Title: Comparing three types of dietary samples for prey DNA decay in an insect generalist predator

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Keywords: diagnostic PCR, molecular diet analysis, prey detection, Carabidae, trophic interactions

Running title: DNA decay in three types of dietary samples

Abstract

The rapidly growing field of molecular diet analysis is becoming increasingly popular among ecologists, especially when investigating methodologically challenging groups such as invertebrate generalist predators. Prey DNA detection success is known to be affected by multiple factors, however the type of dietary sample has rarely been considered. Here, we address this knowledge gap by comparing prey DNA detection success from three types of dietary samples. In a controlled feeding experiment, using the carabid beetle *Pterostichus melanarius* as a model predator, we collected regurgitates, feces and whole consumers (including their gut contents) at different time points post-feeding. All dietary samples were analyzed using multiplex PCR targeting three different length DNA fragments (128 bp, 332 bp and 612 bp). Our results show that both the type of dietary sample and the size of the DNA fragment contribute to a significant part of the variation found in the detectability of prey DNA. Specifically, we observed that in both regurgitates and whole consumers prey DNA was detectable significantly longer for all fragment sizes than for feces. Based on these observations, we conclude that prey DNA detected from regurgitates and whole consumers DNA extracts are comparable, whereas prey DNA detected from feces, though still sufficiently reliable for ecological studies, will not be directly comparable to the former. Therefore, regurgitates and feces constitute an interesting, non-lethal source for dietary information that could be applied to field studies in situations when invertebrate predators should not be killed.

Introduction

DNA-based diet analysis is rapidly being employed as a widespread tool for empirically characterizing diet and trophic interactions in a broad range of vertebrates and invertebrates (Traugott *et al*. 2013; Clare 2015). DNA-based methods for diet analysis typically rely on the detection of short fragments of prey DNA, recovered from predator's gut contents (*e.g.* Leray *et al*. 2015; Mollot *et al*. 2014) or other types of dietary samples such as feces, regurgitates, or whole consumers (*e.g.* Ibanez *et al*. 2013; Kartzinel *et al*. 2015; Thalinger *et al*. 2016; Wallinger *et al*. 2015). The success of DNA-based approaches to analyze trophic interactions is mainly due to the fact that they allow the direct and accurate identification of trophic links from minute amount of starting material, even for very small-sized organisms such as mites (Pérez-Sayas *et al*. 2015) or zooplankton (Durbin *et al*. 2012). Furthermore, the rapid growth of public sequence databases and methodological improvements in detection sensitivity and high-throughput technology, offer time- and cost-effective procedures applicable to a great variety of ecological systems and to large sample sizes (*e.g.* Valentini *et al*. 2009; Pompanon *et al*. 2012; Sint *et al*. 2011).

DNA-based diet analysis is particularly valuable for studying invertebrate generalist predators (Symondson 2012). Indeed, DNA methods offer a sensitive and flexible alternative to traditional behavioural or dissecting techniques that often fail to detect prey that does not leave hard remains in these cryptic liquid feeders (Traugott *et al*. 2013). DNA techniques are, however, also subject to bias and prey DNA detection success could be hampered by a variety of factors among which the type of dietary sample could play an important role (King *et al*. 2008; Pompanon *et al*. 2012; Traugott

70 *et al*. 2013). In the case of arthropods, whole body extracts are usually the most
71 convenient source of dietary DNA that avoid laborious dissections. Besides the
72 drawbacks of a lethal approach (e.g. sacrificing rare or endangered species; affecting
73 population dynamics, etc.), whole body extracts may pose additional challenges
74 especially in the case of DNA metabarcoding diet analysis. As DNA metabarcoding
75 combines general primers and high-throughput sequencing, the concomitant
76 amplification of consumer DNA usually compromises the detection success of scarcer
77 and degraded prey DNA (*e.g.* Shehzad *et al*. 2012; Piñol *et al*. 2014).
78 Waldner & Traugott (2012) demonstrated that regurgitates, a fluid mixture
79 containing semi-digested prey remains and digestive enzymes, obtained from predatory
80 carabid beetles provided superior prey DNA detection rates compared to whole body
81 DNA extracts. Another prospective source of food DNA are feces, although their use as
82 a dietary source in invertebrates is still uncommon (*e.g*. Ibanez *et al*. 2013; Redd *et al*.
83 2014; Sint *et al*. 2015). Usually, both regurgitates and feces seem to provide similar or
84 better detection rates compared to whole body extracts (Durbin *et al*. 2012; Egeter *et al*.
85 2015; Unruh *et al*. 2016), and contain comparatively much less consumer DNA, making
86 them putatively an ideal source for metabarcoding diet analysis. Nevertheless, to date
87 we lack a comparative and quantitative assessment of the respective efficiency in
88 detection success between whole bodies, regurgitates and feces as well as prospective
89 interactions with other sources of non-dietary variation such as the target DNA fragment
90 size.
91 In this study, we address this knowledge gap by comparing the prey DNA
92 detection rates for three types of dietary samples: whole consumers including their gut

