







PCR-based detection of prey DNA in the gut contents of the tiger-fly, *Coenosia attenuata* (Diptera: Muscidae), a biological control agent in Mediterranean greenhouses

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Abstract. The tiger-fly *Coenosia attenuata* Stein (Diptera: Muscidae: Coenosiini) is a generalist predator that preys on several pests of greenhouse crops and is considered a biological control agent in the Mediterranean region. Previous behavioural observations identified its preferred prey, but a more in-depth evaluation will benefit from using Polymerase Chain Reaction amplification of prey DNA remains in the gut of this predator. To evaluate the rate of decay and suitability of this method for use in the field assessments, we carried out a laboratory feeding calibration experiment on 355 females of *C. attenuata*, which were killed at different intervals of time after ingestion (10 time points from 0 to 48 h). The prey species tested were: *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae: Trialeurodini), *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae: Cirrospilini), *Bradysia impatiens* (Johannsen) (Diptera: Sciaridae) and *Drosophila mercatorum* Patterson & Wheeler (Diptera: Drosophilidae: Drosophilini). Based on a probit model, amplification success of prey DNA declined exponentially with increasing time after ingestion. The half-time molecular detection differed between species, ranging from an average of 5 h for *T. vaporariorum* and *D. isaea*, 6 h for *B. impatiens*, 15 h for *L. huidobrensis* to more than 40 h for *D. mercatorum*. This study confirmed the feasibility of using DNA based detection to identify prey species in the gut contents of *C. attenuata* and provided calibration curves for a better understanding of predation activity in this agroecosystem.

INTRODUCTION

Generalist invertebrate predators may be important biological control agents against crop pests, as shown in a number of manipulative field experiments (Symondson et al., 2002). When considering introducing or enhancing a particular generalist predator species for pest control, its prey preference and effect on pests (target) and non-pests (non-target) should be studied (Stilin & Simberloff, 2000; Louda et al., 2003), even when native control agents are considered (Howarth, 2000). Field observations of predator preferences are not always feasible and molecular ana-

lyses of a predator's gut contents provide a practical method for the identification of prey (Chen et al., 2000; Harper et al., 2005; Sheppard & Harwood, 2005; Pompanon et al., 2012). The tiger-fly *Coenosia attenuata* Stein, 1903 (Diptera: Muscidae) has particular characteristics that make it an interesting biological control agent for use in Mediterranean greenhouses. This species is currently distributed worldwide and probably originated in the Mediterranean region (Hennig, 1964; Pohl et al., 2012; Seabra et al., 2015; Bautista-Martínez et al., 2017). The adult tiger-fly preys on adult whiteflies, leafminers, drosophilids, thrips, leaf-

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hoppers, Psocoptera and fungus gnats, and the larva is also a predator, feeding on soil insects, such as, fungus gnat larvae (Kühne, 1998, 2000; Prieto et al., 2005; Martins et al., 2012; Mateus, 2012). This species is adapted to high temperatures (Gilioli et al., 2005), which is important for surviving inside Mediterranean greenhouses. It also kills more prey than it consumes, making it an efficient biological control agent (Morris & Cloutier, 1987; Martinez & Cocquempot, 2000). Laboratory studies indicate that it prefers whiteflies and leafminers to other species usually present in protected crops (Martins et al., 2012). A method for mass rearing of *C. attenuata* has already been developed, which may be used to supplement tiger-fly populations in commercial greenhouses (Martins et al., 2015).

To understand the effectiveness of this predator as a biological control agent in protected crops, it is necessary to identify the prey consumed under greenhouse conditions. This has already been established in the field by collecting and identifying the remains of prey consumed by this predator (Prieto et al., 2005; Mateus, 2012). This is, however, very time-consuming and biased in terms of what species of prey are identified, which is dependent on their daily flight activity patterns (prey is captured by the predator during flight). The analysis of the gut content of arthropod insects by amplification of prey DNA using Polymerase Chain Reaction (PCR) is an efficient method of identifying prey (e.g. Chen et al., 2000; Foltan et al., 2005; King et al., 2011), but this has not yet been done for *Coenosia* species.

Detection times for DNA after ingestion depends on the predator, prey, rates of consumption and digestion and the length of the DNA fragment amplified (Hoogendoorn & Heimpel, 2001; Gagnon et al., 2011). Thus, it is important to evaluate the rate of decay of DNA of different prey after ingestion and the suitability of this method for subsequent field assessments. There are also several challenges in detecting degraded DNA after digestion using PCR methods: lack of sensitivity of primers, short post-ingestion detection periods and cross-amplification of non-target DNA (King, 2008).

Although new methods of mass sequencing are now available and are increasingly used in metabarcoding and metagenomics (e.g., Pompanon et al., 2012; Paula et al., 2016; Macías-Hernández et al., 2018), PCR amplification of prey DNA remains in the gut of a predator followed by gel visualization is less costly and continues to be useful, especially when the potential species of prey are relatively well-known, as is the case in biocontrol, and when calibration based on a large number of specimens is possible. In such experiments, the individual predators, after a period of starvation, are fed the target prey and are then killed at known time intervals after feeding and preserved for molecular gut content analysis. The proportion of predators for which the prey is detected is expected to decline exponentially with time since feeding and the half-life of detectability can be estimated using probit or logistic models (Greenstone & Hunt, 1993; Payton et al., 2003; Greenstone et al., 2014). This information will be important for interpreting future studies on the DNA of prey in the gut

contents of predators collected in greenhouses, whatever the method used to analyse the remains of the prey's DNA.

In this study, we determined the PCR detection time of prey DNA after ingestion in the gut contents of *C. attenuata* adults preying upon individual prey of five different species in the laboratory.

MATERIAL AND METHODS

Laboratory feeding experiment

Adults of *C. attenuata* used in this study came from a culture established at Instituto Superior de Agronomia (ISA) facilities in Lisbon, Portugal. The adults used for establishing the cultures were collected in greenhouses in the Oeste region of Portugal, mainly in Silveira, Torres Vedras municipality, on several occasions from 2010 to 2012, and periodically a few new adults from Silveira were added. Tiger-fly culture was established following Martins et al. (2015): larvae were fed on fungus gnat larvae of *Bradysia impatiens* (Johannsen, 1912) (Diptera: Sciaridae) living in soil containing 90% organic matter and oats inoculated with *Pleurotus ostreatus* (Jacquin) Kummer, 1871 (Basidiomycota: Agaricales: Pleurotaceae); adults were fed *Drosophila mercatorum* Patterson & Wheeler, 1942 (Diptera: Drosophilidae). Cultures were kept in a room at 23–25°C, RH 65 ± 10% and a 12 h photoperiod.

