. Rodent Activity. The activity metric used was the assessment of the above-ground activity at the population (enclosure) level using motion sensor trap lids. The spectral analysis yielded a periodogram, which is a graphical representation of the dominant activity frequency. Figure 2 shows the periodogram resulting from the spectral analysis. In all enclosures, the dominant frequency in the above-ground activity was 2.4 h, which means that motion sensor surveillance suggests a peak in potential foraging behavior every 2.4 h. During the field trial, 24 individual common voles were caught between 1 and 17 times, yielding 179 captures when blood was sampled.

In a natural environment, foraging activity can be influenced by the type and height of vegetation[31] or the presence of predators.[32] In general, temporal feeding patterns in the common vole are characterized by bouts every 2−3 h.[33] Short-term population-level phase locking can be induced by odors of nearby predators[34] as a means to spread the risk of predation, while feeding phases can also be controlled by the circadian system[35] as well as the seasonal variation in photoperiod.[36] Therefore, laboratory-derived feeding dynamics might not reflect a natural feeding rhythm. However, activity results from the enclosure trial indicate that in enclosures the frequency of activity bouts (every 2.4 h) mimics the feeding pattern under natural conditions.[33]

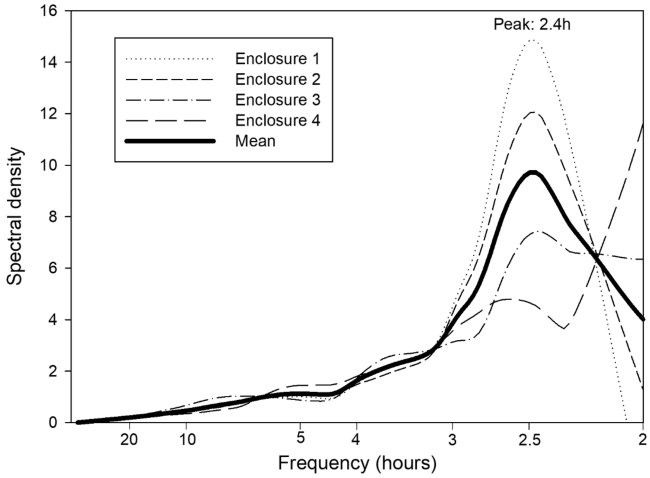

Figure 2. Periodogram of vole activity displaying the frequency (in hours) of above-ground activity in all four enclosures as well as their mean value. Spectral density on the *y*-axis represents the dimensionless signal strength, here above-ground vole activity, in relation to the frequency.

3.3. Blood Residues. Results on the determining factors on individual AI residues are presented in Table 3. In both gavage trials, AI residues significantly decreased with time (single factor effects in Figure 3,BA), though inter-individual variability was high throughout the experimental period. Individual weight did not significantly impact AI residues, while sex did not show a continuous trend throughout gavage doses. When sex was a significant factor, males always tended to have higher blood concentrations.

In all grass feeding trials, except for fludioxonil under laboratory conditions, there was a statistically significant decrease in individual residues over time (Table 3, Figure 3C,D). However, in all grass feeding trials a negative estimate indicates a general trend of declining residues. The confidence intervals were much larger at the start of all grass feeding trials compared to later stages, where they did not deviate substantially from the mean. The influence of individual demographics on AI residues was consistent throughout grass feeding trail. Individual sex was not a significant predictor of residues, while each individual's weight was negatively associated with blood residues for both AIs and all grass feeding trials (Figure 4). The effect of weight seemed to be more pronounced during the laboratory feeding trial compared to the more natural feeding regime in the enclosures and the field, as indicated by the lower estimate (Table 3).

In comparison to the concentrations of cyprodinil and fludioxonil following a dose of 10 mg/kg via iv administration,[25] there were generally lower blood concentrations in the present study. Blood concentrations were higher following single oral gavage administration compared to repeated dietary

