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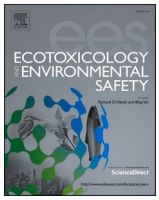

DNA-based seed intake quantification for enhanced ecological risk assessment of small mammals

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ABSTRACT

To prevent the non-acceptable effects of agrochemicals on arable fields, Environmental Risk Assessment (ERA) aims to assess and protect against a wide range of risks due to stressors to non-target species. While exposure to stress is a key factor in ERA models, exposure values are difficult to obtain and rely on laboratory studies with often debatable relevance to field situations. To improve intake estimates, data from realistic field-based scenarios are needed. We developed calibration curves relating known seed numbers of up to 20 onion and carrot seeds consumed by wild-caught wood mice (*Apodemus sylvaticus*) to the seed DNA content in the feces. Based on these inferred quantitative relationships, a field trial was run to determine seed intake in a natural setting using realistic levels of seed spillage. Onion DNA was detected in the fecal samples of the wood mice caught in the field, which resembled a seed intake of up to 1 onion seed. No intake of carrot seeds was detected. This is the first-ever study to quantify seed intake in a realistic field scenario using a DNA-based analysis, showing that accurate seed intake estimates can be obtained. Our approach can help to improve risk assessment models through its minimally-invasive and accurate assessment of seed intake by ERA representative and non-target species, which would otherwise be undetectable with traditional methods. Our novel approach and its results are highly relevant to studies of food intake and diet composition for basic and applied research alike.

1. Introduction

Increased agricultural intensification is considered to be a great threat to terrestrial biodiversity (Stoate et al., 2009). A major part of this threat originates from a loss of appropriate habitats, but has also been linked to the application of agrochemicals (herbicides, insecticides, fungicides, growth enhancers and chemical fertilizer) on arable fields (Tscharntke et al., 2005; Emmerson et al., 2016; Geiger et al., 2010). To prevent non-acceptable effects of agrochemicals, Environmental Risk Assessment (ERA) aims to assess and protect against a wide range of risks to non-target species and ensure a high level of protection of human, animal and environmental health (Storck et al., 2017). Especially when it comes to agrochemical authorization and its usage patterns, a strictly regulated ERA process takes place according to guidance developed by the European Food Safety Authority (EFSA, 2009). An ERA consists of a tiered procedure via a set of toxicity studies, hereby using predicted exposure values to calculate potential risk on multiple endpoints (Storck et al., 2017; Brühl and Zaller, 2019). Such assessments also include safety factors that increase the conservativeness of a potential risk approval (EPRS, 2019). If the risk is deemed acceptable, agrochemicals are allowed on the market and considered safe for a defined period until the next ERA is due for re-registration (Storck et al., 2017; Brühl and Zaller, 2019).

In the models that are used for ERA, exposure is a key factor (EFSA, 2009). However, exposure values are often difficult to obtain and therefore usually based on data and predictions (e.g. extrapolations or

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interpolations) from laboratory studies with often debatable relevance to field situations (Chapmen, 1995; Hart et al., 2003). As an example, estimates on the intake of contaminated food sources by representative focal species in nature are mostly based on invasive and tedious stomach content analyses through visual estimation under a microscope (Hyslop, 1980). Rindorf and Lewy (2004) showed that these traditional methods are prone to bias, with biased intake estimates of up to 150%. Moreover, visually obtained intake estimates may also be hampered by the fact that diet remains are notoriously difficult to identify especially with partially digested material, such as soft plant and animal tissue. Additionally, to our knowledge, very little is known about the consumption of soft plant and animal tissue by small mammals. As a result, these biases may lead to over- or underestimation of exposure which in turn hamper accurate ERA. In addition, ecotoxicologists continue to question the validity and relevance of laboratory studies to field situations, since the artificial conditions in laboratory settings often cannot be simply transferred to field responses (Chapmen, 1995; Vijver et al., 2017; Hilbers et al., 2018). To improve intake estimates, data from realistic field-based scenarios are needed.

DNA-based approaches used in dietary studies (e.g., using feces as a source of DNA of diet constituents) are currently receiving attention (see Creer et al., 2016) and these approaches are now extensively used by ecologists for the qualitative and semi-quantitative assessment of the diet of herbivores (Dell'Agnello et al., 2019; Hibert et al., 2013; Soininen et al., 2015), carnivores (Alberdi et al., 2020; Deagle et al., 2009; Shehzad et al., 2012) and omnivores (De Barba et al., 2014; Robeson et al., 2017). DNA-based diet analysis has the potential to overcome traditional biases in assessing diets and is already able, to a certain extent, to quantify diet constituents in a laboratory setting (see Groen et al., 2022).

