Universiteit Leiden
The Netherlands

What's in the diet? DNA-based analysis for qualitative and quantitative assessment of animal diet

Groen, K.

Citation
Groen, K. (2024, October 9). *What's in the diet?: DNA-based analysis for qualitative and quantitative assessment of animal diet*. Retrieved from https://hdl.handle.net/1887/4094106

Version:	Publisher's Version
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Note: To cite this publication please use the final published version (if applicable).

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

Kevin Groen | Krijn B. Trimbos | Susanne Hein | Astrid I. Blaauw |
Peter M. van Bodegom | Joerg Hahne | Jens Jacob |

Published in **Molecular Ecology Resources**, 2022, *00*, 1-12.
DOI: 10.1111/1755-0998.13609

2.1 | Abstract

DNA-based approaches have highly 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 been struggling to accurately quantify the whole breadth of diet constituents because of methodological biases, such as amplification bias and digestive processes. This study is, to our knowledge, the first diet study that used 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 fecal 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 feces, which would otherwise be undetectable with traditional methods.

2.2 | 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, fecal 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 & 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, Jarman, Deagle, Gales, & Hindell, 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 (for example using feces 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, Kirkwood & Jarman, 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 impacts, see Zarzoso-Lacoste et al., 2016), food web structure (Roslin & Majaneva, 2016) and ecosystem functioning (Nielsen, Clare, Hayden, Brett, & Kratina, 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 dynamics of animal populations (Bowles, Schulte, Tollit, Deagle, & Trites, 2011). Although the advent of DNA-based studies has greatly increased the resolution of dietary breadth (Deagle et al., 2005; Galan et al., 2017; King, Read, Traugott, & Symondson, 2008; Morisset, Štebih, Milavec, Gruden, & Žel, 2013; Murray et al., 2011; Piñol et al., 2014; Pompanon et al.,

2012; Zeale, Butlin, Barker, Lees, & Jones, 2011, and see Creer et al., 2016), DNA-based methods struggled to accurately quantify the whole breadth of diet constituents because of current technical and methodological bias (Clare, 2014; Deagle, Thomas, Shaffer, Trites, & Jarman, 2013; Deagle et al., 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; Deagle et al., 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, Chiaradia, McInnes, & Jarman, 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, Jarman, Haman, Trites and Deagle (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, as droplet digital PCR (ddPCR), are less prone to methodological biases (such as amplification bias) compared to HTS techniques and allow quantitative measurements when analyzing diet components of fecal samples (Bowles et al., 2011; Morisset et al., 2013). Bowles et al. (2011) demonstrated that proportions of diet constituents can be accurately determined from fecal DNA using RT-PCR. Additionally, Murray et al. (2011) compared the two methods for DNA-based fecal dietary analysis and concluded that species-specific qPCR assays have higher sensitivity than HTS approaches. Furthermore, ddPCR is favored over quantitative PCR (qPCR) for DNA quantification since it does not need reference material, is less susceptible to PCR inhibition and is more sensitive towards very small quantities of DNA (Floren, Wiedemann, Brenig, Schütz, & Beck, 2015; Miotke, Lau, Rumma, & Ji, 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 fecal samples of wood mice fed with one or five small vegetable seeds and the relationship between seed intake and seed DNA content in fecal 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 is 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 fecal DNA quantification techniques for small diet 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).

2.3 | Materials and Methods

2.3.1 | Live trapping

Wood mice were live-trapped with Ugglan traps in the surroundings of Muenster, Germany (51.97 N, 7.55 W). 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 pre-baited with rolled oats for 3 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 hours. 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, Lanškroun, Czech Republic), sexed and weighed with a spring scale (Pesola, Schindellegi, Switzerland) to the nearest gram. Mice were housed individually in standard rodent cages with wood shavings, a turned over clay flower pot 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. 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.

2.3.2 | Food pellet preparation

We produced custom-made food pellets (FPs) to deliver known proportions of known diet components to the wood mice (see Appendix S2.1 for details). Components of FPs were wheat kernels (non-target 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 mg ± 0.3 mg (SD), and for carrot 1.8 mg ± 0.3 mg (SD)) at varying composition of components (Table 2.1).

Table 2.1 | Composition of matrix pellets (MP) and the number of wood mice tested per feeding trial. MPs main constituents were wheat kernels (as non-target 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 section 2.4).