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Table 3. Results of Generalized Linear Mixed Modeling for Each Application Methodology and AI[a]

							random effects			
							time		ID:time	
application methodology	AI	factor	estimate	std. error	*t*-value	*p*-value	variance	standard deviation	variance	standard deviation
gavage [100 mg/kg]	Cyp	intercept	5.533	0.750	7.373	<0.001	0.001	0.036	1.252	1.119
		weight	−0.015	0.033	−0.462	0.644				
		sex [m]	0.855	0.332	2.577	0.010				
		time	−0.162	0.021	−7.771	<0.001				
	flu	intercept	4.812	0.724	6.644	<0.001	0.001	0.027	1.167	1.080
		weight	0.026	0.032	0.808	0.419				
		sex [m]	0.514	0.320	1.605	0.109				
		time	−0.188	0.020	−9.357	<0.001				
gavage [200 mg/kg]	cyp	intercept	5.975	0.691	8.642	<0.001	0.008	0.087	1.534	1.238
		weight	0.018	0.035	0.505	0.613				
		sex [m]	0.307	0.377	0.815	0.415				
		time	−0.206	0.023	−8.788	<0.001				
	flu	intercept	6.461	1.147	5.632	<0.001	4.417	2.102	1.385	1.177
		weight	−0.012	0.034	−0.340	0.734				
		sex [m]	0.820	0.361	2.269	0.023				
		time	−0.266	0.098	−2.713	0.007				
dietary (grass)	Cyp	intercept	6.129	0.656	9.346	<0.001	0.328	0.573	0.000	0.000
		weight	−0.114	0.035	−3.268	0.001				
		sex [m]	−0.098	0.182	−0.536	0.592				
		time	−0.015	0.003	−4.557	<0.001				
	flu	intercept	5.462	0.679	8.041	<0.001	0.352	0.593	0.000	0.001
		weight	−0.141	0.036	−3.899	<0.001				
		sex [m]	0.181	0.189	0.957	0.339				
		time	−0.005	0.003	−1.519	0.129				
enclosure (grass)	Cyp	intercept	4.631	0.383	12.080	<0.001	0.001	0.030	0.748	0.865
		weight	−0.036	0.015	−2.428	0.015				
		sex [m]	0.273	0.139	1.964	0.050				
		time	−0.010	0.001	−7.002	<0.001				
	flu	intercept	4.116	0.414	9.939	<0.001	0.000	0.018	0.874	0.935
		weight	−0.046	0.016	−2.863	0.004				
		sex [m]	0.284	0.150	1.890	0.059				
		time	−0.004	0.001	−2.827	0.005				
field (grass)	Cyp	intercept	4.426	0.248	17.853	<0.001	0.004	0.064	0.681	0.825
		weight	−0.031	0.015	−2.000	0.046				
		sex [m]	0.028	0.141	0.196	0.845				
		time	−0.010	0.001	−7.194	<0.001				
	flu	intercept	4.574	0.404	11.310	<0.001	1.871	1.368	0.041	0.202
		weight	−0.051	0.025	−2.047	0.041				
		sex [m]	0.128	0.235	0.546	0.585				
		time	−0.009	0.002	−4.105	<0.001				

[a]Sex was a categorical variable with female as the reference category.

administration of treated grass when sprayed at a rate of 2 kg/ha. This probably reflects the low absolute oral bioavailability of the two compounds determined through semi-natural food intake.

As anticipated, each route of administration (including following iv administration[25]) was characterized by high inter-individual variability in the internal exposure to the two compounds. Contributing factors to the high variability may be genetic diversity, nutritional status, and unknown parasitation or infection in the wild caught/F_1 common voles used in the study. These parameters have not been considered but could also affect the individual food uptake and hence the individual exposure to the residues. This also highlights the difference between the use of standardized laboratory rodent strains and wild animals. The latter is relevant for risk assessment in the plant protection and biocide sector but holds the risk for large variation in experiments even if only seemingly healthy voles of similar regional origin are used.

One assumption made by the current risk assessment is conservative estimates of population-level variability in foraging behavior, food intake, and the resulting variability in dose responses.[6] This study highlights that basic individual demographic factors significantly contribute to the observed inter-individual variability. The laboratory feeding trial showed that lighter individuals had a higher food intake per gram BW of fresh grass compared to heavier individuals. This consequently increased the respective dose of each compound, reflected in the increased residues in lighter individuals. Weight has previously been established as a rough indicator for age in rodents.[37] This translated into higher doses of SWITCH and significantly higher residues of both compounds in younger common voles. Although on higher taxonomic levels energy