Therefore, we aimed to use DNA-based diet analysis to estimate seed intake for wood mice (*Apodemus sylvaticus*) in both laboratory and field settings to create realistic field-based scenarios of exposure to contaminated seeds. Groen et al. (2022) established that seed intake by wood mice can be quantified with DNA markers and showed that sex and age did not affect the detectability of seed intake. However, they only quantified seed intake for a maximum of up to 5 (small) vegetable seeds in the wood mouse diet. Here, we developed calibration curves relating known seed numbers of up to 20 seeds consumed by wild-caught wood mice to the seed DNA content in the feces. On basis of these new findings, a field trial (first-ever) was run to determine small seed intake in a natural setting using realistic levels of seed spillage. This study can help to improve risk assessment models through a less invasive and accurate assessment of seed intake by non-target species including ERA representative focal species. In addition, the results are highly relevant to studies of food intake and diet composition for basic and applied research alike.

2. Material and methods

2.1. Focal species

The wood mouse (*Apodemus sylvaticus*) is used as the representative focal species for the ERA of small omnivorous and granivorous mammals (EFSA, 2009). The wood mouse feeds on (small) seeds and is therefore potentially exposed to seed treatments. Moreover, wood mice inhabit field margins when shelter is available and occur in agricultural fields and can therefore have access to freshly drilled seeds and other parts of plants (Pelz, 1986).

2.2. Live trapping

Wood mice were live-trapped with Ugglan traps in the surroundings of Muenster, Germany (51.97° N, 7.55° E). Traps were set in a forest habitat or along hedgerows and/or tree rows. The trapping regime was identical to previous studies (Chiron et al., 2018; Hein and Jacob, 2019; Groen et al., 2022). In brief, traps were pre-baited with rolled oats for 3 nights before restocking with apple chunks, peanut curls, rodent pellets and rolled oats as bait and wood wool for nesting material. Traps were activated for 3–5 nights and checked about every 12 h. After capture, the individuals were individually marked with a passive integrated transponder (PIT) tag (LUX-IDent, Lanškroun, Czech Republic), sexed and weighed with a spring scale (Pesola, Schindellegi, Switzerland) to the nearest gram. Mice were housed in standard rodent cages with wood shavings, a turned-over clay flowerpot for nesting, standard pellets (Altromin 1324; Altromin, Lage, Germany) as food source and tap water ad libitum at standard holding conditions at reversed day-night cycle. All procedures involving animals were covered by permission of the authorities of the German federal state of North Rhine-Westphalia under permit 84–02.04.2016. A540.

2.3. Pellet preparation and feeding trial

We produced custom-made food pellets (FPs) to deliver known proportions of known diet components to wood mice (see Groen et al., 2022 for details). In short, components of FPs were wheat kernels (non-target seeds), mealworms (invertebrates) and wheat leaves (foliage). These ingredients were selected to represent the main food categories at relevant proportions - seeds 50%, invertebrates 25%, foliage 25% - for wood mice reported in natural habitats (Abt and Bock, 1998). Matrix pellets (MPs) were produced; these were FPs spiked with a given amount of onion (*Allium cepa*) and carrot (*Daucus carota var. sativus*) seeds (mean seed weight for onion 3.8 mg ± 0.3 mg (SD), and for carrot 1.8 mg ± 0.3 mg (SD)) at varying proportions of components of MPs (Table 1). In this study, we focused on onion and carrot seeds because these plant species are frequently grown in European horticulture, with 182,210 ha used for onion and 119,010 ha for carrot production in the EU in 2018 (Eurostat, 2020).

The feeding trial routine was conducted according to the method described in Groen et al. (2022). In short, each feeding trial started with placing randomly selected mice in fresh cages. After a 12-hour fasting phase, 10 individual adult mice (5♂ and 5♀) were fed one MP (t = 0, see Table 1 for the different MPs fed and the replication). All fecal samples 24 h after MP consumption were collected. Droppings per individual were collected in a collection tube, labeled and dried in a drying cabinet (UF 110, Memmert GmbH, Büchenbach, Germany) for 2–4 h at 35 °C to ensure homogenization of the droppings in a bead mill. After the trial, individuals were returned to the cages with wood shavings and a flowerpot. All individuals were weighed before each trial. Captured individuals were re-used in trials after a resting period of at least 3 days. Wood mice, not used anymore for ongoing feeding trials, or after all feeding trials were finished, were released at the place of capture.