content, regurgitates and feces. Using a controlled feeding experiment involving a widespread carabid predator, *Pterostichus melanarius* (Coleoptera: Carabidae) we test the following hypotheses: (i) post feeding prey DNA detection success should be similar or better in regurgitates compared to whole beetles due to lesser degradation of prey DNA in the former; (ii) prey DNA detection success should be lower in feces compared to regurgitates and whole bodies as faecal material represents the final stage of the digestion process; and (iii) prey DNA detection should decrease with increasing DNA fragment size and the time post-feeding for all types of samples.

Material & Methods

Sampling and maintenance of predators

Pterostichus melanarius individuals were collected by dry pitfall traps in two adjacent maize fields situated at the experimental site of INRA Le Rheu (Ille-et-Vilaine, France; GPS coordinates: 48.10744282N; 1.78830482W). Regular 24-hour trapping sessions were conducted in July – August 2013 until a sufficient number of individuals had been collected. All living beetles were brought to the laboratory where they were identified to species level and individually placed in plastic containers filled with loam. Beetles were stored at room temperature and continuously provided with water and food (field-collected earthworms and small pieces of apple).

Feeding experiment

Prior to the feeding experiment, beetles were starved for 96 h in fresh individual plastic Petri dishes (5 cm diameter) containing only a droplet of water. After the starvation period, all beetles were transferred to a new Petri dish and provided with one freshly freeze-killed mealworm (*Tenebrio molitor*, Coleoptera: Tenebrionidae) cut in half. Carabids were allowed to feed for one hour in a dark climatic chamber at 20°. After feeding all beetles which had fully consumed the mealworm were transferred into fresh Petri dishes with no food. Beetles were stored at room temperature and continuously provided with water during the experiment.

For the “whole beetle” treatment, batches of 10 randomly chosen carabids were frozen in 2-mL reaction tubes by immersion in liquid nitrogen at 0, 12, 24, 36, 48, 60, 72 and 96 hours post-feeding. Immersion in liquid nitrogen was necessary as previous tests showed that living beetles do not die immediately after placement at -20°C, leading them to regurgitate into the reaction tube. After immersion, all whole beetles were stored at -20°C. Thirteen starved beetles were never allowed to feed and they were freeze-killed at 0 h to be used as negative controls. For the “regurgitate” treatment, batches of 10 randomly chosen individuals were allowed to regurgitate on a cotton wool tip according to the protocol described in Waldner & Traugott (2012) at 0, 12, 24, 36, 48, 60, 72 and 96 h post-feeding. After regurgitation, all beetles per given time-point were freeze-killed and stored at -20°C. Exactly the same procedure at each time point was applied on a control tip without touching a beetle for checking potential DNA carry-over contaminations. All samples were stored at -20°C prior to DNA extraction and PCR. For the “feces” treatment, 20 carabid beetles were placed in new Petri dishes after feeding with a droplet of clean water. Carabids were continuously checked for feces production

138 at every 6 hours. Detected feces were immediately frozen within the Petri dish at -20°C
139 whereupon the corresponding carabid individual was transferred into a new Petri dish.
140 Feces production was monitored until all beetles died.
141
142 **Molecular diet analysis**
143 Regurgitate and fecal samples were directly lysed in 200 µl TES Lysis Buffer
144 (Macherey-Nagel, Germany) and 5 µl Proteinase K (10 mg/mL) overnight at 56°C. The
145 whole beetles were previously ground using three 4 mm stainless steel beads (Lemoine
146 S.A.S, Rennes, France) within a volume of 620 µl TES Lysis Buffer and 10 µl
147 Proteinase K (10 mg/mL) per beetle. Tissues were disrupted by a 1-minute bead-
148 beating step using a professional paint mixer (Fluid Management Inc., Wheeling, IL,
149 USA). All samples were incubated overnight at 56°C. Respectively 2, 6, and 2 lysate
150 blanks (i.e. no DNA material) were carried out for the whole beetles, fecal and
151 regurgitate treatments. DNA was extracted in batches of 92 samples using the Biosprint
152 96 DNA Blood Kit (Qiagen, Hilden, Germany) on a Biosprint 96 extraction robotic
153 platform (Qiagen) following the manufacturer's instructions. DNA was finally diluted in
154 200 µl TE buffer (0.1 M TRIS, pH 8, 10 mM EDTA) and the extracts were stored at -28
155 °C. To avoid contamination, DNA extractions were done in a separate pre-PCR
156 laboratory using a UVC-equipped laminar flow hood. To check for sample-to-sample
157 cross-contamination, four extraction negative controls (PCR-grade RNase-free water
158 instead of lysate) were included within each batch of 92 samples. All of these controls
159 tested negative using the diagnostic PCR assay described below.

