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Establishment of a faecal DNA quantification technique for rare and cryptic diet constituents in small mammals

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Abstract

DNA-based approaches have greatly improved the applicability of dietary studies aimed at investigating ecological processes. These studies have provided direct insights into, otherwise difficult to measure, interactions between species and trophic levels, food web structure and ecosystem functioning. However, despite these advances, DNA-based methods have struggled to accurately quantify the whole breadth of diet constituents because of methodological biases, such as amplification bias and digestive processes. The present study is, to our knowledge, the first diet study to use droplet digital PCR to quantify diet constituents. We manipulated the diet of wild-caught wood mice (*Apodemus sylvaticus*) by feeding them with a known amount of small vegetable seeds (onion and carrot) and quantified the DNA traces of these diet constituents in faecal samples. The sensitivity of the technique combined with the control on the experimental design allowed mitigation of methodological bias. We were able to accurately determine DNA concentrations of small vegetable seeds in the diet of wood mice. Quantification of target DNA demonstrated significant differences in DNA content when one vs. five seeds were consumed. These differences remained significant when the age, sex and other diet constituents of the mice were altered. Different DNA markers, targeting different parts of the chloroplast, influenced onion DNA detectability. However, all onion and carrot markers showed higher DNA content for higher seed numbers. Overall, the sensitive DNA-based approach developed in this study allows for minimally invasive quantification of small diet constituents in faeces, which would otherwise be undetectable with traditional methods.

KEYWORDS

Apodemus sylvaticus, diet analysis, droplet digital PCR, quantification, rodents, vegetable seeds

1 | INTRODUCTION

Diet studies have been an integral component of research related to the biology and ecology of animals for decades. Traditionally,

morphological and visual approaches, such as gut, stomach, faecal and scat content analyses, are used to determine what a consumer has eaten (Miller & McEwen, 1995; Montague & Cullen, 1985). In this way, a qualitative list of dietary items can be obtained (Miller

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& McEwen, 1995; Montague & Cullen, 1985) and further ecological relationships can theoretically be determined. However, morphological methods are restricted to what is not digested at the time of sampling and therefore lack resolution (Casper et al., 1997; Mumma et al., 2016). Additionally, partly digested diet remains are difficult to identify and the process is time-consuming (Pompanon et al., 2012). The result is, at best, a biased picture of diet choice (Symondson, 2002). Furthermore, studying animal diets using morphological methods has undesirable effects on the sampled individuals and potentially on populations because of invasiveness of the techniques (Murray et al., 2011).

Recently, DNA-based approaches have received attention because of their use in dietary studies (e.g., using faeces as a source of DNA of diet constituents) (Creer et al., 2016). These approaches are now extensively used by ecologists for (qualitatively and semi-quantitatively) assessing the diet of herbivores (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). These studies have utilized the outcome of DNA analyses for investigating ecological processes such as interactions between trophic levels (see Clare, 2014) and species interactions (e.g., predator–prey relationships, see Zarzoso-Lacoste et al., 2016), food web structure (Roslin & Majaneva, 2016) and ecosystem functioning (Nielsen et al., 2017), which are otherwise difficult to measure.

Despite these advances, quantitative determination of diet constituents has remained a major challenge, while this is critical for assessing trophic interactions, predator–prey relationships and the dynamics of animal populations (Bowles et al., 2011). Although the advent of DNA-based studies has greatly increased the resolution of dietary breadth (Deagle et al., 2005; Galan et al., 2018; King et al., 2008; Morisset et al., 2013; Murray et al., 2011; Piñol et al., 2014; Pompanon et al., 2012; Zeale et al., 2011, and see Creer et al., 2016), DNA-based methods have struggled to accurately quantify the whole breadth of diet constituents because of current technical and methodological bias (Clare, 2014; Deagle et al., 2013, 2019; Jusino et al., 2019; King et al., 2008; Mata et al., 2019; Pompanon et al., 2012; Symondson, 2012). According to Clare (2014), tissue-specific differences in DNA content, primer or marker choice, copy number variation, digestive processes, sample pooling and DNA purification are all steps in the quantification process that introduce biases (see Deagle et al., 2013, 2019; Jusino et al., 2019; Mata et al., 2019; Pompanon et al., 2012). Nevertheless, several studies did show positive correlations between proportions of diet constituents consumed and the amount of DNA of the respective diet constituents in scat samples (using high-throughput sequencing [HTS]: Deagle et al., 2010; using quantitative PCR [qPCR]: Bowles et al., 2011; McCracken et al., 2012; for a comparison between HTS and qPCR see Murray et al., 2011). Additionally, Thomas et al. (2014) used HTS and diet-specific correction factors to account for the above-mentioned biases and found promising improvements regarding the accuracy of DNA diet estimates using these corrections.

Real-time PCR (RT-PCR) techniques, such as droplet digital PCR (ddPCR), are less prone to methodological biases (such as amplification bias) compared to HTS techniques and allow quantitative measurements when analysing diet components of faecal samples (Bowles et al., 2011; Morisset et al., 2013). Bowles et al. (2011) demonstrated that proportions of diet constituents can be accurately determined from faecal DNA using RT-PCR. Additionally, Murray et al. (2011) compared the two methods for DNA-based faecal dietary analysis and concluded that species-specific qPCR assays have higher sensitivity than HTS approaches. Furthermore, ddPCR is favoured over qPCR for DNA quantification because it does not need reference material, is less susceptible to PCR inhibition and is more sensitive towards very small quantities of DNA (Floren et al., 2015; Miotke et al., 2014; Morisset et al., 2013; Verhaegen et al., 2016). To illustrate the sensitivity, ddPCR has already been proven to reliably quantify minimal traces of contamination in assays of food and feed samples (Floren et al., 2015; Morisset et al., 2013), plant remains at crime scenes (Coyle, 2014) and malaria parasite densities in human blood (Koepfli et al., 2016). However, despite promising results in other fields, for diet studies ddPCR has not yet been used and quantitative relationships with small quantities of cryptic diet constituents have yet to be established.