Potential differences in digestive patterns between wood mice held in the laboratory and those living outdoors were mitigated by 1) minimizing the period mice spend in the lab before being used in trials and 2)

Table 1
Composition of matrix pellets (MP) and the number of wood mice tested per feeding trial (note that individuals were reused after a break of at least 3 days). MPs main constituents were wheat kernels (as non-target seeds), mealworms (as invertebrates) and wheat leaves (as foliage). O = onion; C = carrot.

MP	Non-target seeds	Invertebrates	Foliage	Target seeds	♂ adult	♀ adult
4.1	50%	25%	25%	0 O + 0 C	5	5
4.2	50%	25%	25%	1 O + 1 C	5	5
4.3	50%	25%	25%	3 O + 3 C	5	5
4.4	50%	25%	25%	5 O + 5 C	5	5
4.5	50%	25%	25%	10 O + 10 C	5	5
4.6	50%	25%	25%	15 O + 15 C	5	5
4.7	50%	25%	25%	20 O + 20 C	5	5

offering diet components in the lab that resemble their natural food, 3) deriving mice from the wild for laboratory trials and 4) using the target species in both settings instead of the classical caged laboratory house mouse. Furthermore, even if the passage time in the wood mice gut was slightly different between lab and field conditions we would assume similar results. As Groen et al. (2022) showed that 95% of DNA already passes the GI tract after 8 h and we covered a sampling period of 24 h in the feeding trial to encompass any variation in the passing of target DNA through the GI tract.

2.4. Field trial

Two field trials were conducted in October 2019 on an agricultural field neighboring a wood strip at the premises of JKI in Muenster, Germany (51.97° N, 7.55° E). Two thousand m^2 of the agricultural field were prepared for sowing by standard agricultural techniques. Forty plots of 50 m^2 (5 ×10 m) were established along a hedge with trees and understory. In the first trial, 7 onion seeds/m^2 and 15 carrot seeds/m^2 (considered as medium density of exposed seeds) were distributed on the surface. In the second trial, 14 onion seeds/m^2 and 30 carrot seeds/m^2 (considered as high density of exposed seeds) were distributed on the surface. Onion and carrot seeds were not sown but distributed on the surface to resemble medium and high densities found in the end rows during field studies under good agricultural practice and are estimates of how much a drilling machine loses at headlands during a U-turn (Roy et al., 2019). The choice of seed density in the field trial was based on the occurrence of seeds on the surface to resemble realistic conditions and not on potential consumption by wood mice. To ensure an equal seed distribution over a plot, an aliquot of seeds corresponding to the required seed density per plot was mixed with sand. The seed-sand mixture was distributed by hand per plot. Directly afterwards, 96 Ugglan multiple capture live traps were set (ca. 2 traps per plot) and equipped with a sensor that immediately indicated a capture of an animal by sending a signal to a pager (Notz et al., 2017). Traps were activated at midnight to allow wood mice (as nocturnal species) time to feed before capture. Caught animals were weighed, sexed and reproductive activity recorded, individually marked with a PIT tag (LUX-I-Dent, Lanškroun, Czech Republic), released at the point of capture and fecal pellets were sampled. Trapping was continued for up to 5 days per trial run. Care was taken to remove all droppings before setting traps again.

2.5. DNA extraction and quantification

Mouse droppings per individual were weighed, and each sample was divided into subsamples to not exceed the maximum starting amount (< 200 mg dry weight) as stated in the protocol of the extraction kit. DNA extraction was performed using the DNeasy Plant Maxi kit (Qiagen) with an introduction of a stool inhibitor removal step (using the INHIBITEX (Qiagen) tablets from the QIAamp DNA Stool kit). For in-depth information regarding the extraction optimization and choice made see Appendix S1. Before sample lysis, the mouse droppings were homogenized in a bead mill using 5 mm stainless steel beads. After DNA extraction, each extract was quantified with four species-specific primer sets. Two sets for each plant species as described by Groen et al. (2022) to accurately account for DNA fragmentation by digestion. This means that for each plant species quantification results are doubled (e.g. one fecal sample results in two measurements for each plant species; one measurement per primer set). DNA quantification was done (*in duplo*) using droplet digital PCR (ddPCR) according to the manufacturer's protocol (ddPCR Supermix for Probes, Bio-Rad). In short, 1 or 5 μL DNA (depending on the MP i.e. depending on whether 1 or 5 target seeds had been used in the feeding trial, to prevent overloading; 5 μL for MPs 4.1–4.3 and 1 μL for MPs 4.4 – 4.7), 11 μL ddPCR supermix for probes (Bio-Rad), 1 μL target primers (10 μM) and 1 μL Taqman probes (5 μM) supplemented to 22 μL total volume with RNAse/DNAse free water were mixed and loaded on to a QX200 droplet generator. After droplet generation, the droplets were transferred to a thermal cycler machine. After PCR, the droplets were read on a QX200 droplet reader (Bio-Rad). Threshold values for determining positive droplets were determined using the Quantasoft software (v1.7, Bio-Rad). Positive droplets of duplicate measurements were merged using the same software to strengthen quantification statistics. The threshold for a positive signal was set based on a positive control sample (*A. cepa* and *D. carota sat.* DNA only). Droplets above the threshold were counted as positive events. No-template controls were used as negative controls for the test samples. Count estimates for each sample were compared to the maximum confidence interval (95%) of the negative controls to determine if DNA concentrations were statistically different from zero. Raw DNA concentrations of droplet digital PCR were given in DNA copies/μL. These were recalculated to total DNA copies in the sample (DNA content) and used for further statistical analyses.