Species used as prey are important pests or biological control agents of protected vegetable crops in the region (Figueiredo et al., 2011): whitefly *Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera: Aleyrodidae) (*Tva*), leafminer *Liriomyza huidobrensis* (Blanchard, 1926) (Diptera: Agromyzidae) (*Lhu*) and the eulophid *Diglyphus isaea* (Walker, 1838) (Hymenoptera: Eulophidae) (*Dis*). In addition, two other species kept in the culture unit, were also included: the fungus gnat *B. impatiens* (*Bim*) and drosophilid *D. mercatorum* (*Dmrc*). The whitefly and leafminer were also reared in ISA facilities on tomato plants, under the conditions indicated above. *D. isaea* was acquired from Biobest (*Diglyphus*-system).

Controlled feeding experiments were carried out during 2012 and 2013, with one adult female tiger-fly, between 2–10 days old, put in a 10 × 10 × 10 cm transparent plastic cage with one or two adult individuals of a particular target species of prey (either *Tva*, *Lhu*, *Dis*, *Bim* or *Dmrc*). Prior to the beginning of each experiment, predators were starved for about 12 h. When each tiger-fly ceased feeding (on one prey), the feeding time was recorded, and the tiger-fly was placed in a box without prey and killed at a specific time after ingestion (0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h or 48 h) (Table S1). From previous observations, we knew that tiger-flies fed *D. mercatorum* would survive for at least 48 h (longest time period of the experiment), but if fed a smaller species of prey, they survive for 24 h. Bearing this in mind, we provided these tiger flies with an adult of *D. mercatorum* after 24 h so that they survived for 48 h (but not in the case of the prey *D. mercatorum*). We aimed at using 8 prey individuals in each experiment (for each period of time and species). However, depending on prey availability at the time of the experiments, the individuals initially included in the assays ranged from 2 to 23 (Table S1). We expected some mortality to occur during the experiments and tried to include more individuals whenever possible. Tiger flies (N = 407) were preserved in absolute ethanol and frozen. The temperature during the experiment was controlled at 25°C.

Molecular analysis

DNA extraction from each *C. attenuata* individual was done using the entire thorax and abdomen, instead of using only the dissected gut, in order to minimize the risk of cross-contamina-

tion in such minute samples. From each predator, we also extracted DNA separately from the head, used as a negative control to ensure that the amplifications obtained from the thorax plus abdomen were not of the predator DNA. DNA extractions from individual predators and prey were done using the EZNA®Tissue DNA Isolation Kit (Omega Bio-tek).

DNA of each prey was obtained from fresh prey insects and the mitochondrial *cytochrome c oxidase subunit I* gene (COI), a DNA barcode region widely used in insects (Hebert et al., 2003), was amplified and sequenced. COI was amplified using primers LEP-F and LEP-R (Hajibabaei et al., 2006), yielding a fragment of 620 bp. Polymerase Chain Reaction (PCR) was carried out in a Perkin Elmer 2700 thermocycler. PCR reaction volume of 12.5 µl contained 1 buffer (Promega), 2 mM of MgCl₂, 0.1 mM of dNTPs, 0.4 µM of each primer, 0.25 U of *GoTaq Flexi* DNA polymerase (Promega) and approximately 10 ng of DNA. PCR conditions were: 94°C for 1 min, 5 cycles of 94°C for 30 s, 45°C for 1 min and 72°C for 1 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1.5 min and 72°C for 1 min and a final extension of 5 min at 72°C. PCR products were purified with SureClean (Bioline) and Sanger sequencing of the forward sequence was done on an ABI3730XL, at Macrogen Europe. DNA sequences were checked and edited using Sequencher version 4.0.5 (Gene Codes Corporation).

For aligning several sequences of this COI fragment from the predator and from different species of target prey, either already available in Genbank or sequenced by us (Genbank accession numbers MT428362 for *Tva*, MT428363 for *Bim* and MT428364 for *Dmrc*), we designed specific primers for each prey species with the help of Amplicon version 02 (Jarman, 2004). We aimed at amplifying short fragments between 100 and 250 bp, which are more likely to be detected in digested samples, in regions that differed between species. Primers for two different fragments for each species of prey (three for *Tva*) were designed. These primers were tested on the target species and the predator. Since all of them amplified, the two (or three) primer sets were kept for this study, allowing replication within each species of prey in order to assess the consistency of amplification.

Polymerase Chain Reactions (PCR) were carried out using positive controls (DNA of prey) and negative controls (DNA from the head of the predator) and a blank. PCR reaction volume of 12.5 µl contained 1 × buffer (Promega), 1.8 mM of MgCl₂, 0.1 mM of dNTPs, 0.24 µM of each primer, 0.25 U of *GoTaq Flexi* DNA polymerase (Promega) and approximately 10 ng of DNA. PCR conditions were: 94°C for 3 min, 30 cycles of 94°C for 1 min, 50°C for 45 s and 72°C for 45 s and a final extension of 7 min at 72°C. PCR products were visualized on 1% agarose gels for checking amplification success (Fig. S1).

Data analysis

Probit models were fitted to the PCR detection success against time after ingestion (as in e.g. Chen et al., 2000; Ma et al., 2005; Greenstone et al., 2007). Probit analysis is a type of regression applied when response variables are binomial and the relationship between the response and the predictors is sigmoid. The fit of each model was tested against a null model (with just the intercept) by calculating the difference between the residual deviance of our model and the null model, and doing a χ^2 test with 1 degree of freedom. The half-time molecular detection, i.e., the time after ingestion that corresponds to 50% of the predators testing positive for the DNA of the target prey, was calculated using the probit model coefficients: Probit(Y) = aX + b; a – decay rate, b – Y-intercept, X – time after ingestion; Y – probit of detection success. Solving for the value of X when Y = 0 (inverse normal of 0.5), gives the half-time. Probit analyses were done using gen-

eralized linear models (GLM) with a probit link function (scripts available in <https://github.com/seabrasg/probit>).

Testing if the regression lines differed between different PCR fragments for the same prey species (PCR-1 and PCR-2, as well as PCR-3 for *Tva*) and between different species of prey, was done by comparing GLM models with and without the interaction term PCR × Time or the interaction Prey × Time (Robertson et al., 2017).