This study specifically aimed for absolute quantification, using species-specific primers, of small vegetable seeds that are hard to quantify by visual observations or stomach content analysis. We investigate the detectability of seed DNA in faecal samples of wood mice fed with one or five small vegetable seeds and the relationship between seed intake and seed DNA content in faecal samples of wood mice. Furthermore, we explore the effect of digestive biases, by looking at the effect that may occur through sex differences (Asarian & Geary, 2013), age differences (Karasov & Douglas, 2013) and diet composition, on establishing quantitative relationships. Additionally, the effect of marker choice on quantification is examined.

For this, we manipulated the diet of wood mice (*Apodemus sylvaticus*) by feeding them small vegetable seeds. Wood mice are distributed across most of Europe and parts of North Africa (Wilson & Reeder, 2005). They prefer forests and other habitats with well-developed woody plants but can also inhabit arable fields if food and shelter are present (Mitchell-Jones et al., 1999), where mainly seeds are consumed (Abt & Bock, 1998). Additionally, wood mice are used as an animal model and a focal species for ecological risk assessment studies (European Food Safety Authority, 2009).

Insights into the effect of methodological biases when quantifying small diet constituents can improve faecal DNA quantification techniques for these constituents for use in realistic field scenarios and ecological research. Additionally, a proof of concept is needed for studies where quantification of other small dietary constituents matters. The application of a sensitive, robust and validated technique is desirable for research in the field of animal ecology (resource use, trophic interaction, competition) and applied research alike (exposure to environmental contaminants, risk assessment).

2 | MATERIALS AND METHODS

2.1 | 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 forest habitat or along hedgerows and/or tree rows. The trapping regime was identical to previous studies (Chiron et al., 2018; Hein & Jacob, 2018). In brief, traps were prebaited with rolled oats for three nights before restocking with apple chunks, peanut curls and rolled oats as bait and wood wool for nesting material. Traps were activated for three to five nights and checked about every 12 h. Live trapping was conducted several times from April to November 2017. After capture, the individuals were individually marked with a passive integrated transponder (PIT) tag (LUX-IDent), sexed and weighed with a spring scale (Pesola) to the nearest gram. Mice were housed individually in standard rodent cages with wood shavings, an upturned clay flower pot for nesting, standard pellets (Altromin 1324; Altromin) as a food source and tap water *ad libitum* at standard holding conditions at a reversed day–night cycle. Subadults were younger than 4 weeks (F_1 generation were captive-born offspring from wild parents). All procedures involving animals were conducted according to relevant legislation and by permission of the authorities of the German federal state of North Rhine-Westphalia under permit 84–02.04.2016. A540.

2.2 | Food pellet preparation

We produced custom-made food pellets (FPs) to deliver known proportions of known diet components to the wood mice (see Appendix S1 for details). Components of FPs were wheat kernels (nontarget seeds), mealworms (invertebrates) and wheat leaves (foliage). These three ingredients were selected to represent the main food

categories at relevant composition—seeds 50%, invertebrates 25%, foliage 25%—for wood mice reported in natural habitats (Abt & Bock, 1998). Matrix pellets (MPs) were produced; these were FPs spiked with a given number of target seeds. These were onion (*Allium cepa*) and carrot (*Daucus carota sativus*) seeds (mean seed weight for onion 3.8 ± 0.3 mg [SD], and for carrot 1.8 ± 0.3 mg [SD]) at varying composition of components (Table 1).

2.3 | Gut passage time

To acquire as much target DNA as possible for accurate quantification of seed intake, we determined the time it took for the target seed DNA to completely pass through the gastrointestinal tract (GIT) of a wood mouse. For this, we fed three adult male and three adult female wood mice MPs with five onion and five carrot seeds. Faecal samples were collected after $t = 8, 24, 36$ and 48 h and analysed for their DNA content. Results showed that >97% of all target seed DNA passed the GIT after $t = 24$ h (see Results, Figure 1) and therefore further analyses were done using $t = 8$ plus $t = 24$ h after feeding only.

2.4 | Feeding trials

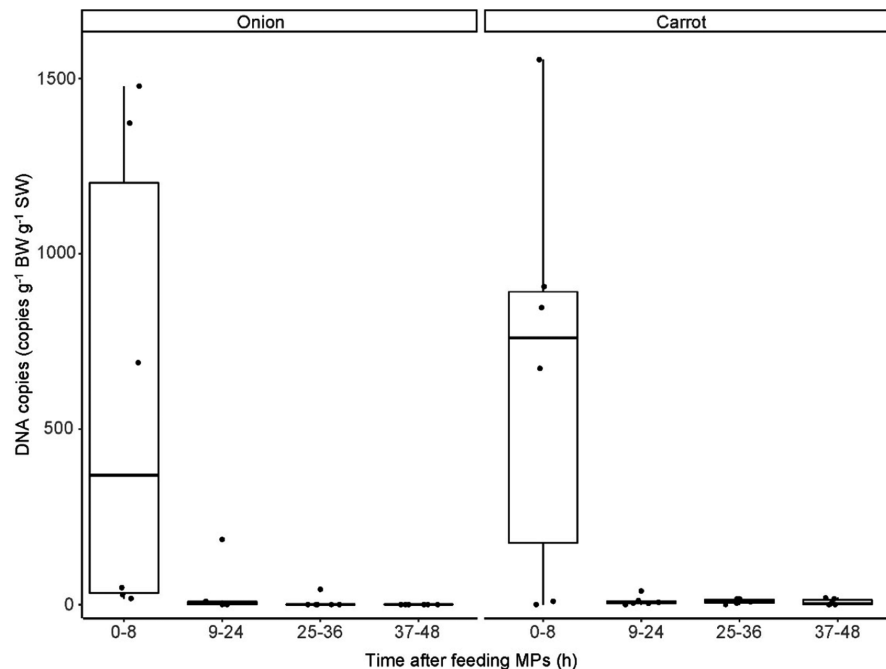
The feeding trial routine was conducted according to the schedule depicted in Table S1. Each feeding trial started with placing randomly selected mice in fresh cages with filter paper as bedding and an upturned clay flower pot for shelter for 3.5 days. After the first day in the new cage, food was switched from the standard pellet diet to an FP diet to allow the GIT get used to the different feed. After 2.5 days, FPs left over were removed and a 12-h fasting phase was conducted overnight (reversed day–night cycle). On day 4, all individuals were fed one MP ($t = 0$) which was provided in a

