Detection of prey DNA in bat feces: Effects of time since feeding, meal size, and prey identity

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Abstract
Molecular analyses of feces are widely used to study the feeding ecology of bats. However, little is known about how detectability of prey DNA from bat feces is influenced by gut retention time, mass of prey eaten, or prey identity, which hampers the interpretation of field data. Here, we address these knowledge gaps by conducting a feeding experiment in which different insect species and meal sizes were offered to two bat species, *Myotis daubentonii* and *Pipistrellus nathusii*. The feeding regime consisted of three main and three side prey species fed in different masses, whereupon feces were collected over 72 h and examined for prey DNA using a species-specific multiplex PCR system. DNA of all three main prey species was detectable from feces produced within 1 h after consumption of a meal. Prey detectability decreased over the following 10–20 h, and after 40 h only spurious detections occurred. For both prey and bats, species identity affected detectability: for example, mealworms were detected for a longer time post-feeding compared to wax moths/house flies, and the latter were detected for a shorter time in *M. daubentonii* than in *P. nathusii*. While DNA from all three side prey species was detectable, signals were weak and detections limited to the first 20 h after consumption. These findings indicate that detectability of prey DNA from bat feces is foremost affected by the mass of prey consumed and time post-feeding, whereas it does not strongly differ between prey species. To increase the detection of prey ingested in low mass, we suggest collecting fecal samples soon after bats return from foraging, as detectability is highest right after feeding occurs, and detections largely depict the previous foraging bout (with detection windows usually <24 h).

KEYWORDS
Chiroptera, diagnostic PCR, diet analyses, molecular detection, multiplex PCR, *Myotis daubentonii*, *Pipistrellus nathusii*, post-feeding detection interval
DNA-based methods are increasingly being used to identify prey in bat feces (e.g., Foo et al., 2017; Krauel et al., 2018; Krüger et al., 2014a; Zeale et al., 2011). The ecological questions addressed so far include, for example, to pin-point dietary differences between males and females in European free-tailed bats Tadarida teniotis (Mata et al., 2016), to assess if bats prey on potential pest insects (Alipoura et al., 2018), or to examine if there is a dietary partitioning in boreal bats (Vesterinen et al., 2018). The strength of such molecular methods is that they increase the likelihood that so far overlooked or rare taxa are detected (e.g., Clare, 2014; Razgour et al., 2011; Vesterinen et al., 2013) by enabling the identification of prey that are strongly digested or the identification of soft-bodied prey and insect larvae (Clare et al., 2009; Razgour et al., 2011). Furthermore, targeting the DNA of prey increases the likelihood of attaining a highly resolved taxonomic description of a bat's diet (Clare et al., 2011; Gordon et al., 2019; Sousa et al., 2019; Vesterinen et al., 2018), given the availability of reference sequences, whereas morphological methods usually only allow prey identity to be resolved the family or order level (Ashrafi et al., 2011; Hayes et al., 2019; Krüger et al., 2012; Pereira et al., 2002).

To make meaningful interpretations of prey DNA detected from field-collected fecal samples (Greenstone et al., 2014) and to not bias results toward the more detectable and/or longest detectable prey, factors that influence post-feeding prey DNA detection intervals, however, need to be accounted for. For example, after consumption of a meal, the detection success of prey DNA has been shown to depend on consumer and prey combinations (e.g., McInnes et al., 2017; Thalinger et al., 2017). Therefore, to resolve temporal and spatial predator-prey dynamics from field-collected fecal samples, it is advisable to conduct feeding experiments to investigate the severity of such biases (Bai et al., 2019; Morris et al., 1994; Oehm et al., 2011; Salvarina et al., 2013; Sousa et al., 2016; Stalinski, 1994; Thalinger et al., 2016, 2017). However, although feeding experiments, where bats were fed prey labeled with undigestible markers, such as pollen, fuchsin, or fluorescence dye, have shown that the majority of the prey consumed pass through the digestive system of bats within a few hours after consumption (Morris et al., 1994; Roswag et al., 2012; Stalinski, 1994), it is questionable if these findings apply to more sensitive DNA-based detection methods.