MP	Non-target seeds	Inverte-brates	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

2.3.3 | 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 purpose, we fed 3 adult male and 3 adult female wood mice MPs with five onion and five carrot seeds. Fecal samples were collected after t = 8, 24, 36 and 48 hours and analysed for their DNA content. Results showed that >97% of all target seed DNA passed the GIT after t=24 hours (see Results, Fig. 2.1) and therefore further analyses were done using t=8 plus t=24 hours after feeding only.

2.3.4 | Feeding trials

The feeding trial routine was conducted according to the schedule depicted in Table S2.1. Each feeding trial started with placing randomly selected mice in fresh cages with filter paper as bedding and a turned over 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 a FP diet to let the GIT get used to the different feed. After 2.5 days, FPs leftovers were removed and a 12-hour 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 small glass Petri dish (see Table 2.1 for the different MPs fed and the replication). MPs were usually consumed within 1-2 hours. All fecal samples during 8 hours 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 fecal 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 hours 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 used anymore for ongoing feeding trials, or after all feeding trials were finished, were released at place of capture.

2.3.5 | 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. In order to investigate quantification variation between primer sets, two species-specific primers were designed on different positions on the chloroplast DNA (see Figure S2.1) to specifically quantify the DNA content of onion and carrot (Table 2.2). 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 & HEX

dyes) were constructed (see Table 2.2). A PCR protocol for a multiplex reaction with both primer sets was constructed and optimized using a thermal gradient (Table S2.2). 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., 2020) (Table S2.3). Additionally, specificity tests were performed ***in vitro*** using DNA extracts of the MPs raw components (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 S2.4). Each of these tests suggested high specificity of the primer sets for the target taxa.

Table 2.2 | Specifications of primers and probes designed and used in this study. All oligonucleotides were designed to target chloroplast DNA of ***A. cepa*** (accession NC_024813) and ***D. carota sat.*** (accession CM004358).

Species	Primer set (marker)	Name	Sequence (5'-3') †	Length (bp)	Amplicon length (bp)	Location on cpDNA (min. – max. bp)
Carrot Primers (*D. carota sat.*)	Dcsat1	Dcsat_F_1	GACGCACTCCTATGAACGTGA	21	127	50858 – 50984
		Dcsat_R_1	AGGAGACATGTCCTAAAGTTCG	22		
		Dcsat_probe_1	[HEX]AAATATACCGGATTAGTCGACTCGA[BHQ2]	25		
	Dcsat3	Dcsat_F_3	TCGGATTGACCCACTACTTT	20	129	49720 – 49848
		Dcsat_R_3	ATCAACCGACATCCACTTCG	20		
		Dcsat_probe_3	[HEX]GTTCAGTCTCTTCCCACGAA[BHQ2]	20		
Onion Primers (*A. cepa*)	AC1	AC_F_1	AAAGGTGAGGGGGACGTA	18	135	47212 – 47346
		AC_R_1	TTGGCTATCCCCTCTCGT	18		
		AC_probe_1	[FAM]CCACCTCTATTGTATGAATCATCTT[BHQ1]	25		
	AC3	AC_F_3	AATTGTTTCCGATTCACCAATTTTT	25	177	111375 – 111551
		AC_R_3	TCGAGCTATTTGGACAAAGCA	21		
		AC_probe_3	[FAM]TTGCTTTTCAAGAAAACAAT[BHQ1]	20		

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

2.3.6 | 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 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 1.2, 2.1-2.3 and 3.1 and 1 µL for all other MPs, see Table 2.1), 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). 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.

2.3.7 | 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: 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 (due to digestion) in its feces. 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, second with the zero's included. The outcomes of both analysis were closely compared for any substantial differences between the two approaches (see Results). Distributions of DNA content were non-parametric and of a small sample size. Therefore, differences between DNA content were tested using non-paired 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, Wheeler & Dinno, 2021). Means and confidence intervals (95%) were determined by bootstrapping (R=10000, 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 non-parametric 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 1 seed only or mice fed 5 seeds only) the result of the overall analysis (based on the full dataset) 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 2.5 and Figure S2.7). 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, 2014).

2.4 | 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 fecal samples collected within 8 hours after feeding five onion and carrot seeds to wood mice (Figure 2.1). Nine to 24 hours 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 fecal samples collected within this time frame. Fecal samples collected 25-36 and 37-48 hours 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). In percentages of the total amount of DNA copies collected within 48 hours, 98.9% of total onion DNA and 97.7% of total carrot DNA had been excreted within 24 hours after feeding.