TABLE 1 Composition of matrix pellets (MP) and the number of wood mice tested per feeding trial

MP	Nontarget seeds (%)	Invertebrates (%)	Foliage (%)	Target seeds	♂ adult	♂ subadult	♀ adult	♀ subadult
1.1	50	25	25	5O + 5C	5	8	5	6
1.2	50	25	25	1O + 1C	5	9	5	5
2.1	50	50	0	1O + 1C	5	0	5	0
2.2	50	0	50	1O + 1C	5	0	5	0
2.3	0	50	50	1O + 1C	5	0	5	0
2.4	50	50	0	5O + 5C	5	0	5	0
2.5	50	0	50	5O + 5C	5	0	5	0
2.6	0	50	50	5O + 5C	5	0	5	0
3.1	50	25	25	1O + 1C	5	0	5	0
3.2	50	25	25	5O + 5C	5	0	5	0

Note: The main constituents of MPs were wheat kernels (as nontarget seeds), mealworms (as invertebrates) and wheat leaves (as foliage). O = onion; C = carrot. MP 1.1 and MP 1.2 were used for testing the effect of sex and age on DNA quantification. MP 1.1–1.2 and 2.1–2.6 were used for testing the effect of varying diet composition on DNA quantification and MP 3.1–3.2 for testing the effect of a DNA marker on quantification. Note that the absence of subadults in MP 2.1–3.2 was based on the results gained in the MP 1.1 and 1.2 trials (see Results).

FIGURE 1 Gut passage time analysis. Total number of onion (left) and carrot (right) DNA copies per gram body weight (BW) and per gram sample weight (SW) in faecal samples excreted by six adult wood mice fed matrix pellets with five onion and five carrot seeds plotted through time after feeding (y-axis). Primer sets used were AC1 and DCsat1



small glass Petri dish (see Table 1 for the different MPs fed and the replication). MPs were usually consumed within 1–2 h. All faecal samples during 8 h after MP consumption were collected ($t = 8$, average number of droppings 59 ± 17 [1 SD], average sample weight 265 ± 108 mg [1 SD]) and individuals were placed in fresh cages with filter paper until the next morning, when another faecal sample was collected ($t = 24$, average number of droppings 50 ± 29 [1 SD], average sample weight 376 ± 129 mg [1 SD]). Droppings per individual were collected in a collection tube, labelled and dried in a drying cabinet for 2–4 h at 35°C. Twenty-four hours after pellet consumption (= day 5), individuals were returned to cages with wood shavings and a flower pot. 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 longer used for ongoing feeding trials or after all feeding trials were finished were released at their place of capture.

2.5 | Primer design

Major DNA fragmentation occurs in the GIT and the fragment size of dietary DNA decreases along the GIT (Rizzi et al., 2012). Different positions on the chloroplast DNA (cpDNA) might therefore be influenced differently by digestion. To investigate quantification variation between primer sets, two species-specific primers were designed on different positions on the cpDNA (see Figure S1) to specifically quantify the DNA content of onion and carrot (Table 2). Each primer set was designed to only amplify one copy (without mismatches) to prevent copy number variation. In addition, to enhance the specificity of the species-specific primer combinations, TaqMan Hydrolysis probes (FAM and HEX dyes) were constructed (see Table 2). A PCR protocol for a multiplex reaction with both

primer sets was constructed and optimized using a thermal gradient (Table S2). To minimize cross-amplification, specificity testing was done *in silico* using GENEIOUS 10.2.6 (<https://www.geneious.com>) and PRIMER-BLAST (Ye et al., 2012) (Table S3). Additionally, specificity tests were performed *in vitro* using DNA extracts of the raw components of MPs (wheat: *Triticum aestivum* and mealworm: *Tenebrio molitor*), wood mouse (ear) tissue, droppings and several plants closely related to onion and carrot (*Allium sativum*, *Anethum graveolens*, *Petroselinum crispum* and *Coriandrum sativum*; see Table S4). Each of these tests suggested high specificity of the primer sets for the target taxa.