Despite the increasingly common usage of DNA-based prey detection in bats (e.g., Lim et al., 2018; Taylor et al., 2017), little is known on how the detectability of prey DNA is affected by prey identity and mass of consumed prey. Therefore, we here address these knowledge gaps in order to aid sampling design of future field studies, as well as interpretation of already available field data (cf. Alberdi et al., 2019) by evaluating how: (a) gut transition time, (b) prey identity, and (c) the mass of consumed prey, affect post-feeding prey DNA detection success in feces of two species of bats commonly occurring in Central Europe.
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regime consisting of six insect species. The bats were fed once a day (9 pm) on a pre-decided diet, such that each day one main prey (fed ad libitum but at least 1 g) was combined with a different, much smaller side prey (four individuals per species). The order of the main prey was kept the same during the three repetitions of the experiment, whereas the side prey was rotated so that each main prey species was combined with each side prey species (Figure 1). The feeding regime consisted of the main prey *T. molitor* (yellow mealworm), *Musca domestica* (common housefly), or *Galleria mellonella* (greater wax moth), and the side prey *Drosophila melanogaster* (common fruit fly), *Sinella curviseta* (springtail), and *Acyrthosiphon pisum* (pea aphid). The average mass per individual of prey species is shown in Table 1. *Tenebrio molitor* and *G. mellonella* were fed as larvae, whereas *M. domestica* was fed as imago or pupa, and the side prey species as imagines. To simplify handling, adult dipterans and aphids were freeze-killed before they were used in the feeding experiment. The remaining insect species (*T. molitor*, *G. mellonella*, and *S. curviseta*) were killed immediately by decapitation or squeezing them prior to feeding to the bats. The main prey *T. molitor*, *M. domestica*, and *G. mellonella* were attained from a pet shop (Megazoo, Rum, Austria), while *D. melanogaster* was provided by the Institute of Molecular Biology, University of Innsbruck. *Acyrthosiphon pisum* and *S. curviseta* were produced from our own stock cultures (Department of Zoology, University of Innsbruck). Water was provided while feeding to all bats. Fecal pellets were collected for each bat individually and all pellets retrieved for a specific time point were placed in 1.5 ml reaction tubes using gloves and disposable plastic spoons. Within the first 9 h after each feeding event, all pellets produced within 1 h were pooled per bat individual, thereafter, pooling was done at 3-h intervals. Overall, 448 samples were collected (220 of *M. daubentonii*, 228 of *P. nathusii*). One sample contained between one and 14 fecal pellets providing a total of 1102 pellets for the whole experiment which included 554 pellets of *M. daubentonii* and 548 pellets of *P. nathusii*. All pellet samples were stored at −28°C upon collection.

2.3 Development of multiplex PCR assay

All samples used to establish the multiplex PCR assay were DNA extracted using the DNeasy blood and tissue kit (Qiagen) following the

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**FIGURE 1** Schematic outline of the feeding trial including two bat species and six insect prey species. (a) Feeding regime during the 12-day feeding trial. Day 1–3: pre-feeding trial to harmonize starting conditions; Day 4–12: feeding trial with a main prey (three species, written in larger font size), combined with a side prey (three species, written in smaller font size). Each feeding trial lasted for 3 days and was repeated three times per bat individual. Note, in each 3-day trial, the feeding sequence of main prey was kept constant, whereas the feeding sequence of side prey was rotated to obtain different prey combinations. (b) Timing of daily sample collection events. During the first 9 h (9:00 pm till 6:00 am), fecal pellets were collected hourly, between 9 and 24 h sampling was done every 3 h. Note, sampling at t = 24 h was conducted prior to the following days feeding (t = 0 h).
the respective primers in the primer mix employed in the multiplex PCR.