In order to explore the relationship between the body sizes of the prey and half-time molecular detection, we calculated non-parametric Spearman correlations between average body length (values for each species from Figueiredo, unpubl. results) and average half-time molecular detection obtained here (average of the two or three primer sets of each species). In our experiments, we recorded the time each predator spent feeding on each prey, and correlated the average value for each species with both body length and half-time molecular detection.

Regression models and correlations were done in R version 3.4.0.

RESULTS

A total of 407 tiger-flies was used in the predation calibration experiments. Two (or three) pairs of primers were designed to amplify two (or three) different fragments of the COI region of each species of prey (Table 1). When the amplification was ambiguous for a particular primer set, it was considered to be missing data (Fig. S1), reducing sample size for some particular primer sets, particularly *Tva-1* that was left with only one individual for some periods of time. In the case of *D. mercatorum*, the samples from the shorter time periods were used initially for PCR using other sets of primers designed to amplify *D. melanogaster* (designed from sequences obtained in GenBank). This occurred because we initially thought we were working with that species. However, the poor amplification obtained with those primers made us suspect we were dealing with a different species. After sequencing COI gene for this prey (as we did for all the other prey) and blasting in NCBI, the

Table 1. Sequence of primers specific for each species of prey and amplicon sizes for each pair of primers. *Tva* – *Trialeurodes vaporariorum*, *Lhu* – *Liriomyza huidobrensis*, *Dis* – *Diglyphus isaea*, *Bim* – *Bradysia impatiens*, *Dmrc* – *Drosophila mercatorum*.

Prey species	Fragment	Primer	5'-3' Sequence of primer	Size of amplicon
<i>Trialeurodes vaporariorum</i>	Tva-1	Tva-125F	CTTAGGTTGGGGCTCCTG	171
		Tva-295R	ATTCCCCCTATGTGACAGC	
	Tva-2	Tva-113F	TGGCTGGTTCCTCTTATGG	144
		Tva-256R	AACAGTCAACCCGTGCCGC	
	Tva-3	Tva-216F	GACTTTTGATTGATAGGGGAAGC	192
		Tva-407R	CCATTTTATGCCAACGCC	
<i>Liriomyza huidobrensis</i>	Lhu-1	Lhu-277F	ACCTCCAGCTCTTACCC	202
		Lhu-478R	CGTATATTAATAATTGTTG	
	Lhu-2	Lhu-327F	CTGGGACAGGATGAACGG	219
		Lhu-545R	AAGAACTGCAGTAATTAG	
<i>Diglyphus isaea</i>	Dis-1	Dis-384F	ATTAGGTCATAGAGGGCC	168
		Dis-551R	TGCAGTTAATAATATAGCCC	
	Dis-2	Dis-323F	CTAGAATATTTATTGGATC	138
		Dis-460R	ACCTATAATTGAAGAAGCC	
<i>Bradysia impatiens</i>	Bim-1	Bdi-411F	CACCTTTTATTAAGTAGAAGG	110
		Bdi-520R	ATAGATCAACAGAGGCCCTGA	
	Bim-2	Bdi-426F	GAAGGTTAGTAGAAGAGGTAC	199
		Bdi-624R	TCAAAAGATATTCCTGGGGC	
<i>Drosophila mercatorum</i>	Dmrc-1	Dmrc-239F	TAGTTCATTAATACTTGG	176
		Dmrc-414R	TCCACCATGAGCAATTCCTG	
	Dmrc-2	Dmrc-379F	TACCACCTTTATCTTCAGG	190
		Dmrc-569R	GCTGTAATTACTACAGATC	