2.6 | DNA extraction and quantification

Mouse droppings per individual were weighed, and each sample was divided into subsamples to not exceed the maximum starting amount (<220 mg) as stated in the protocol of the extraction kit. DNA extraction was performed using the DNeasy Plant Maxi kit (Qiagen) with the introduction of a stool inhibitor removal step using an InhibitEX (Qiagen) tablet. The DNeasy Blood & Tissue kit (Qiagen) was used for DNA extraction of animal tissue samples and the DNeasy Plant Mini kit (Qiagen) for plant tissue samples. Before sample lysis, the mouse droppings were homogenized in a bead mill using 5-mm stainless steel beads. After DNA extraction, the extract was quantified *in duplo* using ddPCR according to the protocol given in the manufacturer's protocol (ddPCR Supermix for Probes; Bio-Rad). In short, 1 or 5 μ l DNA (depending on the MP; i.e., depending on whether one or five target seeds had been used in the feeding trial, to prevent overloading; 5 μ l for MPs 1.2, 2.1–2.3 and 3.1 and 1 μ l for all other MPs, see Table 1), 11 μ l ddPCR supermix for probes (Bio-Rad), 1 μ l target primers (10 μ M) and 1 μ l Taqman probes (5 μ M) supplemented to 22 μ l total volume with RNase/DNase-free water

TABLE 2 Specifications of primers and probes designed and used in this study; all oligonucleotides were designed to target chloroplast DNA of *Allium cepa* (accession NC_024813) and carrot (*Daucus carota sativus* (accession CM004358))

Species	Primer set (marker)	Name	Sequence (5'-3')	Length (bp)	Amplicon length (bp)	Location on cpDNA (min.-max. in bp)
Carrot primers (<i>D. carota sat.</i>)	Dcsat1	Dcsat_F_1	GACGCACTCCTATGAACGTGA	21	127	50,858–50,984
		Dcsat_R_1	AGGAGACATGTCCTAAAGTTTCG	22		
		Dcsat_probe_1	[HEX]AAATATACCGGATTAGTCGACTCG A[BHQ2]	25		
	DCsat3	Dcsat_F_3	TCGGATTGACCCACTACTTT	20	129	49,720–49,848
		Dcsat_R_3	ATCAACCGACATCCACTTCG	20		
		Dcsat_probe_3	[HEX]GTTCAGTCTCTTCCACGAA[BHQ2]	20		
Onion primers (<i>A. cepa</i>)	AC1	AC_F_1	AAAGGTGAGGGGACGTA	18	135	47,212–47,346
		AC_R_1	TTGGCTATCCCTCTCGT	18		
		AC_probe_1	[FAM]CCACCTCTATTGTATGAATCATCTT [BHQ1]	25		
	AC3	AC_F_3	AATTGTTTCCGATTCACCAATTTTT	25	177	111,375–111,551
		AC_R_3	TCGAGCTATTTGGACAAAGCA	21		
		AC_probe_3	[FAM]TTGCTTTTCAAGAAAACAAT[BHQ1]	20		

Note: [HEX] and [FAM] are fluorophores, [BHQ2] and [BHQ1] are black hole quenchers.

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 (version 1.7; Bio-Rad). 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 ddPCR were given in DNA copies μL^{-1} . These were recalculated to total DNA copies in the sample (DNA content) and used for further statistical analyses.

2.7 | Data analysis

To correct for varying body weight (BW) of mice and varying sample weight (SW), the total DNA copies per sample were divided by BW in grams and SW in grams. Ultimately, all steps taken in this laboratory study were performed to quantify seed intake in wood mice in a realistic field scenario. When zero copies of target DNA would be measured in the field, it might reflect two scenarios: (i) the wood mouse in question did not eat the target seed and therefore the DNA is not present in its faeces; or (ii) the wood mouse did eat the target seed but too long ago for target DNA to be present (due to digestion) in its faeces. In the field, it would be impossible to distinguish between the scenarios above. We argue that these measurements are not of any added value towards an application of this study in a realistic

field scenario, where wood mice would be caught in the wild. On the other hand, the zeros measured are true values and should be included. Therefore, we have run the analysis twice, first with the zeros omitted, and second with the zeros included. The outcomes of both analyses were closely compared for any substantial differences between the two approaches (see [Results](#)). Distributions of DNA content were nonparametric and of a small sample size. Therefore, differences between DNA content were tested using nonpaired Wilcoxon tests (Wilcoxon, 1945). Multiple comparisons were tested using Kruskal–Wallis rank sum tests and a *post hoc* Holm-adjusted Dunn test (Dunn, 1964) in the FSA package (Ogle et al., 2021). Means and confidence intervals (95%) were determined by bootstrapping ($R = 10,000$, BCa method) (Carpenter & Bithell, 2000) using the RCOMPANION package (Mangiafico, 2021). The overall effect of a treatment (sex, age, diet composition and marker) on DNA quantification was analysed after correcting for the effect of increasing seed number. For this, linear regressions (DNA copies ~number of seeds fed) were performed (model fits were evaluated based on visual representations of the residuals). The residuals of these models were then used to test for a significant overall effect of treatments on DNA content using the same nonparametric tests as specified above. When single comparisons did show significant differences for a treatment but were only applicable to a subset of the data (e.g., for mice fed one seed only or mice fed five seeds only) the result of the overall analysis (based on the full data set) overruled these partial differences. This suggests namely that these differences were only applicable to a subset of the data. The markers used for quantification showed differences in detectability (see [Results](#), [Figure 5](#) and [Figure S7](#)). Therefore, we chose to base our results on AC1 and DCsat1 only (see [Discussion](#) for further elaboration). If needed for appropriate visualization purposes, data were log transformed. All figures were

produced using the GGPUBR package (Kassambara, 2020). All statistics were performed in R 3.6.3 (R Core Team, 2020).