Note: Provided are the target species, the primer name, the primer sequences, the respective fragment size per primer pair, and the concentration of the respective primers in the primer mix employed in the multiplex PCR.

| Species            | Target species | Sequence (5′-3′)                          | Fragment size (bp) | Concentration (µM) |
|--------------------|----------------|-------------------------------------------|--------------------|--------------------|
| Galleria mellonella| Acy-pis S709   | TGAACATTATATCCACCTTATGCACAA              | 95                 | 0.2                |
|                    | Acy-pi A709    | GATGAAATTCCCTGCTAGGTGTAAG               |                    |                    |
| Musca domestica    | Mus-dom S708   | TGATTACCTCTTCCTGATATTACCTC              | 114                | 0.2                |
|                    | Mus-dom A708   | TCCACCATGACAAATATTGAT                    |                    |                    |
| Tenebrio molitor   | Ten-mol S705   | ACATAGCTTTTCCCCCGACTT                    | 152                | 0.2                |
|                    | Ten-mol A704   | TCTACCTCTTCGCTAGGCGAATATTAG            |                    |                    |
| Sinella curviseta  | Sin-cur S710   | GTATCTTCTCCCTCATATAAAC                 | 229                | 0.2                |
|                    | Sin-cur A710   | TTCTATATATCTTTCTGGG                  |                    |                    |
| Drosophila melanogaster| Dro-mel S706 | GAATTAGGACATCTCTGGGACC                  | 246                | 0.1                |
|                    | Dro-mel A706   | TCTCTTTTCAACTATTTCTCTTTACTAAATAG      |                    |                    |

Note: Provided are the target species, the primer name, the primer sequences, the respective fragment size per primer pair, and the concentration of the respective primers in the primer mix employed in the multiplex PCR.
2.4 | Screening of bat fecal samples for prey DNA

The newly developed multiplex PCR assay was used to screen the fecal samples for insect prey DNA. Each sample was lysed in 400 µl TES-buffer (0.1 M TRIS, 10 mM EDTA, 2% sodium dodecyl sulfate; pH 8) and 10 µl proteinase K (20 mg/ml). For mechanical fragmentation, 4–5 glass beads (Ø 2 mm) were added to each reaction tube and pellets were crushed by a tissue homogenizer Precellys (BertinCorp) for 2 times at 5590 g for 30 s. Afterward, samples were centrifuged at 21,400 g for 1 min and incubated overnight at 56°C on a rocking platform. DNA extraction was carried out with the BioSprint 96 robotic platform, program “BS96 DNA Tissue” (Qiagen) using the BioSprint 96 DNA blood Kit (Qiagen) in accordance with the manufacturer's instructions. Up to 90 fecal sample lysates, two lysate negative controls and four extraction controls were processed per 96-well plate extraction. To check for DNA contamination, all control samples were tested with the multiplex PCR assay and all of them turned out to be negative.

PCR products were separated by the QIAxel Advanced automatic capillary electrophoresis system (Qiagen) with the corresponding software QIAxel ScreenGel (Method AM320; Qiagen). All DNA fragments which exhibited the expected fragment size and produced a signal strength ≥0.1 RFU were deemed as positive detections. Out of the 448 samples, 433 were positive for at least one of the prey species and in 91 samples more than one prey species was detected.

2.5 | Statistical analysis

To test how prey DNA detection success varies over time between prey and bat species, we fit a generalized linear mixed model with a binomial (logit) link function to the feeding trial detection data. Additionally, to show if amplification strength (RFU value) declined predictably over time and could be used to approximate the time since consumption, we fit a linear mixed model to those samples that were positive for prey DNA. Models were fitted using the glmer/lmer functions available in the “lme4” package (Bates et al., 2015).