160 The DNA extracts were screened with a multiplex PCR assay targeting three
161 overlapping COI mtDNA fragments of *T. molitor,* i.e. 128 bp, 332 bp and 612 bp. The
162 primer mix contained 6 µM of primers Ten-mol-S210 (5'-
163 TACCGTTATTCGTATGAGCAGTAT-3') and Ten-mol-A212 (5'-
164 CGCTGGGTCAAAGAAGGAT-3') as well as 2 µM of primers Ten-mol-S232 (5'-
165 TAATAAGAAGAATTGTAGAAAACGGG-3') and Ten-mol-S231 (5'-
166 TCATTTTTGGAGCGTGATCC-3') (Oehm *et al*. 2011; Sint *et al*. 2011). Each 10 µl PCR
167 consisted of 1.5 µl template DNA, 5.0 µl of 2x Multiplex PCR Kit reaction mix (Qiagen),
168 1.0 µl of primer mix, 0.5 µl of bovine serum albumin (BSA, 10 mg ml^{-1}), and 2.0 µl of
169 PCR-grade RNase-free water (Qiagen) to adjust the volume. Thermocycling was
170 conducted in Eppendorf Mastercyclers (Eppendorf, Hamburg Germany) and cycling
171 conditions were 15 min at 95 °C, 35 cycles of 30 sec at 94 °C, 90 sec at 63 °C, 1 min at
172 72 °C, and final elongation 10 min at 72 °C. To check for amplification success and
173 DNA carry-over contamination, two positive (mealworm DNA) and two negative controls
174 (PCR water instead of DNA) were included within each PCR, respectively.
175 The PCR products obtained were visualized using QIAxcel, an automated capillary
176 electrophoresis system (Qiagen), with method AL320. The results were scored with
177 Biocalculator Fast Analysis Software version 3.0 (Qiagen) and the threshold was set to
178 0.07 relative fluorescent units. Samples above this threshold and showing the expected
179 fragment length were counted as positives. All DNA extracts that were tested negative
180 in the first run were re-tested with general primers (Folmer *et al*. 1994) in a second PCR
181 to check for any amplifiable DNA (all of these samples tested positive). To ensure

182 contamination-free conditions, PCR preparation and visualization of PCR products were
183 done in two separate laboratories (workflow: from pre- to post-PCR areas).
184
185 **Statistical analyses**
186 A generalized linear mixed model was built to fit a logistic regression on the DNA
187 detection data. We integrated three fixed effects into the model: two qualitative factors,
188 the marker size (128 bp, 332 bp, 612 bp) and the sample type (regurgitates, feces or
189 whole body DNA extracts), and one continuous variable, the time post-feeding. To
190 compensate for non-independence in collection of feces individuals were included as a
191 random effect. The model was fitted using the *glmm* function from the R package
192 "*glmm*" (https://cran.r-project.org/web/packages/glmm). Models were fit using a Monte
193 Carlo sample size of 1024 with 10,000 iterations. The distribution of each of the model
194 parameters was approximated to a normal distribution using the maximum goodness-of-
195 fit estimation with the "*fitdist*" function available in the R package "*fitdistrplus*"
196 (Delignette-Muller & Dutang 2015). The variance in detectability rates explained by the
197 model was estimated using the coefficient of determination method (Tjur 2009). Tests of
198 the differences between mean detectability rates for each of the qualitative factors
199 (marker length and sample type) were conducted using a Z-test. The time point for a
200 prey detection probability of 50% (i.e. the time point at which on average half of the
201 individuals show positive for the target prey) was determined for each dietary sample
202 and DNA fragment size. To compensate for false discovery rate in multiple testing
203 comparisons between fragments were based on 95% confidence limits (CI) as