3 | RESULTS

We detected an average of 606 onion DNA copies g^{-1} BW g^{-1} SW and 665 carrot DNA copies g^{-1} BW g^{-1} SW in the faecal samples collected within 8 h after feeding five onion and carrot seeds to wood mice (Figure 1). Nine to 24 h after feeding, we measured 34 onion DNA copies g^{-1} BW g^{-1} SW and 11 carrot DNA copies g^{-1} BW g^{-1} SW in the faecal samples collected within this time-frame. Faecal samples collected 25–36 and 37–48 hr after feeding showed only marginal numbers of DNA copies g^{-1} BW g^{-1} SW (7 and 0 onion DNA copies g^{-1} BW g^{-1} SW, 9 and 7 carrot DNA copies g^{-1} BW g^{-1} SW, respectively). As percentages of the total amount of DNA copies collected within 48 h, 98.9% of total onion DNA and 97.7% of total carrot DNA had been excreted within 24 h after feeding.

Seed DNA was detectable in faecal samples even when only one onion and carrot seed was fed to a wood mouse although with large variation. Mean onion DNA copies in faecal samples of mice fed with one onion seed was 345 DNA copies g^{-1} BW g^{-1} SW (CI: 151–746 copies g^{-1} BW g^{-1} SW) and mean carrot DNA copies in faecal samples of mice fed with one carrot seed was 1.6×10^3 DNA copies g^{-1} BW g^{-1} SW (CI: 0.8×10^3 – 4.5×10^3 copies g^{-1} BW g^{-1} SW). Moreover, a significant difference in total onion and carrot DNA copies in faecal samples was found between wood mice fed one onion and carrot seed and wood mice fed with five onion and carrot seeds (Figure 2). Droppings of mice fed with five onion seeds (mean = 18.1×10^3 DNA copies g^{-1} BW g^{-1} SW, CI: 5.0×10^3 – 61.1×10^3) contained 52.5 times more onion DNA copies compared to droppings of mice fed with one onion seed ($W = 332$, $p < .0005$). Droppings of mice fed with five carrot seeds (mean = 9.3×10^3 DNA copies g^{-1} BW g^{-1} SW, CI: 3.8×10^3 – 21.3×10^3) contained 5.8 times more carrot DNA copies compared to faecal samples of mice fed with one carrot seed ($W = 427$, $p < 0.005$).

Subadult mice fed with one carrot seed excreted droppings that contained 3.4 times more carrot DNA copies compared to adult mice fed one carrot seed (mean subadults 2.2×10^3 , CI: 0.9×10^3 – 6.8×10^3 carrot DNA copies g^{-1} BW g^{-1} SW; mean adults 0.7×10^3 , CI: 0.2×10^3 – 2.0×10^3 carrot DNA copies g^{-1} BW g^{-1} SW; $W = 28$, $p < .05$) (Figure 3). This effect was not apparent for subadult and adult mice fed with one and five onion seeds and with five carrot seeds, respectively. Overall, there was no general effect of age on carrot DNA quantification ($\chi^2 [1, N = 47] = 1.4$, $p = 0.2$).

There was no effect of sex on the number of onion and carrot DNA copies in faeces for mice fed one seed or five seeds (Figure S2). However, the number of onion DNA copies found in faecal samples of mice that had been fed with MPs consisting of one onion seed varied with MP composition ($\chi^2 = 12.5$, $df = 3$, $p < .05$) (Figure 4a). Onion DNA content of faecal samples from mice fed with MPs of a “0% nontarget seeds/50% invertebrates/50% foliage” composition and MPs with a “50/50/0” composition were 17.0 and 12.5 times higher, respectively, compared to onion DNA content of faecal samples from mice fed with MPs with a “50/25/25” diet composition (Dunn test, 0/50/50–50/25/25, $Z = 2.8$, $p < 0.05$; 50/25/25–50/50/0, $Z = -3.1$, $p < .005$). This effect was not apparent for adult mice fed with five onion seeds and with one and five carrot seeds, respectively. Overall, a general effect of diet composition on DNA quantification was not found for onion ($\chi^2 [3, N = 62] = 3.1$, $p = .4$) or for carrot seeds (Figure 4b).

DNA content in faecal samples of mice fed with one or five onion seeds did not show significant differences when quantified with marker AC1 compared to AC3 (Figure 5a). DNA content in faecal samples of mice fed with one or five carrot seeds also showed no significant differences between markers DCsat1 and DCsat3 (Figure 5b). However, when combining all onion data and removing the variance explained by increasing seed number, the overall effect of marker on onion DNA quantification was statistically significant ($W = 180$, $p < .05$). Combining all carrot data and removing the variance explained by increasing seed number, the overall effect of the carrot marker used for quantification did not significantly influence the number of carrot DNA copies found ($W = 202$, $p > .05$).

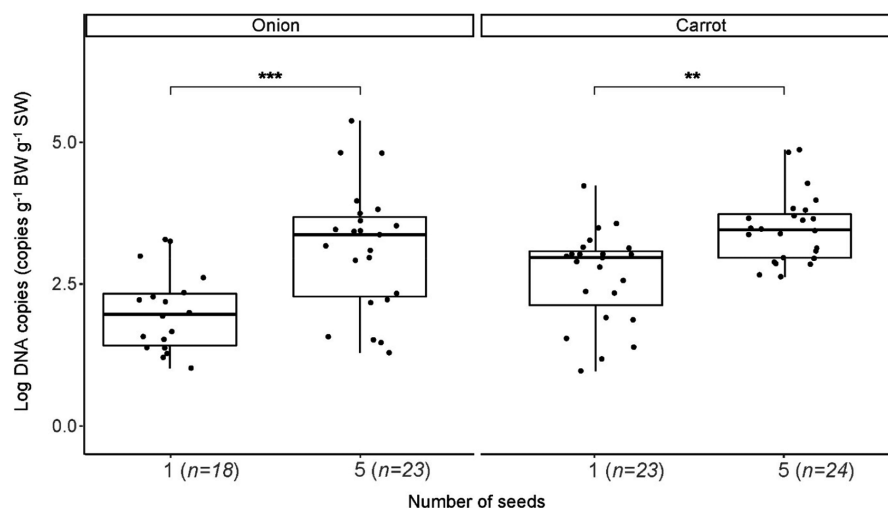


FIGURE 2 Boxplot showing the difference in the number of log DNA copies per gram body weight (BW) and per gram sample weight (SW) in faecal samples of wood mice fed with one onion and one carrot seed (MP1.2) or five onion and five carrot seeds (MP1.1). Mice of different sex and age were included. Primer sets used were AC1 and DCsat1. Zeros were removed from this analysis ($n = 8$ out of 96). Statistically significant differences are depicted (*** $p < 0.0005$, ** $p < 0.005$)