2.6. Data analysis

The total number of DNA copies per sample was normalized for varying body weight (BW; in grams) of mice and varying sample weight (SW; in grams) as this may otherwise affect quantification (e.g. see Stunkard, 1983 for effects of BW on diet metabolism). Distributions of DNA content were non-normal and of a relatively small sample size. Means and confidence intervals (95%) were therefore determined by bootstrapping (R=10000, BCa method) (Carpenter and Bithell, 2000) using the *rcompanion* package (Mangiafico, 2021). Multiple comparisons between seed numbers (0–20 seeds) and seed density (medium vs. high seed density) were tested using pairwise Wilcoxon tests adjusted with Benjamini-Hochberg correction (Wilcoxon, 1945; Benjamini and Hochberg, 1995). Linear regressions of log-transformed data (log(DNA copies)+ 1 ~ number of seeds fed) were performed to get estimates and a linear equation for both the calibration curve of onion and carrot seeds. We assessed the compliance to model assumptions by means of graphical validation of the normality of the residuals. Due to large variation of the DNA content within seed number groups and the non-normal distribution of the residuals, we log-transformed the data to acquire normality. Subsequently, seed intake estimates of wild-caught wood mice in the field trial were predicted by using the average value of DNA content found in the fecal samples of all mice from the field trial against the linear equation from the calibration curve of onion and carrot seeds using the *chemCal* package (*inverse.predict* function, Massart et al., 1997; Ranke, 2022). All figures were produced using the *ggpubr* package (Kassambara, 2020) and, if needed for appropriate visualization purposes, data were presented log-transformed. All statistics were performed in R 3.6.3 (R Core Team, 2014).

All steps taken in the laboratory study and the data analyses were performed to be able to quantify seed intake in wood mice in a realistic field scenario. Zero copies of target DNA measured in the field might reflect three scenarios: 1) the wood mouse in question did not eat the target seed and therefore the DNA is not present in its feces; 2) the wood mouse did eat the target seed but too long ago for target DNA to be present in feces (due to digestion); 3) an experimental error. In the field, it was impossible to distinguish between the scenarios above. We therefore, argue that these zero values are not of any added value towards an application of this study in a realistic field scenario, where wood mice are caught in the wild. However, the zeros measured are true values and should not be neglected. Therefore, we have run the complete analysis twice, first with the zeros omitted (Results), and second with the zeros included (Appendix S2). The samples that contained zeros copies of target DNA, because mice were indeed fed zero seeds (MP4.1), were used in both analyses. The outcomes of both analyses were compared for any substantial differences between the two approaches (see Results).

3. Results

We quantified and plotted DNA content (DNA copies/BW/SW) in the fecal samples of wood mice fed from 0 up to 20 seeds (Fig. 1, see Table S1 for booted means). Multiple comparisons between DNA content per seed number showed that onion and carrot DNA content differed from zero seeds for each seed number > 0 consumed. There was an increase in DNA content from 0 to 10 seeds but a plateau with no statistically discernible differences for higher seed numbers (up to 20) for onion and carrot DNA ($p > 0.2$). DNA content did not differ between 1 and 3 and 3 and 5 seeds for onion and carrot DNA. Hence, calibration was possible for up to 10 seeds in increments of 5 for both onion and carrot DNA. Therefore, linear regression models were developed, for 0 to 10 seeds 0–10 seeds only, to obtain calibration curves per seed species (Fig. 2). The calibration curve for log-transformed onion DNA content showed a strong positive trend with a regression coefficient of 0.31 onion DNA copies g^{-1} BW g^{-1} SW per seed number (Adj. $R^2 = 0.58$, F (82 df) = 116.4, $p < 0.0001$). The calibration curve for log-transformed carrot DNA content also showed a strong positive trend with a regression coefficient of 0.26 carrot DNA copies g^{-1} BW g^{1} SW per seed number (Adj. $R^2 = 0.57$, F (98 df) = 131.6, $p < 0.0001$).