204 suggested by Greenstone et al (2013). All statistical analyses were conducted using the
205 R software (R Core Team 2013).
206
207 **Results**
208 Detectability of mealworm DNA in *P. melanarius* decreased with increasing post-
209 feeding time and prey DNA fragment length for the three dietary samples (Fig. 1, small
210 *vs* medium and small vs large fragments: $p<0.001$; medium *vs* large fragment: $p=0.08$),
211 with post-feeding detection time intervals being longest for the shortest DNA fragment
212 (Fig. 1 A, B, C). We also observed a significant effect of the dietary sample type, with
213 prey DNA detection success being significantly lower in feces compared to regurgitates
214 and whole beetles for all the three fragment sizes (Fig. 1, in all cases $p<0.001$). There
215 was also a tendency for longer post-feeding detection periods in regurgitates compared
216 to whole beetles (Fig. 1A, B) but differences were not significant ($p=0.6$). Our model
217 fitted the data well for all of the three dietary samples: regurgitates (Fig. 1A), whole
218 beetles (Fig. 1B) and feces (Fig. 1C), and explained 50% of the variance in DNA
219 detectability. Raw data are presented in Table 1. For the small prey DNA fragment, 50%
220 detection time was the highest for regurgitates (94 hours) but the value significantly
221 dropped by more than half for the medium fragment (42 hours) and was significantly
222 shortest for the largest prey DNA fragment (30.6 hours; Table 2). In feces 50%
223 detection probabilities were the lowest for all the three DNA fragment sizes, with only 19
224 hours for the largest DNA fragment (612 pb) and a significantly shorter detection
225 probability for the medium prey DNA fragment when compared to the regurgitate
226 samples (Table 2).

Discussion

How long prey DNA can be detected in a sample is determined by a range of interacting factors related to the environment, the predator-prey system and the molecular techniques used. These might affect results, but how is difficult to disentangle without conducting comprehensive experiments that explicitly account for factor multiplicity. Here, we compared multiple dietary samples from one species of invertebrate consumer, in a controlled feeding experiment, and assessed how the combined effects of the type of dietary sample and DNA fragment size will affect the prey DNA detection probability over time since feeding. Our results show that each of these factors significantly affects the rate at which the probability of detecting prey DNA decreases over time. Consistent with our hypothesis, the time during which DNA could be detected was the longest for regurgitates, for each of the three tested prey DNA fragment sizes. While this was not significantly different from DNA detected from whole beetles, prey DNA contained in feces was detectable for a significantly shorter time for all three fragment sizes.

Our results support the general assumption that regurgitates constitute a good alternative source of prey DNA (Waldner & Traugott 2012; Wallinger *et al*. 2015). Such an alternative could be particularly useful in manipulative food web experiments, where the removal and killing of the targeted predators during sampling could disturb the system under study. As 79% of predaceous land-dwelling arthropods use extra-oral digestion (Cohen 1995), this approach is potentially applicable to a large array of taxa and ecological situations. Furthermore, by containing comparatively less predator DNA,

250 regurgitates could also be a valuable source of dietary data in DNA metabarcoding
251 studies involving the use of general primers (Waldner & Traugott, 2012).
252 Nevertheless, the use of regurgitates could entail some additional limitations
253 such as the detection of only the most recent diet items, and probably represents
254 merely a narrow fraction of individual's diet, especially in generalist feeders with
255 frequent switching behavior such as carabids (Lövei & Sunderland, 1996). Thus, the
256 choice of the most appropriate dietary sample will most likely consist in a trade-off
257 between DNA detection rates and representativeness in terms of diet according to the
258 focus of interest.
259 Fecal samples could provide a more integrated picture of individual's diet. Our
260 results show that overall prey DNA detection was lower compared to regurgitates and
261 whole bodies. Note, however, that this was true only for the medium sized fragment
262 when considering the 50% prey DNA detection probability, with significantly lower post-
263 feeding interval found in feces compared to regurgitates. This indicates that feces
264 overall are a good source of dietary DNA at least in *P. melanarius* beetles. Similarly,
265 earlier study in wolf spiders showed that prey DNA was detectable in spider feces albeit
266 in lower rates compared to whole body DNA extracts (Sint *et al*. 2015). As spiders
267 represent an important group of generalist feeders that typically do not regurgitate, the
268 sole non-lethal dietary sample that could be collected are feces. Also, we cannot rule
269 out the possibility that in our case DNA prey detection success in feces was lower
270 simply due to the constraints of the experiment. As carabids were checked for feces
271 every 6 hours, feces deposited earlier within that timeframe could have experienced

272 higher DNA degradation due to longer exposure to ambient temperature, thus resulting
273 in increased variability in DNA prey detection success.