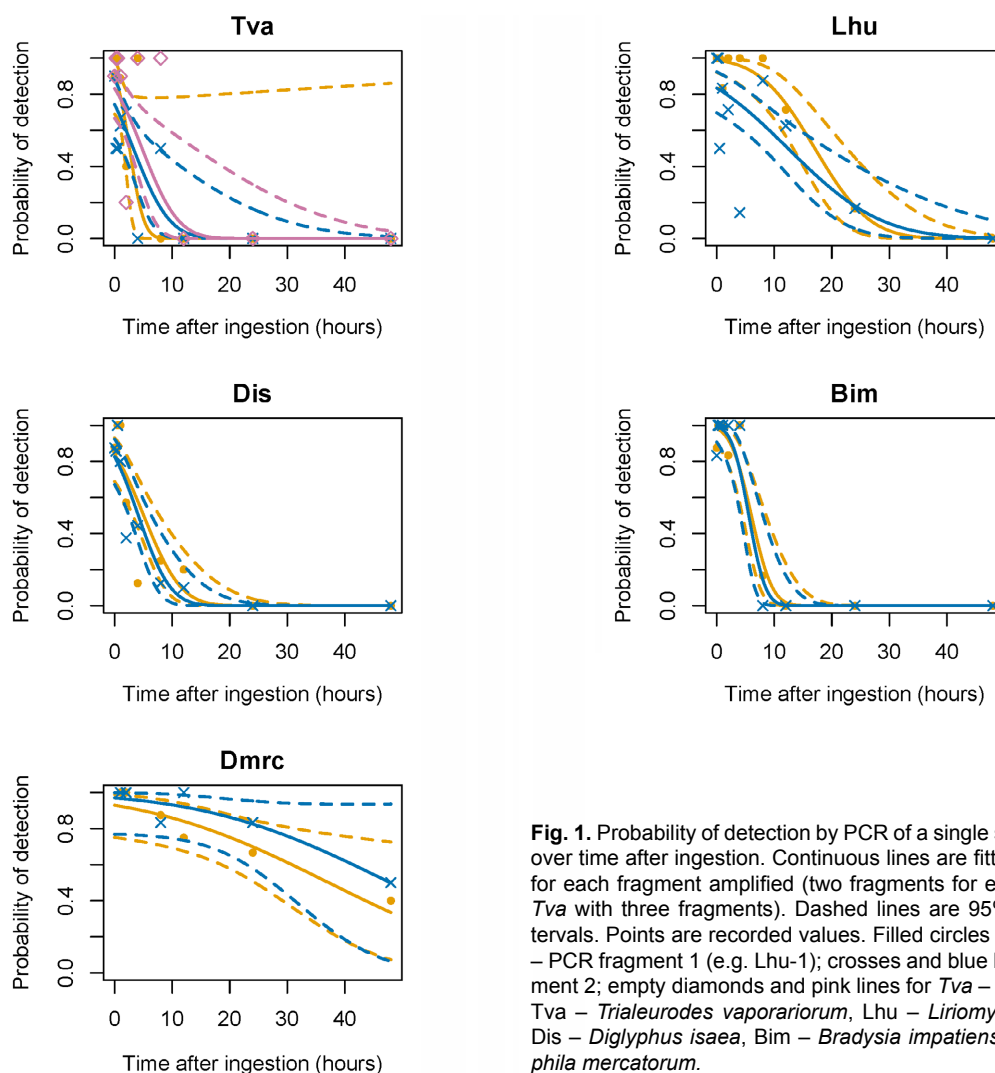


Fig. 1. Probability of detection by PCR of a single specimen of prey over time after ingestion. Continuous lines are fitted probit models for each fragment amplified (two fragments for each prey, except *Tva* with three fragments). Dashed lines are 95% confidence intervals. Points are recorded values. Filled circles and orange lines – PCR fragment 1 (e.g. *Lhu*-1); crosses and blue lines – PCR fragment 2; empty diamonds and pink lines for *Tva* – PCR fragment 3. *Tva* – *Trialeurodes vaporariorum*, *Lhu* – *Liriomyza huidobrensis*, *Dis* – *Diglyphus isaea*, *Bim* – *Bradysia impatiens*, *Dmrc* – *Drosophila mercatorum*.

best hit was with *D. mercatorum*. We designed the new primers for this species and used them in this study. Since at 1 h and 2 h every sample was detected, and later on the amplification rate continued to be high, the shorter periods would likely give full or very high proportion of positive PCRs and therefore we did not perform them. Although the estimates of the probit model may be adversely affected by very low sample sizes, as seen in the large confidence interval estimated for *Tva*-1, there was a good model fit for most of the cases (see below). Comparing the regression lines obtained in PCR-1 and PCR-2 for each species of prey (and PCR-3 for *Tva*), they were not significantly different (F-tests, $p > 0.1$), except for *Lhu* ($p = 0.041$) (Fig. 1; Table S1). Amplification success of the DNA of prey by PCR declined exponentially with increasing time after ingestion and was well described by the probit models in all cases (highly significant model fit, $p < 0.0001$), except for *D. mercatorum* (*Dmrc*-1, $p = 0.011$, *Dmrc*-2, $p = 0.087$) (Table S1), for which after 48 h there was still a substantial detection (Fig. 1; Table S1).

According to the fitted models, the estimated half-time molecular detection ranged from 3 h to 6 h for *T. vaporariorum*, *B. impatiens* and *D. isaea* (lower limits of confidence intervals ranged from 0.42 to 2.63 h and the upper

limits ranged from 10.76 to 18.76 h). The half-time was estimated to be 17.04 h (95% CI 8.03–37.86) and 12.13 h (95% CI 4.05–30.67) for *L. huidobrensis*'s *Lhu*-1 and *Lhu*-2, respectively. For *D. mercatorum*, the estimated half-times were longer (37.23 h, 95% CI 10.02–259.19 for *Dmrc*-1 and 47.90 h, 95% CI 9.93–567.24 for *Dmrc*-2). The wider confidence intervals reflect the lack of data for after 48 h.

The regression lines differed significantly between species (PCR-1 was used for each species) (F-tests, $p < 0.05$), except between *Dis* and *Tva* ($p = 0.075$), between *Bim* and *Tva* ($p = 0.332$) and between *Dis* and *Lhu* ($p = 0.118$).

The time each predator spent feeding on prey ranged from 1 to 33 min and the median time differed significantly (at 0.05 level) between species of prey (Kruskal-Wallis = 126.64, $p < 2.2e-16$, Fig. S2). Pairwise comparisons using Wilcoxon rank sum test revealed significant differences between all species ($p < 0.001$), except between *Dis* and *Bim* ($p = 0.14$). The predator spent longer feeding on the drosophilid prey (*Dmrc*). The average half-time molecular detection of each species of prey was positively and significantly correlated with the average time spent feeding on each prey ($r_s = 1.0$, $p = 0.017$, $N = 5$) (Fig. S3). These two variables were positively but not significantly correlated

(at 0.05 level) with average body size of each species of prey ($r_s = 0.9$, $p = 0.083$, $N = 5$ in both cases) (Fig. S3).

DISCUSSION

The calibration feeding experiments carried out in this study allowed the determination of the probability of detecting the DNA of prey over time in the predator *C. attenuata*. The calibration regression lines between the two (or three) fragments of DNA analysed for each species of prey were consistent, which gives support to the results. For these particular sizes of DNA fragments, from 140 to 220 bp, most species of prey were detected up to 8, 12 or 24 h after ingestion. Only *D. mercatorum* was still detected after 48 h in about half of the predators, which means that it is likely that it would remain detectable for longer than two days. The ability to detect prey remains (DNA included) in the gut content of predators depends on several factors, such as the time after ingestion, meal size and digestion rates, which are influenced by ambient temperature and predator activity levels (e.g., Hagler & Naranjo, 1997; Hoogendoorn & Heimpel, 2001; Greenstone et al., 2014). In this study there was a positive but non-significant correlation between body size (at 0.05 level) and half-time detection, with the substantially bigger *D. mercatorum* having much longer half-time detection than the smaller species. However, size is clearly not the only factor involved since *L. huidobrensis* is slightly smaller on average than *B. impatiens* and it has longer detection times (average 15 h in *Lhu* against 6 h in *Bim*) and *D. isaea* is smaller than *B. impatiens* and they have similar detection times (6h for *Bim* and 5 h in *Dis*). In fact, a predator may not consume an entire prey and so the amount of time spent feeding may not be related to prey size. The strong positive correlation (non-parametric Spearman correlation = 1) here recorded between time spent feeding and half-time detection may indicate that feeding time is indeed a more reliable indicator of the time DNA will remain detectable in the gut contents of a predator.

Longer or shorter detection intervals may each have advantages and disadvantages when analysing feeding periodicity in the field (Hagler & Naranjo, 1997). If prey DNA remains detectable for long periods, the chances of detecting DNA of prey are increased, but the power to identify timing patterns of feeding is reduced. For example, in the case of a tiger-fly sampled in a greenhouse for which *D. mercatorum* DNA is detected in its gut content, it may have fed at any time between several days to just before being collected. For smaller prey detection intervals, it is possible to have a better discrimination and identification of feeding periodicity.