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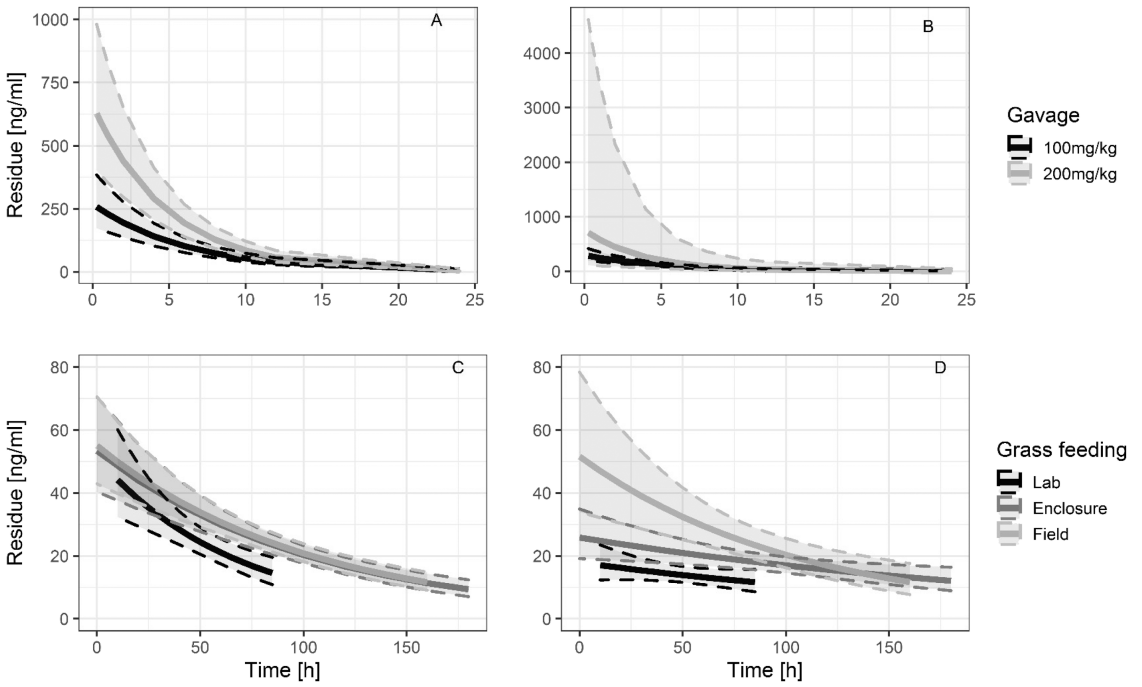

Figure 3. Single factor effects of time on individual AI residues (A,C cyprodinil; B,D fludioxonil) from different application methodologies (A,B oral gavage; C,D grass feeding) as a result of generalized linear mixed modeling. Differences in the temporal scale of model projections reflect different trial lengths.