274 In a recent paper Unruh et al. (2016) even show that there is no difference in
275 DNA detection between whole bodies and feces in the insect predator *Forficula*
276 *auricularia*. While the authors do not discuss the possible mechanisms behind this
277 observation, results tend to suggest that feces could be at least as good dietary source
278 as whole body extracts for predatory insects such as *F. auricularia*. Hence, feces
279 remain a viable non-lethal dietary source in certain situations, as detection rates are
280 generally high.

281 Yet, the question of the time it takes for prey DNA to travel through the digestive
282 tract of insects and how this varies across different taxa remains. Having a better
283 understanding about the temporal aspects of digestion in insects in general, and
284 particularly in carabid beetles is important. Carabids are generalist, mobile feeders with
285 frequent switching behavior (Lövei & Sunderland, 1996) meaning that frequent diet
286 shifts but long prey DNA retention periods may result for instance in an overestimation
287 of consumption rates or in a mismatch between diet composition and estimations of
288 prey availability at the place where dietary samples were collected. We also usually do
289 not consider whether this problem could be exacerbated in herbivorous species as the
290 digestion process of plant DNA in insects can last much longer as compared to animal
291 DNA (Staudacher *et al*. 2011; Wallinger *et al*. 2013, 2015). For instance, results have
292 shown that ^{14}C-inulin labelled prey in carabid beetles could still be detected in feces up
293 to five days post-feeding (Cheeseman and Gillott 1987). It will be interesting to confront
294 these findings with observations about prey DNA transit.

Here, we also show that prey DNA detection continuously decreases over time for all the three types of dietary samples, with longer fragments (332-612 bp) decaying more rapidly compared to the shorter one (128 bp). These results meet our expectations and corroborate the general idea that digested DNA molecules break down relatively quickly and that the size of the targeted prey DNA fragment affects post-feeding prey DNA detection (Agustí *et al*. 2003; von Berg *et al*. 2008). In line with previous studies, our results support the idea that targeting short to medium size DNA fragments in DNA diet analysis is essential in order to maximize the prey detection (Deagle *et al*. 2006; Valentini *et al*. 2009). Nonetheless, if a recent feeding event is the focus, then targeting longer fragments might actually be a better strategy to ensure that only the most recent prey items are detected. Additionally, as in DNA metabarcoding diet analysis there is generally a trade-off between DNA fragment length and taxonomic resolution, targeting longer DNA fragments – within a certain range - could indeed improve the taxonomic discrimination of prey species (Pompanon *et al*. 2012). In this study, the most important observed source of variation in terms of prey DNA detection, besides time post feeding, is DNA fragment size. This could have profound implications in metabarcoding studies where the DNA fragment size usually needs to be optimized in order to meet criteria for both optimal detectability and taxonomic resolution (Taberlet *et al*. 2012). It would be interesting to simultaneously explore the decay rate of a larger array of DNA fragments of different lengths in order to assess whether a general relationship between DNA length and detectability can be drawn despite the many other sources of variability detected in previous studies. One might speculate that a consistent relationship between DNA detection success and DNA fragment size could

318 be further used as a raw predictor of post feeding prey DNA detection intervals based
319 solely on prey DNA fragment length.

320 In general, our findings show that quantitative analyses of diet based on different
321 DNA fragment sizes or on different dietary samples are not directly comparable. Our
322 study suggests that for estimating and comparing consumption rates for the same
323 species between studies using different DNA fragment sizes or different dietary samples
324 (whole beetles/regurgitates vs feces), values should be corrected after taking into
325 account differences in detection probabilities (e.g. Greenstone *et al*. 2010).
326 Nevertheless, prey DNA detection depends on numerous additional factors including
327 species identity of the prey or the predator (Hosseini *et al*. 2008; Wallinger *et al*. 2013),
328 the feeding mode (Greenstone *et al*. 2007, 2013), the time since the last meal, the
329 number/size or the quality of prey consumed (Hoogendoorn & Heimpel 2001; Harper *et*
330 *al*. 2005; Eitzinger *et al*. 2014), which we did not investigate here. The next step
331 therefore would be the integration of multiple sources of variation in a complex
332 multispecies, multifactorial experimental design where the different sources of variation
333 could be quantified at once, and hierarchized (Welch *et al*. 2014).
334

335 **Acknowledgements**

336 We thank Paul Abrams for his invaluable help with early analyses of data presented
337 here. This study was founded by the French National Research Agency (Peerless
338 project ANR-12-AGRO-0006). S.K. was funded by the Atlantic Canada Opportunities
339 Agency. R.M. was supported by a grant of the regional government of Tyrol (“Land
340 Tirol”) provided by the Mountain Agriculture Research Unit of the University of

Innsbruck. We also thank the three anonymous referees for their pertinent suggestions which helped improve the present manuscript.