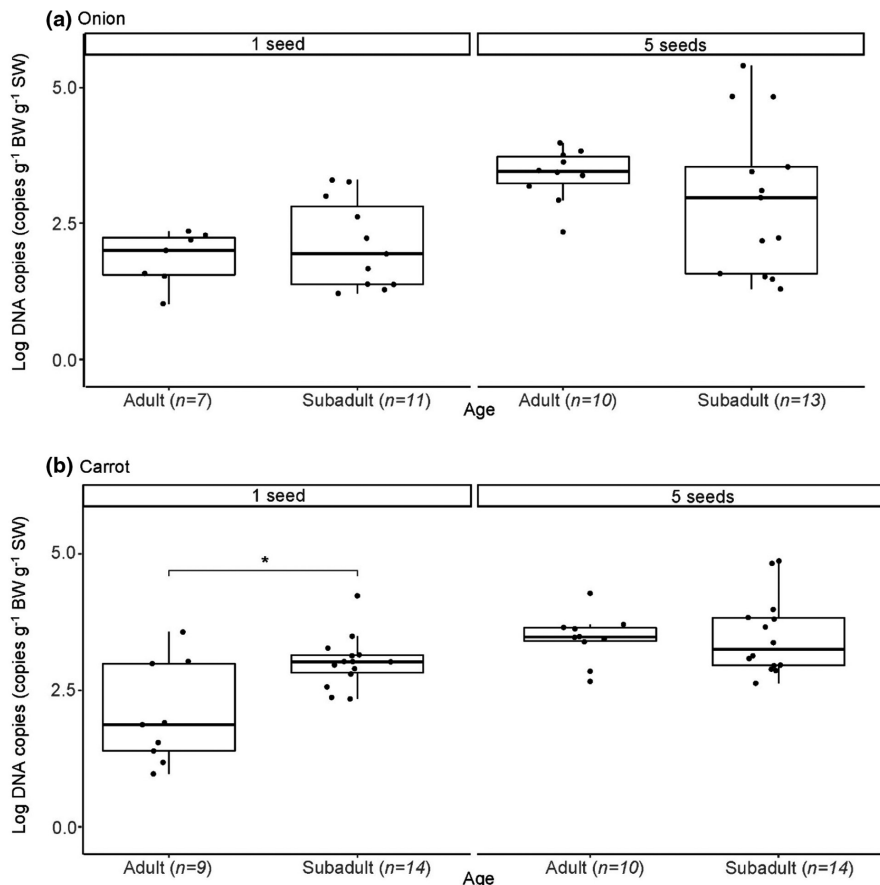


FIGURE 3 The number of log-transformed onion (a) and carrot (b) DNA copies per gram body weight (BW) and per gram sample weight (SW) in faecal samples of adult and subadult wood mice fed one seed (MP1.2) or five seeds (MP1.1). Primer sets used were AC1 and DCsat1. Zeros were removed from this analysis ($n = 8$ out of 96). Statistically significant differences are depicted ($*p < 0.05$)

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). Aside from the differences within treatment groups (last paragraph of Appendix S2), the overall effects of the treatments found in this analysis were the same as the overall effects found in the analyses where the zeros were excluded.

4 | DISCUSSION

Quantification of DNA content in wood mice faeces was experimentally validated and the method was able to discern between one and five seeds fed. Furthermore, factors such as age, sex and other diet constituents did not alter the digestion patterns. The results here can enhance seed intake estimates, which is valuable information in multiple scenarios. These include risk assessments for small mammals that eat seeds on which plant protection products are applied as coating, studying seed predation, food preference and food competition.

Marker choice did significantly influence onion DNA quantification, although all markers were designed to produce only one amplicon and the same samples were measured with each marker. This result could be due to the presence of NUPTs (nuclear cpDNA) (Richly & Leister, 2004). NUPTs arise when organellar DNA infiltrates nuclear DNA (Richly & Leister, 2004). Ayliffe et al. (1998) stated that

NUPTs vary interspecifically in size and copy number as well as intra-specifically in species, within individual plants or even within different tissues of the same individual. Each marker targeted a different part of the chloroplast locus and could therefore potentially result in targeting different or additional NUPTs. However, both NUPTs and cpDNA are amplified and can be used as indicators of target DNA. It is important to note that NUPTs also may cause differences in detectability of target DNA. AC3 did not detect any DNA in six out of 10 one-onion seed samples. When these zero measurements were included, marker AC3 scored consistently lower in onion DNA copies compared to AC1 for all seed numbers, although this was nonsignificant. This does suggest that reliable quantification of marginal quantities can only be done by first investigating how different markers influence detectability (see Di Bernardi et al., 2021). In this study, we therefore based our results on AC1 and DCsat1 only.