Seed DNA was detectable in fecal samples even when only one onion and carrot seed was fed to a wood mouse although with large variation. Mean onion DNA copies in fecal 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 fecal 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 fecal samples was found between wood mice fed one onion and carrot seed and wood mice fed with five onion and carrot seeds (Figure 2.2). 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 ($W = 332$, $p < 0.0005$). 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 fecal samples of mice fed with one carrot seed ($W = 427$, $p < 0.005$).

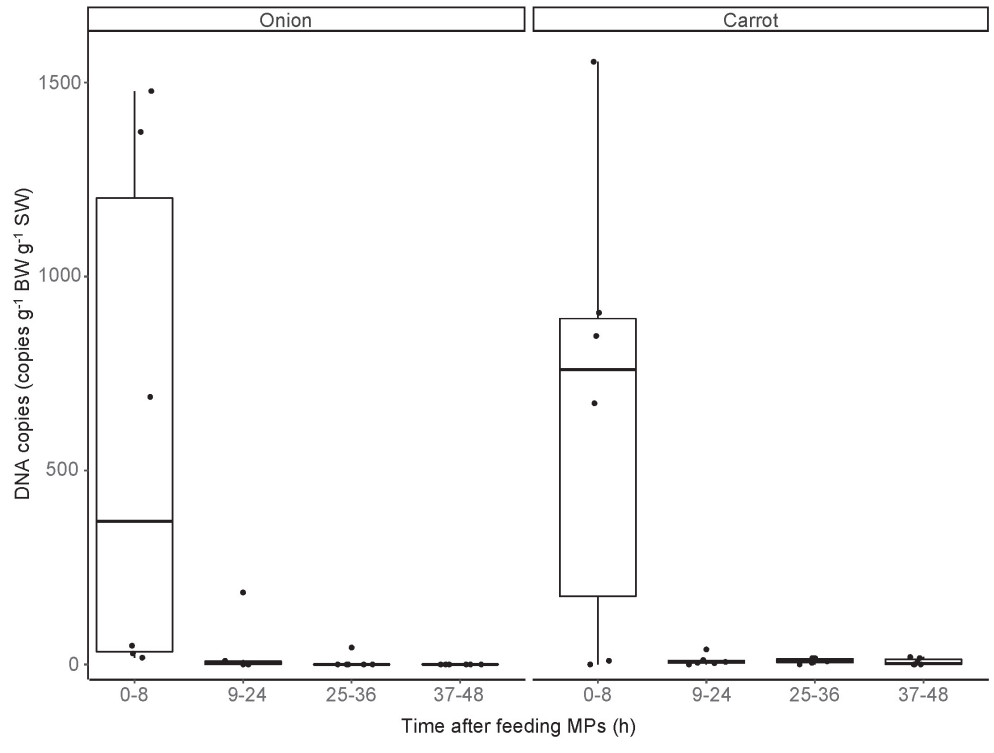

Figure 2.1 | Gut passage time analysis. Total number of onion (left) and carrot (right) DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples excreted by six adult wood mice fed matrix pellets with five onion and five carrot seeds plotted through time after feeding (y-axis). Primer sets used were AC1 and Dcsat1.

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; $W = 28$, $p < 0.05$) (Figure 2.3). 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 (χ^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 feces for mice fed one seed or five seeds (Figure S2.2). However, the number of onion DNA copies found in fecal samples of mice that had been fed with MPs consisting of one onion seed varied with MP composition ($\chi^2 = 12.5$, df = 3, $p < 0.05$) (Figure 2.4, A). Onion DNA content of fecal samples from mice fed with MPs of a ‘0%