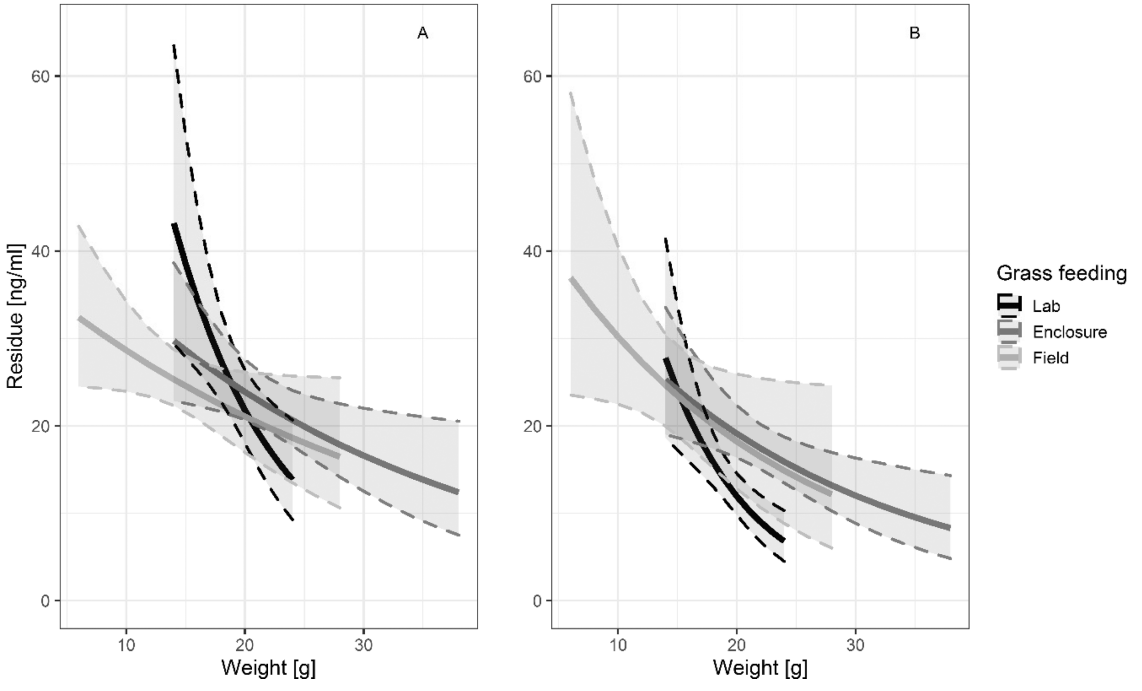

Figure 4. Single factor effects of individual weight on AI residues (A cyprodinil; B fludioxonil) from different application methodologies as a result of generalized linear mixed modeling. Differences in the temporal scale of model projections reflect different weight distributions within the population.

demands clearly increase with body mass,[38] within-species variability is likely related to reproductive activities and behaviorally mediated.[38] Dietary requirements in rodents differ between age groups[39] and young individuals face high nutritional requirements to sustain somatic growth[40] as well as sexual maturation.[41,42] Optimizing both processes is a key factor in determining an individual's own reproductive output.[43] Consequently, malnutrition, especially in juveniles, has been associated with dispersal from natal grounds in order to establish more suitable territories.[44] In older individuals, caloric restrictions benefit longevity and have been demonstrated to increase the reproductive output of older females.[45]

Grasses have limited nutritional value and digestibility, and this might represent a challenge to supply the increased energy

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demands of juveniles/subadults.[46] This might explain why in the present study juvenile/subadult individuals consumed more grass per gram BW compared to older individuals. The extension of this approach into natural feeding pattern in the enclosure and field trial confirmed the observation from the laboratory trial. Here, a similar trend emerged and younger individuals tended to have significantly higher residues compared to older individuals. Age-dependent differences in food intake and subsequently higher doses of the respective PPP have been a constant feature at all experimental levels: standard laboratory feeding trials, semi-natural enclosures, and wild populations. As a consequence of age dependency in PPP residues, it can be assumed that exposure risk varies within population and is inversely associated with age. The age structure of a common vole population is subject to many seasonal and multiannual fluctuations. Seasonal changes have long been established, with wintering populations generally being the oldest.[47] In the spring, new recruitment is dependent on the start of the breeding season gradually replacing over-wintered adults, increasing the number of individuals susceptible to PPP residues. In addition, selective predation is also known to modulate age structure.[48]

In conclusion, many factors can contribute to heterogeneity in dose responses between individuals and within a population, which is a characteristic of wild populations. So far, such ecological realism is often missing from risk assessment procedures.[49] ESFA guidance on risk assessment for birds and mammals is based on estimating the food intake rate (FIR) for a focal species, derived from estimates of daily energy expenditure, assimilation efficiency, food energy, and moisture content of different food items.[5] For a 25 g vole, with a diet of primarily grasses and cereal shoots, the FIR is then calculated as 1.33 times BW. There is an implicit assumption that the proportion of a dose reaching the systemic circulation (bioavailability) for a gavage dose is the same as from the dose consumed via fresh diet in the field. As we have shown, this can be a very conservative assumption. Standard toxicity tests also go to great lengths to provide controlled conditions to laboratory-reared and inbred mouse or rat strains. Allowing for a more natural feeding regime would incorporate a key element of population-level variability into laboratory trials. Population modeling has long been suggested as a toolbox to introduce incorporate natural variability into the ecological risk assessment.[50] The danger is that, as described above, many factors can have direct and indirect effects on one life history trait (here: feeding pattern) and that modeling all complexities might actually increase uncertainties.[51] The key is to identify relevant factors to be included in future modeling approaches to manage uncertainties in a biological meaningful way.[52] In our case, demographic factors proved to be crucial in predicting residues and future approaches could establish dose−residue relationships that are reflective of natural food intake rates in wild common vole populations in order to develop more physiologically based toxicokinetic models.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c00620.

Weather condition enclosure study, grass residues enclosure and field study, and grass consumption under laboratory condition (PDF)

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