We found that seed DNA passed the wood mice gut within 24 h without variation, which is within the range of passage time in mammals (Lambert, 1998). Tsuji et al. (2015) showed that gut passage time in herbaceous/omnivorous mammals accelerates with increased food intake. This suggests that increasing the number of seeds consumed further may require additional calibration. In terms of detectability, this study has shown that as low as one ingested seed was detectable in wood mice faeces. Even trace amounts of DNA of up to two copies per measured PCR sample (22 μ l) were detectable, demonstrating extremely high sensitivity using this faecal DNA quantification technique.

FIGURE 4 Effect of diet composition (in percentages nontarget seeds/invertebrates/foilage) on the number of log-transformed onion (a) and carrot (b) DNA copies per gram body weight (BW) and per gram sample weight (SW) in faecal samples of adult wood mice fed one seed or five seeds. MP 1.1–1.2 and 2.1–2.6 were used. Primer sets used were AC1 and DCsat1. Zeros were removed from this analysis ($n = 23$ out of 160). Statistically significant differences are depicted ($*p < 0.05$)

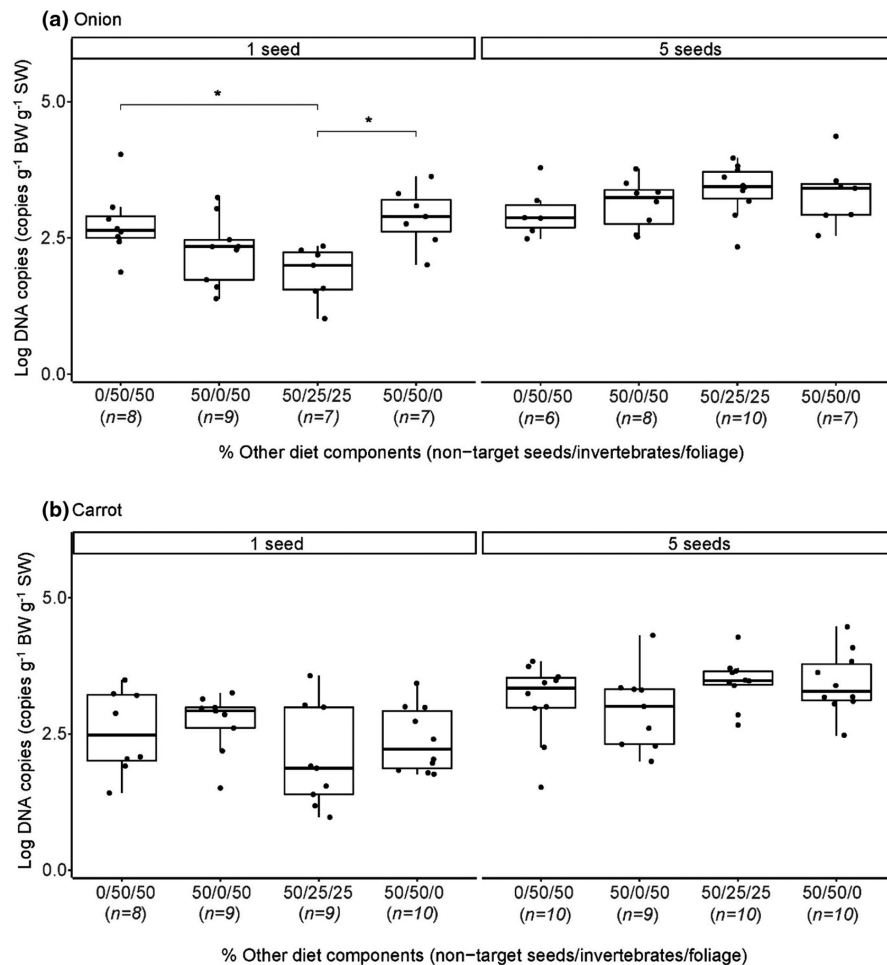
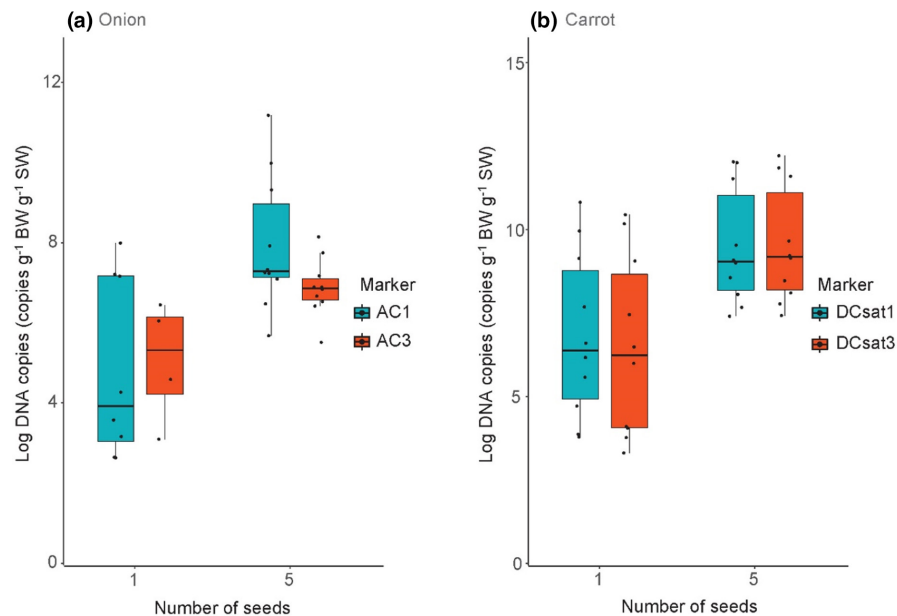