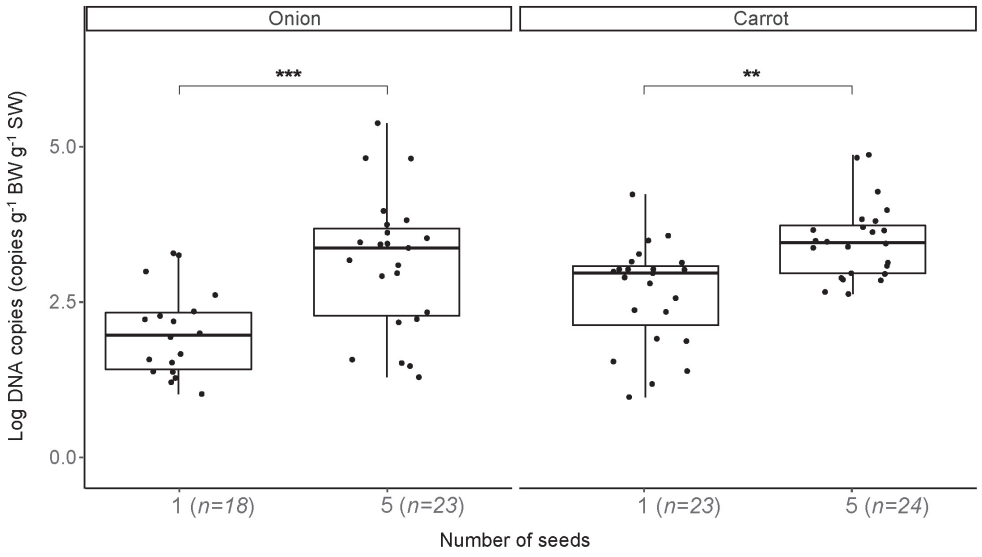

Figure 2.2 | Boxplot showing the difference in the number of log DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples of wood mice fed with one onion and one carrot seed (MP1.2) or five onion and five carrot seeds (MP1.1). Mice of different sex and age were included. Primer sets used were AC1 and Dcsat1. Zeros were removed from this analysis (n = 8 out of 96). Statistically significant differences are depicted ($p < 0.0005$ '***', 0.005 '**').

non-target 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 fecal 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, $Z = -3.1$, $p < 0.005$). 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 neither found for onion (χ^2 (3, N = 62) = 3.1, $p = 0.4$) nor for carrot seeds (Figure 2.4, B).

DNA content in fecal samples of mice fed with one or five onion seeds did not show significant differences when quantified with markers AC1 compared to AC3 (Figure 2.5, A). DNA content in fecal samples of mice fed with one or five carrot seeds also showed no significant differences between markers Dcsat1 and Dcsat3 (Figure 2.5, B). 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 ($W = 180$, $p < 0.05$).