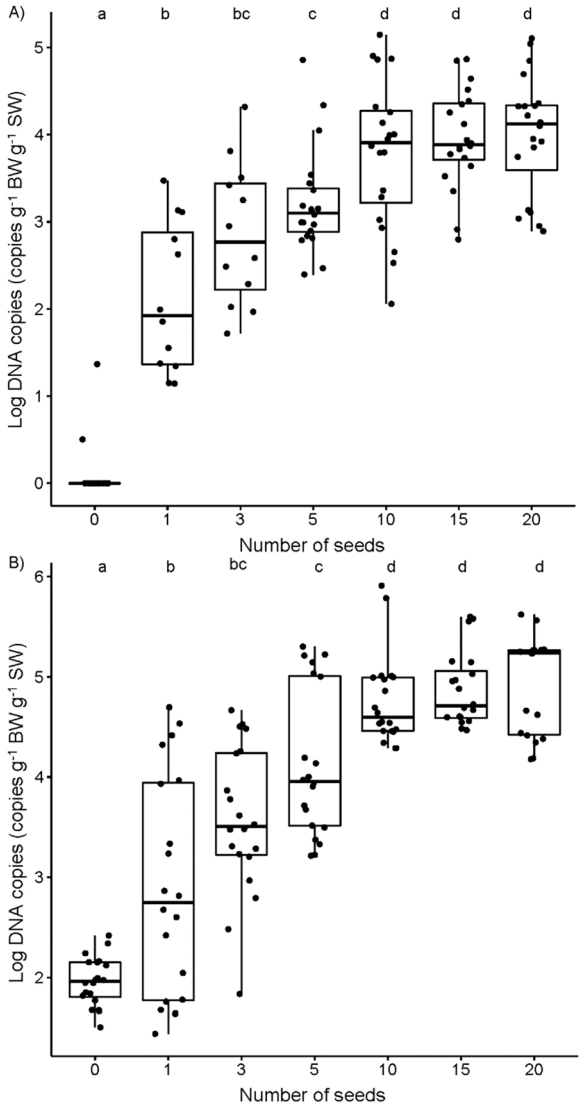

Fig. 1. Log-transformed onion (A) and carrot (B) DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples of wood mice fed different numbers of seeds (x-axis). Different letters a – d indicate a statistically significant difference between seed numbers in multiple comparisons ($p < 0.05$). N = 20 for each seed number except for graph A for 1 seed and 3 seeds N = 12 and for graph B for 20 seeds N = 18. Negative measurements (zeros) were removed from this analysis, except for seed number 0.