Data Accessibility

All the data used in this manuscript are included in the figure and the table presented within the paper.

Author Contributions

MT, SK and MP designed the experiment. SK realized the field work and the feeding experiment. RM carried out molecular analyses. ORR and EC realized data analyses. SK wrote the manuscript with input from all the authors.

Conflicts of Interest: The authors have declared that no competing interests exist.

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524

525 **Figure 1** Prey DNA detection success in the predatory carabid beetle *Pterostichus*
526 *melanarius* for regurgitates (A), whole bodies (B) and feces (C). Detection rates are
527 provided for the different time points examined within each dietary sample and for the
528 three target DNA fragment sizes. Circles and dashed lines indicate actual measures.
529 Bold solid lines indicate the logistic regressions estimated from the glmm model and the
530 shaded area the 95% confidence interval envelopes of the fit. The horizontal line
531 represents the 50% prey DNA detection probability. Corresponding lower and upper
532 95% confidence limits are presented in Table 2.
533
534 **Figure 2** Estimated time points post-feeding for a 50% prey DNA detection probability
535 for the different types of dietary samples and DNA fragment sizes. Provided are the
536 50% prey detection probabilities in hours post-feeding.
537

538 **Table 1** Detection rates of small (128 bp), medium (332 bp) and large (612 bp) prey
539 DNA fragments of the mealworm *Tenebrio molitor* fed to the carabid *Pterostichus*
540 *melanarius* in whole beetles, regurgitates, and feces. *N* is the number of samples
541 analyzed per digestion time.
542