To compensate for non-independence in the collection of feces due...
to repeated sampling from the same individual, the identity of each bat was included as a random factor. That model assumptions were met was assessed using diagnostic plots (Zuur et al., 2010). The most parsimonious model, that was selected based on Akaike information criterion, was one that included full factorial interactions between all included parameters. To show pairwise differences in prey DNA detection between bat and prey species, pairwise tests were conducted after which reported p values were corrected for false discovery rate. As only one sample was positive for the springtail S. curviseta, we excluded this prey species from the analysis.

3 | RESULTS

The greatest number of fecal pellets was deposited between 2 and 3 h after feeding (Figure 3). Defecation decreased over time, which meant that the majority of the tested fecal pellets were collected within the first 9 h of the feeding trial (72% and 67% of the pellets produced by M. daubentonii and P. nathusii, respectively; Figure 3). DNA of all three main prey species was detectable in pellets produced within 1 h after feeding. The likelihood of detecting each prey, however, decreased during the first 10–20 h post-feeding, and after 40 h only spurious detections occurred for T. molitor (Figure 4). Prey DNA was overall detected for a shorter time post-feeding in M. daubentonii than in P. nathusii (t = 2.492, p < 0.01, Figure 4).

However, this difference was only present for the two main prey G. mellonella (z = 2.364, p < 0.05, Figure 4) and M. domestica (z = 3.105, p < 0.01, Figure 4), whereas no differences between bat species were evident for T. molitor (Figure 4). Despite this, DNA from T. molitor was detectable for a longer period of time in both bat species compared to the other two main prey G. mellonella (P. nathusii: z = 4.167, p < 0.001; M. daubentonii: z = 3.651, p < 0.001, Figure 4) and M. domestica (P. nathusii: z = 4.689, p < 0.001; M. daubentonii: z = 3.458, p < 0.001, Figure 4). In fact, T. molitor was the only prey where spurious DNA detections occurred during the entire course of the 72 h feeding trials (Figures 4 and 5). Furthermore, as RFU values declined over time (t = 17.86, p < 0.001; Figure 5), these spurious detections always had a low RFU value, indicating low amounts of prey DNA present in the fecal samples.

DNA from all three side prey species was detected, but RFU values were weak for all of them and detections limited to the first 20 h after feeding (Figure 6). This made a meaningful test of differences impossible. Nevertheless, most of prey DNA detections occurred for the larger side prey D. melanogaster (Figure 6), whereas A. pisum was only detected within the first 6 h after feeding (Figure 6) and only one single detection of the smallest prey, S. curviseta, occurred (after 4 h; data not shown).

4 | DISCUSSION

With DNA-based analysis of trophic interactions, it is well known that differences in how well prey can be detected from fecal samples will bias results toward the prey that is detectable the longest (Greenstone et al., 2014). However, despite that a multitude of studies have been using DNA-based methods to assess the dynamics between bats and their prey (e.g., Aizpurua et al., 2018; Gordon et al., 2019; Hayes et al., 2019; Mata et al., 2016; Weier et al., 2019), the severity of such biases has not been investigated. To bridge this knowledge gap, we here show how the post-feeding prey DNA detection interval depends on a range of interacting factors such as the species of prey, the mass of prey that was consumed, and the species of bat. Of these factors, the influence of the mass of prey consumed was the strongest, where the side prey, offered as the smallest prey with factor of 10^3-10^4 lower mass than the main prey, was almost undetectable or detectable only for a few hours post-feeding. Whereas, the larger prey mass of the main prey species was easily amplifiable for ~24 h post-consumption. The poorest detection success of all prey species offered in the current feeding experiment was for the collombolan S. curviseta, which was the species with the lowest mass, corroborating that how much prey mass is consumed is a key factor that determines detection success. We also found that pellets