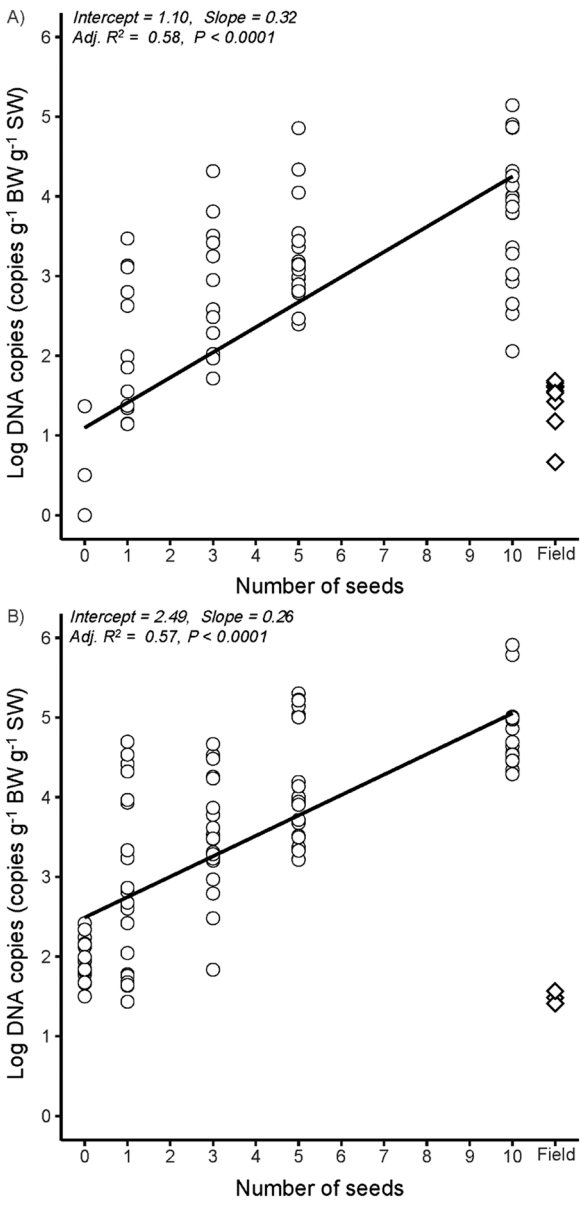

Fig. 2. Linear regression of log-transformed onion (A) and carrot (B) DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples of wood mice and seed numbers fed to wood mice in the laboratory feeding trial (0–10 seeds, circles). Black lines resemble linear trend lines. Statistic test results are given in the graphs for each seed species. Additionally, the DNA content of the fecal samples collected in the field trials (tilted squares) are also given to show the comparison between the field and laboratory feeding trial data. For laboratory feeding trial samples, N = 20 for each seed number for onion and carrot DNA except for graph A for 1 seed and 3 seeds N = 12. For field trial samples, N = 9 for onion DNA and N = 3 for carrot DNA. Negative measurements (zeros) were removed from this analysis, except for seed number 0.

In the field trials, 17 fecal samples were collected (from 11 different wood mouse individuals) and target DNA was quantified. DNA content in the wood mice feces was similar for trials done with medium and high seed density for both onion (W = 3, p = 0.33) and carrot seeds (W = 1, p = 1.00) and both densities were pooled (Fig. 3). Fig. 2 also presents the results of the DNA content found in the field trial samples in comparison to the measured onion and carrot DNA content during the

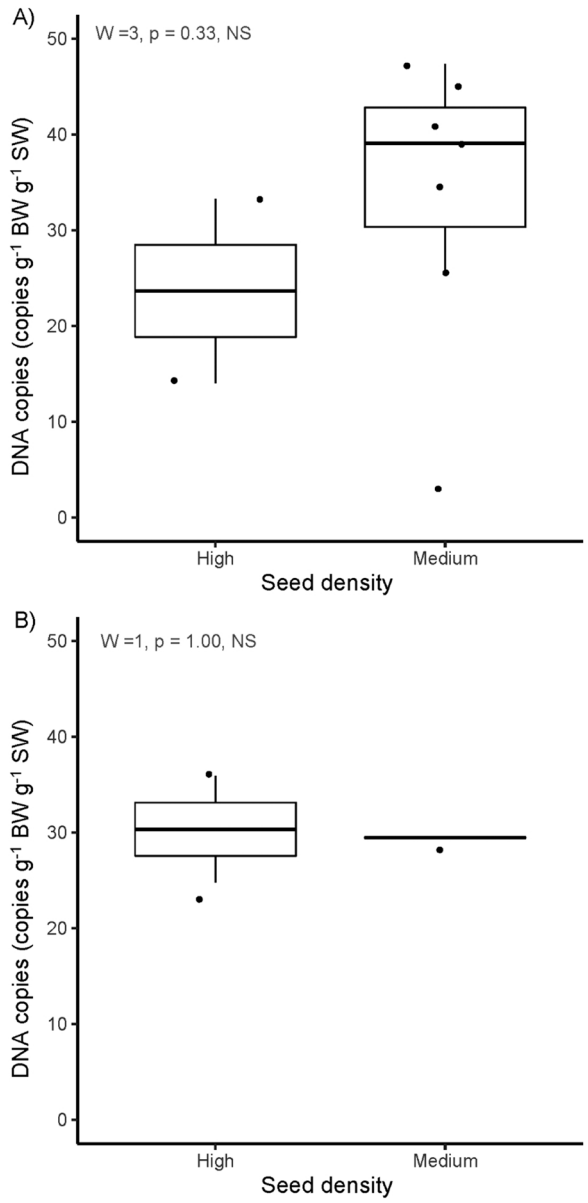

Fig. 3. Onion (A) and carrot (B) DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples collected in the field trial with different seed densities (high and medium seed density) distributed on the field. Statistic test results are given in the graphs for each seed species. No significant (NS) differences were detected. In graph A, N = 2 for high density and N = 7 for medium density measurements. In graph B, N = 2 for high density and N = 1 for medium density measurements. Negative measurements (zeros) were removed from this analysis.