Based on previous observations this predator is very voracious and able to capture large numbers of prey. In some situations, only some of them are actually consumed (Morris & Cloutier, 1987; Moreschi & Süss, 1998) and this can reduce detection times. In a 24-h period, tiger-fly females can each consume up to 12.0 adult sciarids, or 23.8 *Bemisia tabaci* (Gennadius) whiteflies, or 17.25 adult *Liriomyza trifolii* (Burgess in Comstock) leafminers (Tellez et al.,

2009) or 5.7 adult *D. mercatorum*, 10.7 *Trialeurodes vaporariorum* whiteflies or 11.2 *Diglyphus isaea* (Figueiredo et al., 2016). The higher the number of prey consumed the expectation is that the detectability times for particular prey species is increased, which means that the detection times obtained in this study are likely underestimated comparing to what happens in field settings. Another factor to be considered is the ambient temperature, which is usually higher inside greenhouses than in the laboratory, which may have the opposite effect in reducing the detection time because of the resultant increased rate of digestion.

Our aim in this study was to test DNA detectability and decay. In order to use these specific primers in field studies we would additionally need to test for cross-amplifications, in order to guarantee the specificity of the primers. These primers may then be used to study prey preferences and prey-switching behaviour. Despite all the possible factors affecting detectability, this study proved the feasibility of a DNA based detection and identification of preyed species in the field, which could be used to increase our knowledge about the predator's diet (a more complete prey list) and feeding periodicity, more efficiently than observations of behaviour in the field. Even if future studies on the diet of *C. attenuata* in several greenhouse settings and over time will use DNA metagenomics shot-gun sequencing of gut contents (Paula et al., 2016), without the need for specific PCR primers for each prey species, the information that we obtained here on the detectability and decay times of DNA provide the basis for better planning and interpreting such studies. This approach will be dependent on the availability of reference sequence databases for prey taxa.

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REFERENCES

- BAUTISTA-MARTÍNEZ N., ILLESCAS-RIQUELME C.P. & GARCÍA-ÁVILA C.J. 2017: First report of “hunter-fly” *Coenosia attenuata* (Diptera: Muscidae) in Mexico. — *Fla Entomol.* **100**: 174–175.
- CHEN Y., GILES K.L., PAYTON M.E. & GREENSTONE M.H. 2000: Identifying key cereal aphid predators by molecular gut analysis. — *Mol. Ecol.* **9**: 1887–1898.
- FIGUEIREDO E., MEXIA A., MATEUS C., GODINHO M. & RODRIGUES S. 2011: Integrated pest management in vegetable protected crops in the Oeste region. — *Acta Hort.* **917**: 93–101.
- FIGUEIREDO E., MARTINS J., NUNES R., GARCIA A., FRANCO J.C. & MATEUS C. 2016: The tiger-fly *Coenosia attenuata* as a potential biological control agent in vegetable protected crops. In Gerling D., Steinberg S. & Coll M. (eds): Proceedings of the 9th Meeting “Pest Management in an Ever-Changing World” at the Robert H. Smith Faculty of Agriculture, Food & Environment, The Hebrew University of Jerusalem, Rehovot (Israel), October 11–15, 2015. — *IOBC-WPRS Bull.* **119**: 32–33.