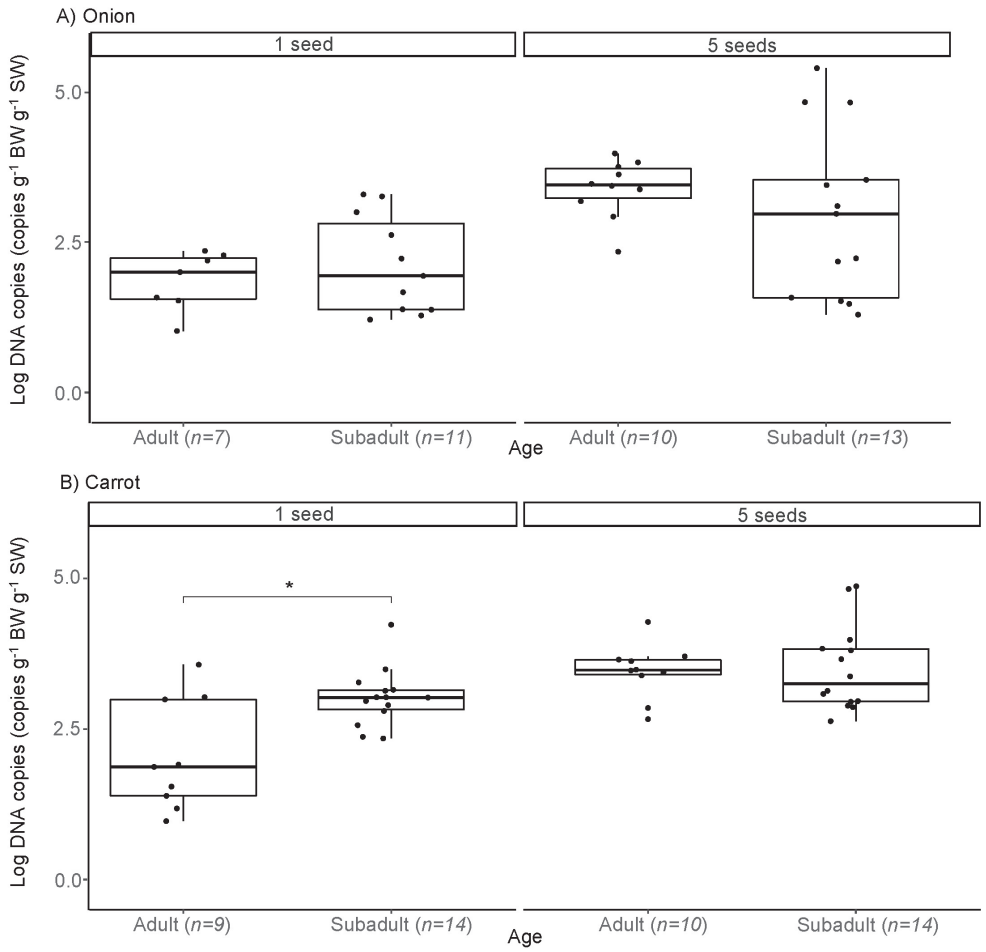

Figure 2.3 | The number of log-transformed onion **(A)** and carrot **(B)** DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples of adult and subadult wood mice fed one seed (MP1.2) or five seeds (MP1.1). Primer sets used were AC1 and Dcsat1. Zeros were removed from this analysis (n = 8 out of 96). Statistically significant differences are depicted ($p < 0.05$ '*').

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 ($W = 202$, $p = 1.0$).

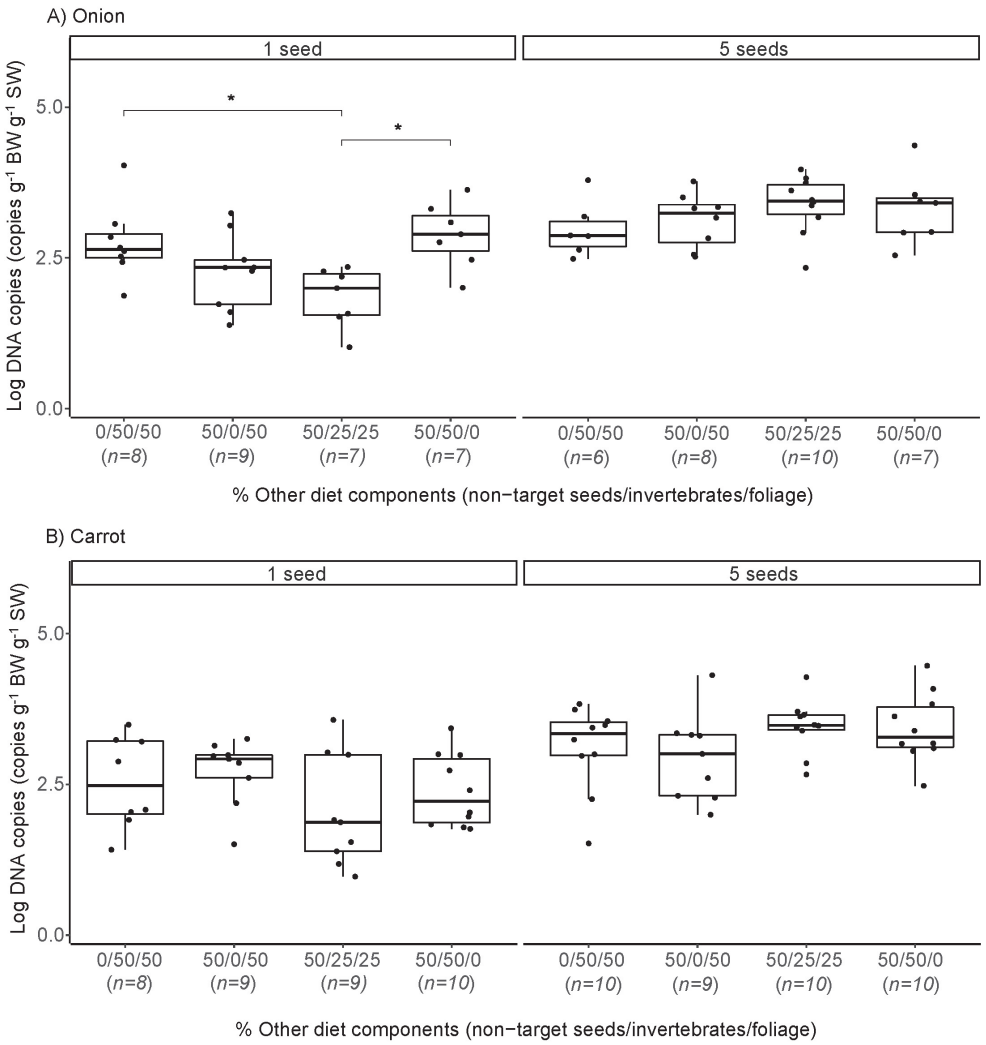

Figure 2.4 | Effect of diet composition (in percentages non-target seeds/invertebrates/foliage) on the number of log-transformed onion **(A)** and carrot **(B)** DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples of adult wood mice fed one seed or five seeds. MP 1.1 -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 ($p < 0.05$ '*').