laboratory feeding trials. We found that 9 out of the 17 fecal samples (52.9%) contained onion DNA. Applied to the number of mouse individuals caught, this accounted for 7 out of the 11 individuals (63.6%) that had consumed onion seeds. We found that only 3 out of the 17 fecal samples (17.6%) contained carrot DNA. This accounted for 3 out of the 11 (27.3%) mouse individuals caught that had consumed carrot seeds. The total number of onion DNA copies g^{-1} BW g^{-1} SW quantified in fecal samples of wild-caught wood mice during the field trial was on average 31.5 (CI: 19.5–39.2, N = 9) and resembled the number of copies found when wood mice were fed 1 onion seed during the feeding trial. The total number of carrot DNA copies g^{-1} BW g^{-1} SW quantified in fecal samples of wild-caught wood mice during the field trial was on average 30.1 (CI: 24.8 – 35.9, N = 3) and was even lower than the number of copies found for wood mice that were given no carrot seeds (0 carrot seed number, Fig. 1B) during the feeding trial. Using the calibration curves of Fig. 2 and filling in the log-transformed onion and carrot DNA copies g^{-1} BW g^{-1} SW of the field trial samples; the model predicted a seed intake of 1.08 (SE: 1.15) onion seeds and 0.00 (SE: 1.93) carrot seeds.

The above analyses were also performed including measurements that did not yield any onion or carrot DNA copies (see Appendix S2 for all the results including figures and statistics). A difference between the two analyses was found within a comparison between seed numbers 3 and 5 (Fig. S4) for onion DNA content only (which was not apparent in the analyses with zeros excluded). Another difference between the two analyses was found in the total number of onion DNA copies g^{-1} BW g^{-1} SW found in fecal samples of wild-caught wood mice during the field trial. With zeros included, onion DNA copy number resembled the number of copies found when wood mice were fed 0 onion seeds during the feeding trial (instead of 1 in the analyses with zeros excluded). In this case, the calibration curve models predicted a seed intake of 0.00 (SE: 0.76) onion seeds and 0.00 (SE: 1.29) carrot seeds. This thus reflects no intake of onion and carrot seeds in the field, whereas the data with zeros excluded did.

4. Discussion

To our knowledge this is the first-ever study to quantify seed intake in a realistic field scenario using a DNA-based analysis. Calibration curves from 0 to up to 10 onion and carrot seeds were developed for the relation of seed numbers eaten by wood mice and DNA content of these seeds in feces, experimentally validated in a laboratory setting and applied in a field trial to quantify seed intake in a wild wood mouse population. Onion DNA was detected in the fecal samples of the wood mice caught in the field, which resembled a seed intake of up to 1 onion seed. Carrot DNA was also detected, although minimally, in the fecal samples of the mice caught in the field. However, unlike onion seed intake, the amount of carrot DNA copies was not sufficiently high to suggest any intake of carrot seeds by wood mice in the field.

This result can be explained by the fact that fecal samples of wood mice fed 0 carrot seeds in the laboratory trials did also contain (minimal) traces of carrot DNA (~100 DNA copies g^{1} BW g^{-1} SW). We allowed a 3-day resting period between each feeding trial run plus an extra fasting phase of 1 day to avoid detecting remnant target seed DNA in later trials. Therefore, it seems unlikely that such remnants biased results. Additionally, Groen et al. (2022) showed that > 97% of the DNA already passed the gut 24 h after feeding carrot seeds. However, minimal traces of DNA (1.5%) did pass 37–48 h after feeding wood mice 5 carrot seeds (Groen et al., 2022). We chose to add these 0 seed quantification values to the calibration curve and prevented the curve to intercept at the origin to correct for any of such 'late' gut passage occurrences. Importantly, the number of carrot DNA copies found in the field samples was even significantly lower than the number of copies found for mice fed 0 seeds which clearly indicates 0 carrot seed intake in the field.

To our knowledge seed intake has never been quantified in a realistic field scenario using DNA-based methods. This study is thus a proof of concept that DNA-based methods are applicable and useful to estimate seed intake (although with some experimental error/variation). Based on only the fecal samples of mice that did contain onion or carrot DNA, quantifiable seed intake was detected for onion seeds only. The inclusion of zeros (measurements where no target DNA was present) resulted in substantially lower average seed intake estimates of (near) zero seeds. This difference was expected with fecal samples from the field trial where almost 50% of samples did not contain onion DNA and more than 80% did not contain carrot DNA.