FIGURE 5 Log-transformed onion (a) and carrot (b) DNA content (copies per gram body weight (BW) and per gram sample weight (SW) of faecal samples of wood mice fed different numbers of seeds (x-axis). MP 3.1–3.2 were used. $N = 10$ for each number of onion and carrot seeds fed for each marker. Except for mice fed with one onion seed and marker AC1 where $n = 8$ and marker AC3 where $n = 4$. Zeros were removed from this analysis ($n = 8$ out of 80)



Although sex and age differences in food intake and digestion patterns are known (Karasov & Douglas, 2013), effect sizes are usually very small and highly variable (Asarian & Geary, 2013). This was in concordance with our findings of large intragroup variation and a lack of general effects of sex and age. Although samples sizes are

small for each group ($n = 7$ –14), even if sample size were to increase, an overall effect of sex and age on quantification is not expected due to the variable nature of these effects (Asarian & Geary, 2013). The absence of sex or age effects on quantification imply that there is no need to differentiate between sex and age when wood mice are

not preselected, for instance when wood mice were to be caught in the field.

Furthermore, there was no congruent and overall no significant effect of diet composition on target seed DNA content in faecal samples. Theoretically, Karasov and Douglas (2013) suggest that the expression of digestive enzymes and nutrient transporters approximately matches the dietary load of their respective substrates. As such, changing diet composition in wood mice to a more invertebrate-rich or more cellulose-rich diet would suggest an adaptation in digestibility of their GIT. Additionally, Hetland and Svihus (2001) found that there is a great ability of organisms to adjust the gut passage time and increased gut volume to increasing feed consumption. These adaptations together may change the digestibility of diet constituents and this could explain why there was no effect of diet composition on DNA quantification as these adaptations could have potentially levelled the differences in respective substrates. This result implies that quantification of seed intake in, for example, field conditions is possibly independent of the diet eaten in the field. This suggests that when wild wood mice are caught in a realistic field scenario, there is no need to account for what a wood mouse might have eaten during, after or before intake of onion and/or carrot seeds, if this was even possible.

Advances in DNA methodologies have led to improvements in the ability to detect species and communities in less favourable (due to rapid DNA degradation) sampling environments such as aquatic environments, GITs and faeces (Andres et al., 2021). Mitochondrial or plastid DNA is mostly favoured for quantification research since it is present in higher copy numbers, but the ability to detect trace amounts of DNA also opens up the possibility to target nuclear DNA. Andres et al. (2021) have documented the application of multiple amplicon-based methods to obtain intraspecific nuclear genetic information from environmental DNA (eDNA) samples and to estimate the absolute abundance of a species in eDNA samples. Quantification using nuclear DNA approaches has the capacity to estimate the number of (exact) genetic contributors in a DNA sample and thereby might overcome the correlative relationships between DNA concentrations and relative species abundance, which is frequently challenged by large variation (Andres et al., 2021; Iversen et al., 2015) and heavily impacted by taxon-specific amplification biases (Andres et al., 2021; Kelly et al., 2019).

In this study, we used newly designed species-specific primers to quantify cryptic diet constituents. In theory, our approach can also be used with universal primers that would target a whole taxon. However, in practice this would make absolute quantification problematic. The use of a universal primer would be beneficial in terms of utility, costs and effort but quantification would almost always be hampered by taxon-specific amplification biases (Piñol et al., 2018). Furthermore, a direct comparison with metabarcoding approaches is not valid because metabarcoding practices have to date never been shown to be fully quantitative, but semiquantitative at best, and is more suited to produce whole dietary breadth taxonomic lists and relative read abundances (Pringle & Hutchinson, 2020).

Apart from taxon-specific amplification biases, copy number variation biases are also illustrated within this study, as shown by the differences in target seed DNA ratios present in faeces of mice fed one vs. five target seeds (for onion the ratio between DNA copies was 1:52.5 and for carrot DNA copies 1:5.8). This entails that for every new target, a calibration study like this study would be needed and the DNA sequence of the target needs to be known upfront. This study may act as a guideline for setting up these target adjustments to be able to quantify other cryptic diet constituents.

We have shown reliable quantification of one and five onion and carrot seeds, with no detectable bias by sex, age or diet composition, which suggests that further quantification of increased seed numbers should be possible with this approach. Moreover, the same trend for all onion and carrot markers in terms of seed fed vs. DNA content was found (i.e., higher seed numbers led to higher DNA content). This proof of concept allows future studies to examine the possibility of building calibration curves with ingested seeds vs. DNA content. This opens up the possibility to use these calibration curves to estimate intake of seeds (or other small diet constituents) from wild wood mice and potentially other small mammals in a natural setting. Enhanced insights into seed intake (e.g., minimum number of seeds eaten) can, for example, be beneficial to risk assessments studies where seed coating procedures are applied or exposure to environmental contaminants need to be measured, and to studies of animal ecology (resource use, seed predation, diet choice, trophic interaction, competition).

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CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

K.G., K.T., S.H., J.H. and J.J. conceived and designed the experiments. K.G., S.H., A.B. and J.J. performed the experiments. K.G. and A.B. analysed the data. K.G., K.T., P.v.B., J.H. and J.J. interpreted the results. K.G. wrote the paper. All authors revised the manuscript draft and approved the final manuscript for publication.

OPEN RESEARCH BADGES



This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://doi.org/10.5061/dryad.pnvx0k6nz>.

DATA AVAILABILITY STATEMENT

Raw onion and carrot DNA copy counts as measured by ddPCR per mouse fed different MPs were uploaded to Dryad. These counts include mouse morphological data (body weight, sex and age). Furthermore, a data file was uploaded to estimate gut passage time of target seed DNA. Both files are available at Dryad: <https://doi.org/10.5061/dryad.pnvx0k6nz>. All collaborators are included as co-authors.

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SUPPORTING INFORMATION

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