- FOLTAN P., SHEPPARD S., KONVICKA M. & SYMONDSON W.O.C. 2005: The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. — *Mol. Ecol.* **14**: 4147–4158.
- GAGNON A.E.Á., DOYON J., HEIMPEL G.E. & BRODEUR J. 2011: Prey DNA detection success following digestion by intraguild predators: influence of prey and predator species. — *Mol. Ecol. Resour.* **11**: 1022–1032.
- GILIOLI G., BAUMGARTNER J. & VACANTE V. 2005: Temperature influences on functional response of *Coenosia attenuata* (Diptera: Muscidae) individuals. — *J. Econ. Entomol.* **98**: 1524–1530.
- GREENSTONE M.H., ROWLEY D.L., WEBER D.C., PAYTON M.E. & HAWTHORNE D.J. 2007: Feeding mode and prey detectability half-lives in molecular gut-content analysis: an example with two predators of the Colorado potato beetle. — *Bull. Entomol. Res.* **97**: 201–209.
- GREENSTONE M.H., PAYTON M.E., WEBER D.C. & SIMMONS A.M. 2014: The detectability half-life in arthropod predator-prey research: what it is, why we need it, how to measure it, and how to use it. — *Mol. Ecol.* **23**: 3799–3813.
- HAGLER J.R. & NARANJO S.E. 1997: Measuring the sensitivity of an indirect predator gut content ELISA: detectability of prey remains in relation to predator species, temperature, time, and meal size. — *Biol. Contr.* **9**: 112–119.
- HAJIBABAEI M., JANZEN D.H., BURNS J.M., HALLWACHS W. & HERBERT P.D.N. 2006: DNA barcodes distinguish species of tropical Lepidoptera. — *Proc. Nat. Acad. Sci. USA* **103**: 968–971.
- HARPER G.L., KING R.A., DODD C.S., HARWOOD J.D., GLEN D.M., BRUFORD M.W. & SYMONDSON W.O.C. 2005: Rapid screening of invertebrate predators for multiple prey DNA targets. — *Mol. Ecol.* **14**: 819–827.
- HEBERT P.D.N., CYWINSKA A., BALL S.L. & DEWAARD J.R. 2003: Biological identifications through DNA barcodes. — *Proc. R. Soc. (B)* **270**: 313–321.
- HENNIG W. 1964: Muscidae: Gattung *Coenosia* Meigen, section 63b. In Lindner E. (ed.): *Die Fliegen der Palaearktischen Region*. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, pp. 518–619.
- HOOGENDOORN M. & HEIMPEL G.E. 2001: PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. — *Mol. Ecol.* **10**: 2059–2067.
- HOWARTH F.G. 2000: Non-target effects of biological control agents. In Gurr G. & Wratten S. (eds): *Biological Control: Measures of Success*. Springer, Dordrecht, pp. 369–403.
- JARMAN S.N. 2004: Amplicon: software for designing PCR primers on aligned DNA sequences. — *Bioinformatics* **20**: 1644–1645.
- KING R. 2008: Molecular analysis of predation: a review of best practice for DNA-based approaches. — *Mol. Ecol.* **17**: 947–963.
- KING R.A., MORENO-RIPOLL R., AGUSTÍ N., SHAYLER S.P., BELL J.R., BOHAN D.A. & SYMONDSON W.O.C. 2011: Multiplex reactions for the molecular detection of predation on pest and non-pest invertebrates in agroecosystems. — *Mol. Ecol. Resour.* **11**: 370–373.
- KÜHNE S. 1998: Open rearing of generalist predators: a strategy for improvement of biological pest control in greenhouses. — *Phytoparasitica* **26**: 277–281.
- KÜHNE S. 2000: Räuberische Fliegen der Gattung *Coenosia* Meigen, 1826 (Diptera: Muscidae) und die Möglichkeit ihres Einsatzes bei der biologischen Schädlingsbekämpfung. — *Stud. Dipterol. (Suppl.)* **9**: 1–78.
- LEORA SOFTWARE 1987: *POLO-PC: A Users Guide to Probit or Logit Analysis*. LeOra Software, Berkeley.
- LOUDA S.M., PEMBERTON R.W., JOHNSON M.T. & FOLLETT P.A. 2003: Nontarget effects – The Achilles' heel of biological control? Retrospective analyses to reduce risk associated with biocontrol introductions. — *Annu. Rev. Entomol.* **48**: 365–396.
- MA J., LI D., KELLER M., SCHMIDT O. & FENG X. 2005: A DNA marker to identify predation of *Plutella xylostella* (Lep., Plutellidae) by *Nabis kinbergii* (Hem., Nabidae) and *Lycosa* sp. (Araneae, Lycosidae). — *J. Entomol. Nematol.* **129**: 330–335.
- MACÍAS-HERNÁNDEZ N., ATHEY K., TONZO V., WANGENSTEEN O.S., ARNEO M. & HARWOOD J.D. 2018: Molecular gut content analysis of different spider body parts. — *PLoS ONE* **13**(5): e0196589, 16 pp.
- MARTINEZ M. & COCQUEMPO C. 2000: La mouche *Coenosia attenuata*, un nouvel auxiliaire prometteur en culture protégée. — *PHM Rev. Hort.* **414**: 50–52.
- MARTINS J., DOMINGOS C., NUNES R., GARCIA A., RAMOS C., MATEUS C. & FIGUEIREDO E. 2012: *Coenosia attenuata* (Diptera: Muscidae), um predador em estudo para utilização em culturas protegidas. — *Revta Ciênc. Agrár.* **35**: 229–235.
- MARTINS J., MATEUS C., RAMOS A.C. & FIGUEIREDO E. 2015: An optimized method for mass rearing the tiger-fly, *Coenosia attenuata* (Diptera: Muscidae). — *Eur. J. Entomol.* **112**: 470–476.
- MATEUS C. 2012: Bioecology and behaviour of *Coenosia attenuata* in greenhouse vegetable crops in the Oeste region, Portugal. — *Bull. Insectol.* **65**: 257–263.
- MORESCHI I. & SÜSS L. 1998: Osservazioni biologiche ed etologiche su *Coenosia attenuata* Stein e *Coenosia strigipes* Stein (Diptera: Muscidae). — *Boll. Zool. Agrar. Bachicolt.* **30**: 185–197.
- MORRIS D.E. & CLOUTIER C. 1987: Biology of the predatory fly *Coenosia tigrina* (Fab.) (Diptera: Anthomyiidae): reproduction, development, and larval feeding on earthworms in the laboratory. — *Can. Entomol.* **119**: 381–393.
- PAULA D.P., LINARD B., CRAMPTON-PLATT A., SRIVATHSAN A., TIMMERMAN M.J.T.N., SUJJI E.R., PIRES C.S.S., SOUZA L.M., ANDOW D.A. & VOGLER A.P. 2016: Uncovering trophic interactions in arthropod predators through DNA shotgun-sequencing of gut contents. — *PLoS ONE* **11**(9): e0161841, 14 pp.
- POHL D., KÜHNE S., KARACA I. & MOLL E. 2012: Review of *Coenosia attenuata* Stein and its first record as a predator of important greenhouse pests in Turkey. — *Phytoparasitica* **40**: 63–68.
- POMPANON F., DEAGLE B.E., SYMONDSON W.O.C., BROWN D.S., JARMAN S.N. & TABERLET P. 2012: Who is eating what: diet assessment using next generation sequencing. — *Mol. Ecol.* **21**: 1931–1950.
- PRIETO R., FIGUEIREDO E. & MEXIA A. 2005: *Coenosia attenuata* Stein (Diptera: Muscidae): prospecção e actividade em culturas protegidas em Portugal. — *Bol. Sanid. Veget. Plagas* **31**: 39–45.
- ROBERTSON J.L., JONES M.M., OLGUIN E. & ALBERTS B. 2017: *Bioassays with Arthropods*. 3rd ed. CRC Press, Taylor & Francis Group, Boca Raton, 212 pp.
- SEABRA S.G., BRÁS P.G., MARTINS J., MARTINS R., WYATT N., SHIRAZI J., REBELO M.T., FRANCO J.C., MATEUS C., FIGUEIREDO E. & PAULO O.S. 2015: Phylogeographic patterns in *Coenosia attenuata* (Diptera: Muscidae), a widespread predator of insect species associated with greenhouse crops. — *Biol. J. Linn. Soc.* **114**: 308–326.
- SHEPPARD S.K. & HARWOOD J.D. 2005: Advances in molecular ecology: tracking trophic links through predator – prey food-webs. — *Funct. Ecol.* **19**: 751–762.
- STILIN P. & SIMBERLOFF D. 2000: The frequency and strength of nontarget effects of invertebrate biological control agents of

plant pests and weeds. In Follett P.A. & Duan J.J. (eds): *Non-target Effects of Biological Control*. Springer Science + Business Media, New York, pp. 31–44.

SYMONDSON W.O.C., SUNDERLAND K.D. & GREENSTONE M.H. 2002: Can generalist predators be effective biocontrol agents? — *Annu. Rev. Entomol.* 47: 561–594.

TELLEZ M.D.M., TAPIA G., GAMEZ M., CABELLO T. & VANEMDEN H. 2009: Predation of *Bradysia* sp. (Diptera: Sciaridae), *Liriomyza trifolii* (Diptera: Agromyzidae) and *Bemisia tabaci* (Homoptera: Aleyrodidae) by *Coenosia attenuata* (Diptera: Muscidae) in greenhouse crops. — *Eur. J. Entomol.* 106: 199–204.