This result does imply that very few target seeds are consumed and if so, onion seeds are preferred over carrot seeds. This might be explained by the bigger size of onion seeds (more than 2 times heavier in weight) as this might potentially correspond to higher nutritional values and

thus a more favorable tradeoff between effort and gain when foraging (Hernández et al., 2019). Furthermore, no effect of seed density was found on the number of DNA copies found in wood mice fecal samples for both onion and carrot seeds. This suggests that a higher number of these seeds available does not necessarily lead to higher consumption.

The calibration curves in this study were obtained by running linear regressions for known seed numbers and their corresponding DNA copies. Regular calibration curves tend to use single-point estimates (Hart et al., 2003). Here we chose to use probabilistic approaches as distributions and regression to get the best fit for our data. Our measure for goodness of fit obtained in this way provides a more complete and balanced description for the accuracy of risk assessment models (Van Loco et al., 2002; Hart et al., 2003). Although large variation within seed number DNA copies is apparent and inherent to digestive processes, which mostly favor random processes under the influence of the gut microbiome (Cresci and Bawden, 2015), our models explain a substantial part of the variation in our data ($R^2 > 0.55$). Nonetheless, larger sample sizes per seed number would increase the power of these regressions and would improve calibration power (making the now large confidence intervals smaller). Additionally, less variation would theoretically allow for an extension of the calibration curve up to more than 10 seeds. However, in light of our field trial results demonstrating seed numbers below 5, improving the calibration curve for more than 10 seeds was considered irrelevant for this study.

Estimates of seed intake of the whole wood mouse population that were only based on (and averaged over) the positive samples led to higher estimates of seed intake. In the laboratory trials, only $< 1\%$ of the measurements for carrot DNA and $< 5\%$ of the measurements for onion DNA did not result in the detection of DNA content although mice had consumed 1, 3, or 20 (20 seeds only applicable to carrot DNA) target seeds (see Table S2, Appendix S2). Therefore, considering positive samples only may be preferred in an ERA, even if this potentially leads to an overestimation. With the aim of ERA, an overestimation might be preferred over an underestimation, since this could contribute to an improved protection of wild animals from the risk of exposure.

The quantification approach used in this study may be extended to other diet constituents that are consumed similar to the foraging of seeds by wood mice. A foraging behavior where delayed consumption occurs, e.g. some rodents collect large seeds that they find in their environment and deposit it in a hoard, rather than directly consuming them (Jones et al., 1990; Quy et al., 2005), would not favor accurate quantification in a real field scenario. It is unlikely that hoarding also occurs for small seeds, although this should be further investigated. Furthermore, note that to create an applicable DNA-based protocol for a new model organism or diet constituent new calibration curves are required for each that govern time and costs. Also, note that sampling feces should be conducted in the same period, or only shortly after, sowing occurs. If feces were to be sampled too long after sowing (when sprouting has already started), non-seed material of the same plant species (e.g. sprouts) is present and intake of this non-seed material will increase DNA copies in the feces causing an overestimation of the number of ingested seeds.

This study has proven that a less invasive assessment of seed intake by a relevant focal species can be obtained through DNA-based analysis of fecal samples. While we did not test this explicitly, when seed intake is combined with treatments by agrochemicals, our approach can be used to assess the exposure through feed intake by those agrochemicals in the European ERA process. This method can also be suitable for other exposure scenarios in other species and could be used as a less invasive tool in risk assessment and for a multitude of purposes in basic and applied research. Ultimately, this approach could optimize exposure models ensuring an improved level of protection for wild animals.

CRediT authorship contribution statement

Kevin Groen: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft. **Jens Jacob**: Conceptualization, Methodology, Data curation, Writing – review & editing. **Susanne Hein**: Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing. **Emilie Didaskalou**: Methodology, Data curation, Formal analysis. **Peter M. van Bodegom**: Writing – review & editing. **Joerg Hahne**: Conceptualization, Review. **Krijn B. Trimbos**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Krijn Trimbos reports financial support was provided by Bayer CropScience AG. Joerg Hahne reports a relationship with Bayer CropScience AG that includes: employment.

Data availability

Raw onion and carrot DNA copy counts as measured by ddPCR in the fecal samples per mouse fed different MPs and the field trial samples can be found at doi: 10.17632/r7pfjmxxcd.1.

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Data accessibility and benefit-sharing statement

Raw onion and carrot DNA copy counts as measured by ddPCR in the fecal samples per mouse fed different MPs and the field trial samples can be found at doi:10.17632/r7pfjmxxcd.1. All collaborators are included as co-authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115036.

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