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**Figure 4** Decline in prey DNA detection probability over time for Myotis daubentonii and Pipistrellus nathusii split between the three main prey species Tenebrio molitor, Musca domestica, and Galleria mellonella. Shaded area shows 95% confidence intervals.
produced shortly after a feeding event had the highest probability to show the “full” diet spectrum of bats, highlighting the need of standardizing collection times of feces (i.e., to collect feces which have been produced within a certain time post-feeding) to avoid biasing results by mixing samples with different prey DNA detection probabilities. Therefore, we would suggest that one possibility to minimize this bias is to ensure that defecation has occurred as close to feeding processes as possible and to capture bats directly after they return from foraging trips. This should be possible as for many bat species it is known when their time of emergence and foraging peaks is occurring (Duvergé et al., 2000; Jones & Rydell, 1994; Rydell et al., 1996). However, it should be considered that this behavior differs between species and that bats have many feeding episodes per night with different prey species. Therefore, feces from bats from different species may have to be collected at different timepoints.
We found that gut transition of prey DNA was very rapid for both *M. daubentoni* and *P. nathusi*. This rapid transition allowed prey DNA to be detected from feces already within the first hour after feeding and corroborates findings from studies using marker labeled prey (Morris et al., 1994; Roswag et al., 2012; Stalinski, 1994). However, as prey DNA can consistently be detected for 24 h, prey is detectable for a considerably longer time with molecular methods than the maximum of 24 h that has previously been reported by studies that were investigating prey hard parts (e.g., Stalinski, 1994). In fact, we could show that the probability of detecting prey DNA did not decrease until after another meal had been consumed on the second day of the feeding trials. After this period (>24 h), detections of the first prey species decreased rapidly to the point where the prey was no longer detectable, except for *T. molitor* that was sparsely detected up until the third day after consumption. This rapid gut transition and defecation are common among flying vertebrates such as insectivorous bats (Morris et al., 1994; Neuw, 2000; Roswag et al., 2012; Stalinski, 1994) and correspond to the morphology of the digestive tract which is an adaption to the aerial lifestyle (Price et al., 2015; Stutz & Ziswiler, 1984). It should be noted that during our experiment the movements of the bats were limited to changes in hanging position. This is comparable to bats resting in roosts (usually this is the source of fecal samples collected by bat dietary studies (e.g., Andriollo et al., 2019; Vesterinen et al., 2018). This likely slowed bats’ metabolism (Bai et al., 2019; Buchler, 1975) and therefore the current results may not directly apply to samples obtained from flying bats caught to collect feces.

We also found that the differences in detectability between types of meals (i.e., species of prey) were small. Even though spurious detections outside of the predicted detectability window were found for *T. molitor* mealworms, the most hard-bodied prey in our feeding experiment, this was likely an effect of the thick exoskeleton of the mealworms remaining a longer period within the guts of bats than more easily digestible prey. This is analogous to observations of voles fed diets of either green plant parts or grain, where the latter were detected much longer post-feeding than the former (Kostelecka-Myrcha & Myrcha, 1964). Whether the hypothesis holds true, that less easily digestible insect prey can be detected for longer times post-feeding than soft-tissue prey, needs to be tested in future work.

While differences in prey DNA detection success did occur between bat species, these were small and only present for *M. domestica* and *G. mellonella*, but not for *T. molitor*. Therefore, the current experiment suggests that differences in detectability between these two bat species are much smaller than differences due to prey identity or mass of prey consumed. Nevertheless, the lack of observed differences between the two bat species may not be generalizable to all bat species, as the selected bats were of the same sex, are very similar in size and diet, and belong to the same family (Vespertilionidae). Feeding experiments conducted with labeled prey indicate that such interspecific differences are likely, for example, Roswag et al., 2012 observed quite different median maximum retention times between 10 different Vespertilionid bat species using fluorescence-marked mealworms. Thus, even though we did not find large differences in post-feeding prey DNA detection intervals between the two investigated bat species, we cannot conclude that species identity effects will not bias the DNA-based diet results of field studies. Another confounding factor, which might have affected prey DNA detection intervals, is the treatment of the prey before feeding them to the bats: dipterans and aphids were freeze-killed, while the other insects were fed fresh. Freezing might have released the DNA through breaking up of cells, and hence, affected the digestibility of the prey. Despite these possible effects, the current data does not indicate that the freeze treatment has affected our results.