543

544

545

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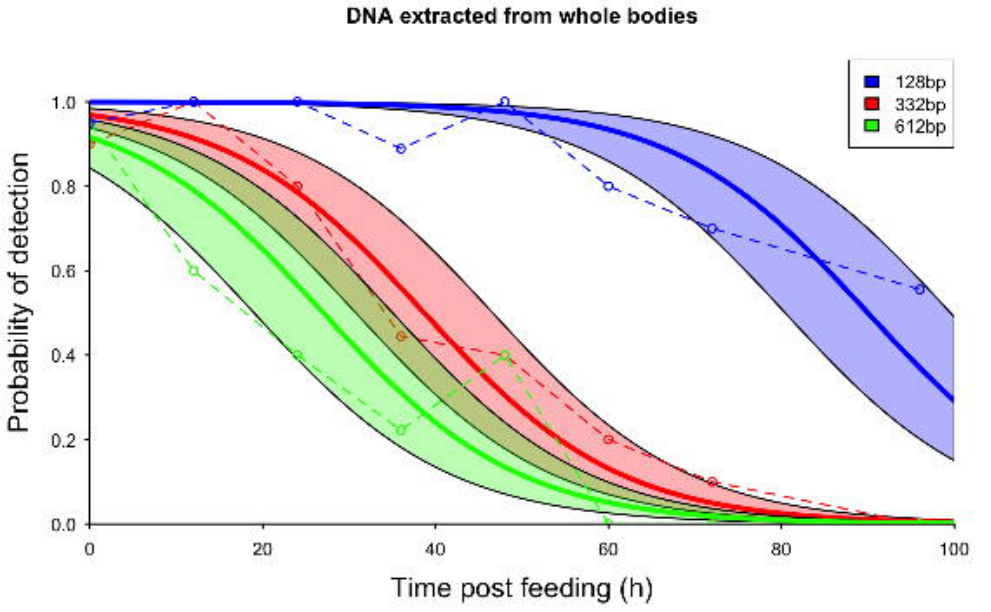
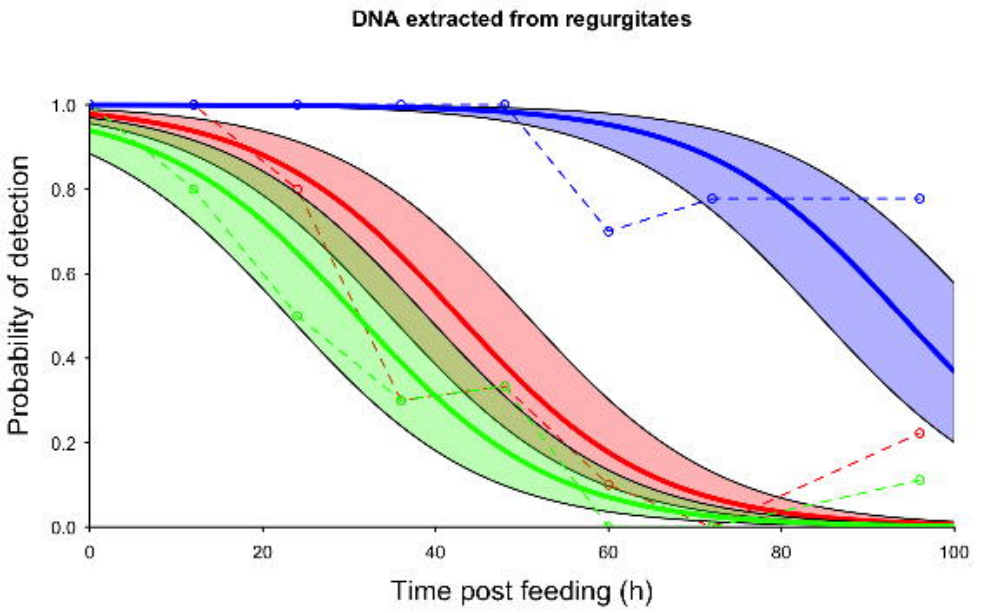
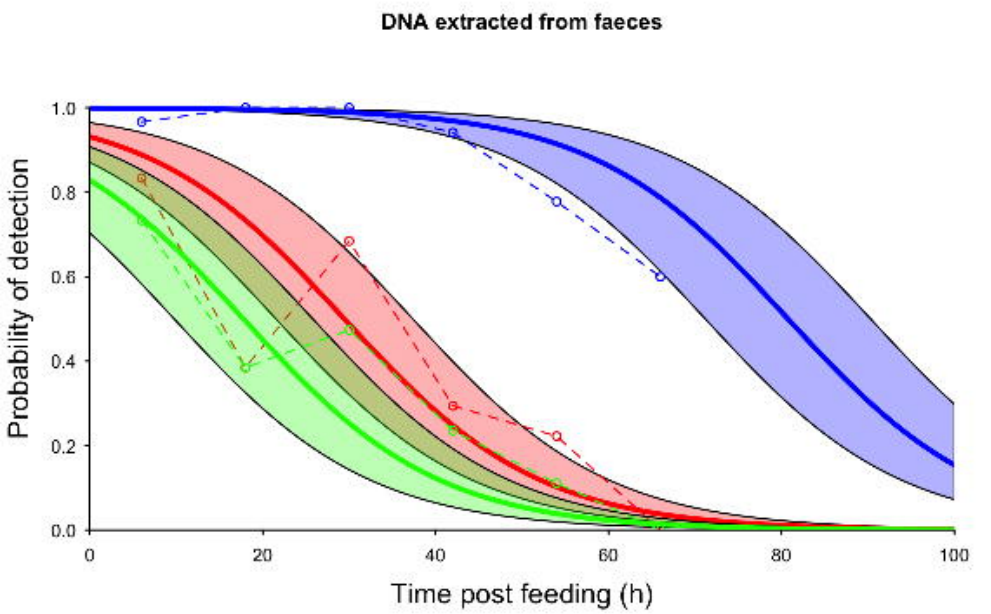
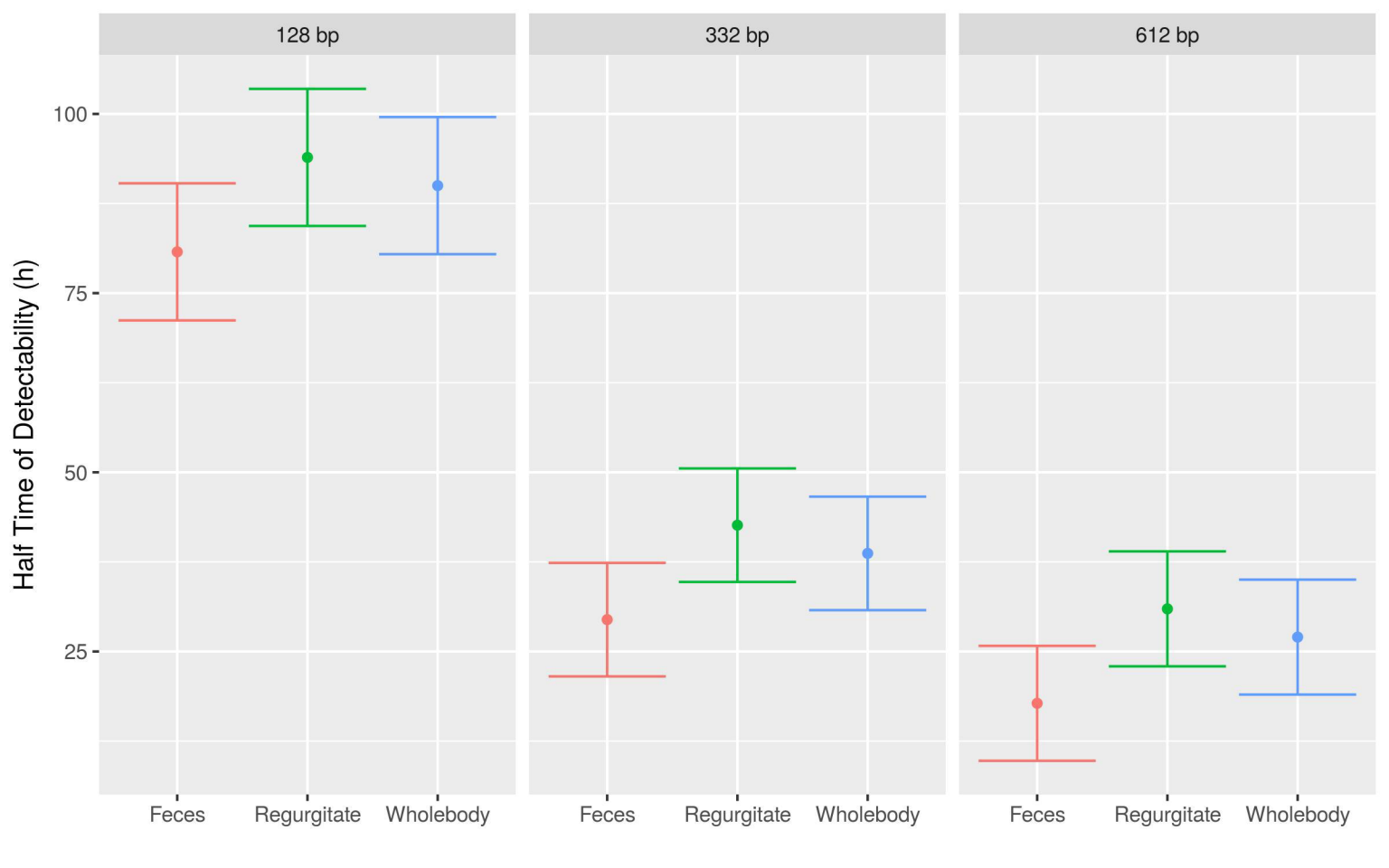