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Table S1. Proportion of PCR positive detections (Prop) for each fragment amplified for each species of prey recorded in each time period after ingestion. N – number of *C. attenuata* tested. Results are shown for: Probit models; significance of fit compared to a null model (with just an intercept); half-time molecular detection; and comparisons between regression lines for each PCR fragment. Tva – *Trialeurodes vaporariorum*, Lhu – *Liriomyza huidobrensis*, Dis – *Diglyphus isaea*, Bim – *Bradysia impatiens*, Dmrc – *Drosophila mercatorum*.

Time (Hours)	<i>T. vaporariorum</i>						<i>L. huidobrensis</i>						<i>D. isaea</i>						<i>B. impatiens</i>						<i>D. mercatorum</i>					
	Nind	Tva-1		Tva-2		Tva-3		Nind	Lhu-1		Lhu-2		Nind	Dis-1		Dis-2		Nind	Bim-1		Bim-2		Nind	Dmrc-1		Dmrc-2				
		N	Prop	N	Prop	N	Prop		N	Prop	N	Prop		N	Prop	N	Prop		N	Prop	N	Prop		N	Prop	N	Prop	N	Prop	
0	10	10	0.90	10	0.90	10	0.90	12	9	1	9	1	8	8	0.88	8	0.88	8	8	0.88	6	0.83	5	-	-	-	-			
0.25	2	1	1	2	0.50	2	1	8	8	1	7	1	8	7	0.86	7	0.86	8	8	1	8	1	4	-	-	-	-			
0.5	3	2	1	2	0.50	3	1	3	2	1	2	0.50	8	8	1	8	1	8	8	1	8	1	5	-	-	-	-			
1	10	9	0.89	8	0.63	10	0.90	8	6	0.83	6	0.83	6	6	1	5	0.80	8	6	1	4	1	5	5	1	5	1			
2	10	10	0.40	10	0.70	10	0.20	8	8	1	7	0.71	10	7	0.57	8	0.38	8	6	0.83	5	1	5	3	1	3	1			
4	6	1	1	6	0	4	1	8	8	1	7	0.14	9	8	0.13	9	0.44	8	7	1	7	1	6	-	-	-	-			
8	4	1	0	4	0.50	1	1	8	5	1	8	0.88	8	8	0.25	8	0.13	6	6	0.17	6	0	8	8	0.88	6	0.83			
12	4	4	0	4	0	4	0	11	7	0.71	8	0.63	10	10	0.20	10	0.10	8	7	0	7	0	8	8	0.75	4	1			
24	4	4	0	4	0	4	0	12	12	0.17	12	0.17	11	11	0	11	0	9	9	0	9	0	10	9	0.67	6	0.83			
48	10	9	0	9	0	9	0	14	14	0	14	0	16	8	0	8	0	23	23	0	23	0	8	5	0.40	2	0.5			
Total	63	51		59		57		92	79		80		94	81		82		94	88		83		64	38		26				

PROBIT MODEL RESULTS

	Tva-1	Tva-2	Tva-3	Lhu-1	Lhu-2	Dis-1	Dis-2	Bim-1	Bim-2	Dmrc-1	Dmrc-2
Intercept	1.311	0.652	0.961	2.262	0.970	0.984	0.935	2.105	2.425	1.471	1.875
CI 95% Lower limit	0.602	0.152	0.454	1.535	0.538	0.510	0.464	1.393	1.551	0.727	0.880
CI 95% Upper limit	2.188	1.183	1.520	3.216	1.439	1.496	1.439	3.012	3.615	2.321	3.179
Coefficient estimate (Time)	-0.507	-0.201	-0.202	-0.133	-0.080	-0.195	-0.227	-0.350	-0.436	-0.040	-0.039
CI 95% Lower limit	-1.012	-0.358	-0.389	-0.191	-0.120	-0.287	-0.337	-0.530	-0.693	-0.073	-0.089
CI 95% Upper limit	-0.152	-0.081	-0.081	-0.085	-0.047	-0.115	-0.134	-0.215	-0.261	-0.009	0.006
z value	-1.97	-2.76	-2.66	-4.86	-4.23	-4.22	-4.24	-4.45	-3.98	-2.47	-1.67
P	0.049	0.006	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.096

MODEL FIT

Null deviance	70.681	80.413	78.861	101.468	110.703	111.288	111.274	121.812	114.085	41.6030	18.597
Degrees of freedom	50	58	56	78	79	80	81	87	82	37	25
Residual deviance	32.547	52.899	45.721	30.359	71.034	61.957	60.646	25.203	16.906	35.0860	15.672
Degrees of freedom	49	57	55	77	78	79	80	86	81	36	24
Difference in deviance between null and residual	38.134	27.515	33.139	71.109	39.670	49.331	50.628	96.609	97.179	6.517	2.924
Difference in df between null and residual	1	1	1	1	1	1	1	1	1	1	1
P-value	6.60E-10	1.56E-07	8.58E-09	3.38E-17	3.01E-10	2.16E-12	1.12E-12	8.45E-23	6.33E-23	0.011	0.087
LogLikelihood	-16.274	-26.449	-22.861	-15.179	-35.520	-30.978	-30.323	-12.601	-8.453	-17.543	-7.836
Heterogeneity factor (deviance/df residual)	0.664	0.928	0.831	0.394	0.911	0.784	0.758	0.293	0.209	0.975	0.653
HALF-TIME	2.59	3.24	4.76	17.04	12.13	5.05	4.12	6.02	5.57	37.23	47.90
CI 95% Lower limit	0.59	0.42	1.17	8.03	4.50	1.77	1.38	2.63	2.24	10.02	9.93
CI 95% Upper limit	14.44	14.64	18.76	37.86	30.67	13.05	10.76	14.03	13.84	259.19	567.24

COMPARISON OF REGRESSION LINES obtained for different primer sets for each prey (GLM models with and without the interaction term PCR x Time)

Difference in deviance		1.309		4.255		0.289		1.698		0.0002
Difference in df		2		1		1		1		1
P-value		0.273		0.041		0.591		0.194		0.9885