The above analyses were also performed including measurements that did not yield any onion or carrot DNA copies (see Appendix S2.2 for all the results including figures and statistics). Aside from the differences within treatment groups (last paragraph of Appendix S2.2), 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.

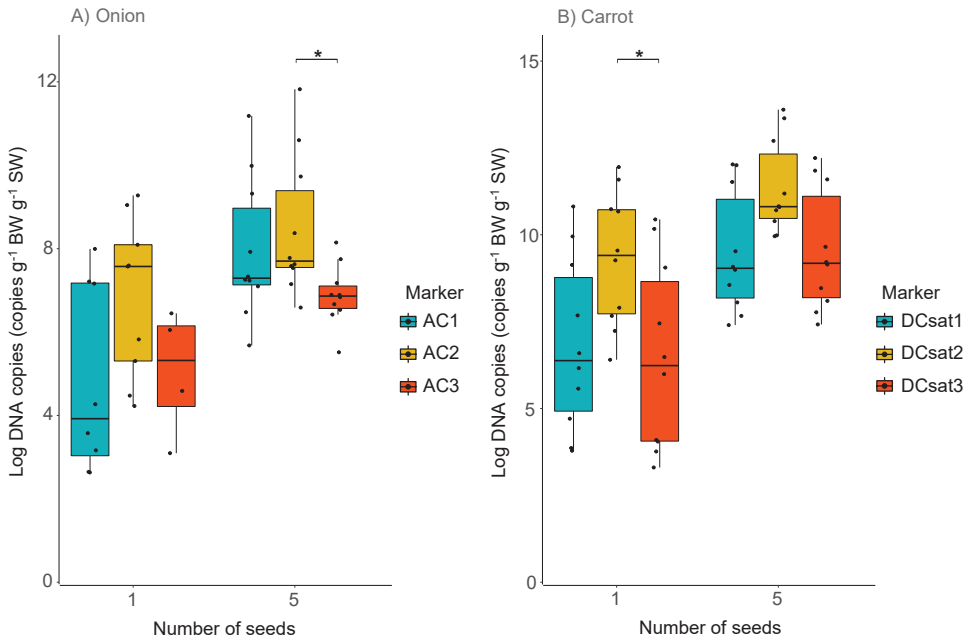

Figure 2.5 | Log-transformed onion **(A)** and carrot **(B)** DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples of wood mice fed different numbers of seeds (x-axis). MP 3.1 – 3.2 were used. N = 10 for each number of onion and carrot seeds fed for each marker. 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).

2.5 | Discussion

Quantification of DNA content in wood mice feces 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, Scott and Timmis (1998) stated that NUPTs vary interspecifically in size and copy number as well as intraspecifically 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, NUPTS and cpDNA are both 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 6 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 non-significant. 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 hours without variation, which is in the range of passage time in mammals (Lambert, 1998). Tsuji, Miura, Kotoge, Shiraishi and Murai (2015) proved 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 feces. Even trace amounts of DNA of up to 2 copies per measured PCR sample (22 µL) were detectable, demonstrating extremely high sensitivity using this fecal DNA quantification technique.

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 would 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 pre-selected, 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 fecal 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 that there was no effect of diet composition on DNA quantification as these adaptations could have potentially leveled the differences in respective substrates. This result implies that quantification of seed intake in e.g. field conditions is possibly independent of the diet eaten in the field. This suggests that when wild wood mice would be 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 even possible.

Advances in DNA methodologies have led to improvements in the ability to detect species and communities in less-favorable (due to rapid DNA degradation) sampling environments such as aquatic environments, GITs and feces (Andres, Sethi, Lodge, & Andrés, 2020). Mitochondrial or plastid DNA is mostly favored 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. (2020) have documented the application of multiple amplicon-

based methods to obtain intraspecific nuclear genetic information from 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 an 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., 2020; Iversen, Kielgast, & Sand-Jensen, 2015) and heavily impacted by taxon-specific amplification biases (Andres et al., 2020; Kelly, Shelton, & Gallego, 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, Senar, & Symondson, 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 semi-quantitative 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 feces 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 and 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 versus 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 versus 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).

2.6 | Supporting information and data accessibility

Additional supporting information can be found in the online version of the article at the publisher's website (scan the QR code below). 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.