As RFU values can be used to approximate the amount of prey DNA amplified during PCRs (Thalinger et al., 2019), and therefore, serve as a proxy for the (relative) amount of prey tissues consumed (Deagle et al., 2019), we also tested how RFU values decrease over time for each of the three main prey species. However, while results showed that observed RFU values on average do decrease over time, they only did so predictably for very low DNA concentrations. Arguably, this is because at higher DNA concentrations the amount of amplicon generated during PCR plateaus (Thalinger et al., 2019).

Most of the studies assessing the diet of bat species using DNA-based approaches have employed a metabarcoding approach (e.g., Foo et al., 2017; Krauel et al., 2018; Krüger et al., 2014 a; Mata et al., 2016, Vesterinen et al., 2018; Zeale et al., 2011). Prey metabarcoding, however, entails the use of general arthropod/insect primers, many of them with well-known amplification biases, meaning that they will preferably amplify DNA from certain taxa whereas others are missed (Alberdi et al., 2019). Moreover, the metabarcoding approaches can be biased when it comes to the detection of rare DNAs in a sample, as the most dominant DNA types are typically sequenced (Rennstam-Rubmark et al., 2019). What prey sequences can finally be retrieved from a dietary sample therefore depends largely on the primers employed and the sequencing depth per sample. Due to these methodological biases, we in this study opted to detect the DNA of each of the six prey species using a newly designed multiplex PCR assay. This approach has the advantage that it will detect the DNA of the different prey species independently of the amount of other (prey) DNA present in the sample, and therefore, provide the most accurate information on which target prey DNA is present in a particular sample. The detection sensitivity was balanced across prey species (see Sint et al., 2012) to avoid introducing differences in detection sensitivity across the prey taxa. Based on the current findings, we would suggest that when diagnostic PCR approaches are used to detect specific prey, it will increase the possibility to detect prey taxa consumed in small quantities. Moreover, this will balance detectability windows between different amounts of prey when fecal samples are pooled to increase the total amount of DNA contained in samples before they are subjected to PCR. Note, however, that this will not be the case with prey metabarcoding approaches, where pooling has been shown to decrease the diversity of prey taxa which can be retrieved from bat fecal samples (Mata et al., 2019).
5 | CONCLUSIONS

While prey DNA detectability can be influenced by a range of factors outside of the ones tested by this study (Traugott et al., 2021), our findings show that prey DNA detectability windows in feces are most strongly affected by the mass of insect prey consumed by bats. On the other hand, differences between bat and prey species were so small that they are unlikely to strongly bias the results of field studies. To most completely capture the insect prey which has been consumed, i.e., for also detecting prey which has been consumed in very small quantities (here side prey), fecal pellets produced shortly after feeding provide the best results. Therefore, we would suggest that one way to minimize biases is to ensure that defecation has occurred as close to feeding processes as possible by capturing bats directly after they return from foraging trips. This will allow to avoid biasing results by mixing samples with different prey DNA detection probabilities. Our experiments have also shown that the prey DNA retrieved from fecal pellets typically stems from the prey consumed within the previous night (or foraging bout), indicating that field-derived results do usually not contain a molecular imprint of meals taken several nights before, which could complicate the analysis of temporal patterns in bat-prey relationships.

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AUTHOR CONTRIBUTIONS

PS, SAR, and MT designed the study. PS, SAR, and MT conducted feeding experiment. PS and MT conducted the molecular analyses. PS, ORR, SAR, and MT interpreted the obtained data. PS, ORR, and MT wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data generated for this study is available on DRYAD (https://doi.org/10.5061/dryad.d51c5b033).

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