DNA extracted from whole bodies
128bp
332bp
612bp
Probability of detection
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time post feeding (h)


DNA extracted from regurgitates
Probability of detection
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time post feeding (h)


DNA extracted from faeces
Probability of detection
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time post feeding (h)


128 bp
332 bp
612 bp
Half Time of Detectability (h)
100
75
50
25
Feces
Regurgitate
Wholebody
Feces
Regurgitate
Wholebody
Feces
Regurgitate
Wholebody

			Detection rate per fragment size (%)		
Dietary sample	Digestion time (h)	*n*	Small (128 bp)	Medium (332 bp)	Large (612 bp)
Whole bodies	0	20	95	90	95
	12	10	100	100	60
	24	10	100	80	40
	36	9	78	44	22
	48	10	100	40	40
	60	10	80	20	0
	72	10	70	10	0
	96	9	56	0	0
Regurgitates	0	11	100	100	100
	12	10	100	100	80
	24	10	100	80	50
	36	10	100	30	30
	48	9	100	33	33
	60	10	70	10	0
	72	9	78	0	0
	96	9	78	22	11
Feces	3	8	100	87,5	100
	6	13	92	69	69
	9	5	100	100	60
	12	4	100	100	50
	15	7	100	43	57
	21	2	100	50	50
	24	4	100	25	0
	27	12	100	75	50
	30	2	100	50	50
	33	5	100	60	40
	39	9	100	22	11
	42	3	67	0	0
	45	2	100	0	0
	48	3	100	100	100
	51	4	100	0	25
	54	2	50	0	0
	57	1	100	100	0
	58	1	100	100	0
	60	1	0	0	0
	64	1	100	0	0
	70	3	67	0	0
	76	1	0	0	0