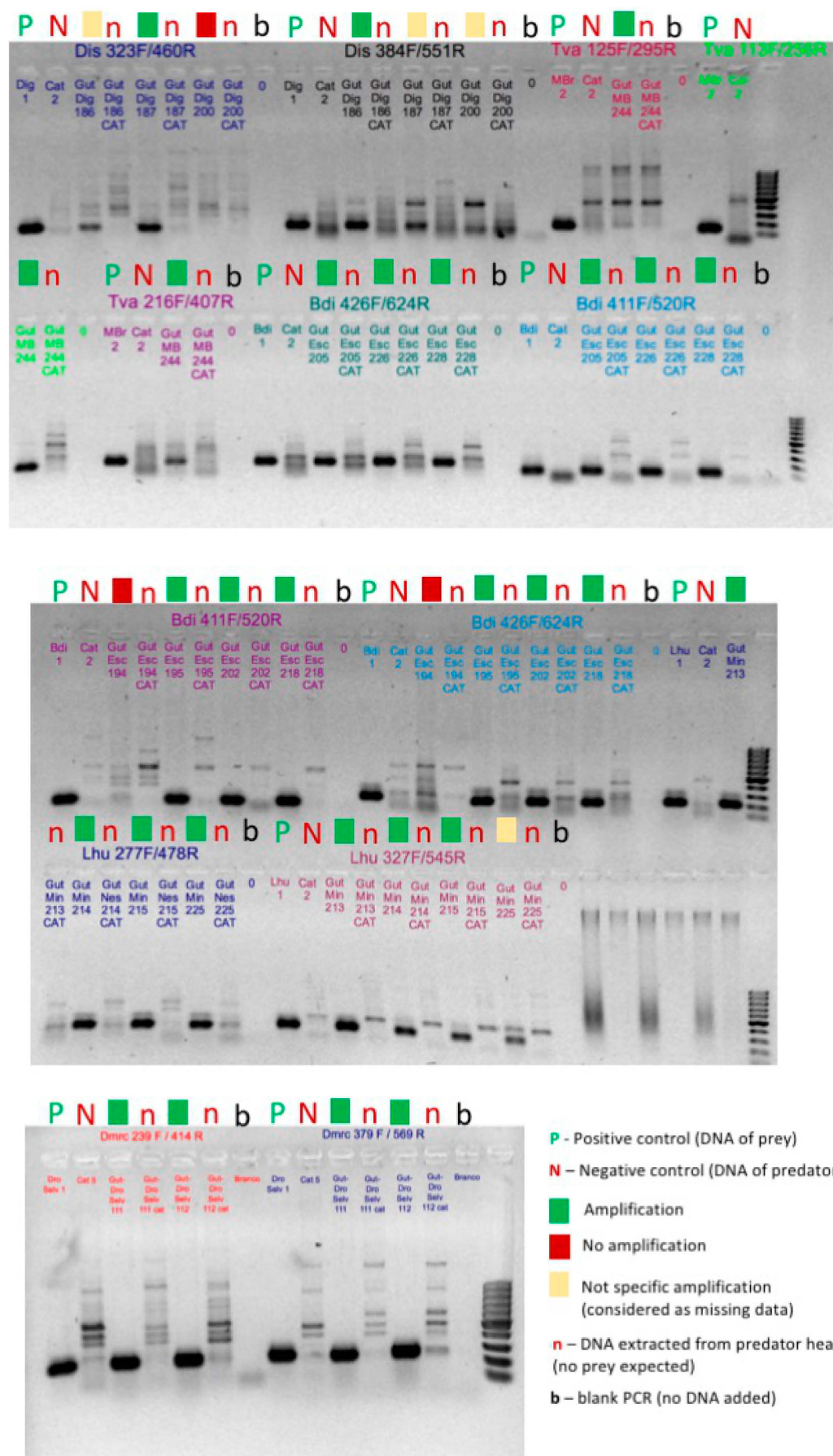


Fig. S1. Agarose gel images of PCR products after electrophoresis. Primer pairs are indicated at the top of the wells and sample identification below each well. The DNA ladder on the right of each figure ranges from 100 bp to 1000 bp fragments.

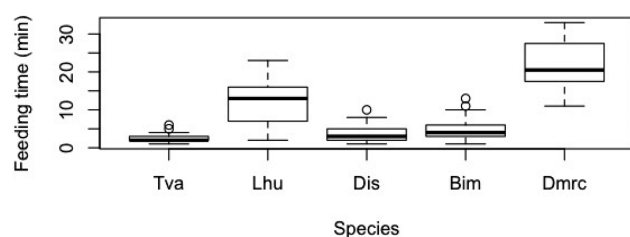


Fig. S2. Distributions of time each predator spent feeding on each species of prey. Tva – *Trialeurodes vaporariorum*, Lhu – *Liriomyza huidobrensis*, Dis – *Diglyphus isaea*, Bim – *Bradysia impatiens*, Dmrc – *Drosophila mercatorum*.

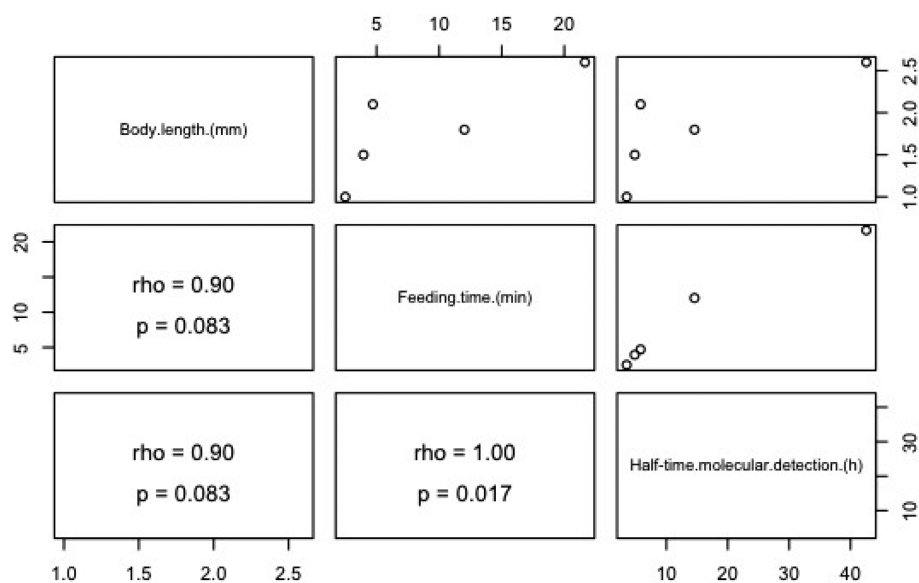


Fig. S3. Scatterplots and estimated Spearman correlation indices (ρ) and corresponding p-values between pairs of the following variables: body length, feeding time and half-time molecular detection.