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Insect pest consumption by bats in macadamia orchards established by molecular diet analyses

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ABSTRACT

The diet of insectivorous bat species is difficult to study and the least invasive tool to gain information on these predators' foraging preferences is the study of their faecal pellets. The aim of this study was to determine whether bats consumed insect pest species in macadamia orchards, with the additional goal of incentivising farmers to adopt a more integrated pest management approach (IPM). We used a molecular approach to provide insight into insectivorous bat diet, analysing pellets with fluorescent-labelled and species-specific primers (COI). Faecal pellets were collected from captured individuals or from trays installed underneath bathouses and roosts between July 2015 and April 2017 in the Levubu region, Limpopo, South Africa. Four of the main insect pests, two moth (Lepidoptera: Tortricidae) and two stinkbug (Hemiptera: Pentatomidae) species, were collected for species-specific primer development and assay optimisation. We extracted DNA from the faecal pellets and amplified the target regions of the four target pest species present. To verify the results of the fragment analyses we also sequenced all PCR products.

All the species or families of bats from which pellets were collected foraged on at least one of the four major insect pests, with insect pest sequences obtained and confirmed from 57 out of 103 samples (55%). Bats consumed insect pests throughout the macadamia growing seasons and are much more generalist and presumably opportunistic feeders than previously assumed. Nearly all species and families of bats analysed foraged on both the Lepidopteran and Hemipteran insect pest species. In conclusion, bats appear to be important for pest control and we suggest that farmers should maintain or restore (semi-) natural vegetation inside and adjacent to their farms. Adding water sources and roosting opportunities, and minimizing pesticide treatments may furthermore promote bat activity.
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1. Introduction

A growing body of literature emphasizes the value of the ecosystem service insectivorous bats provide in agricultural landscapes (Boyles et al., 2011; Cleveland et al., 2006; Kunz et al., 2011; Lopez-Hoffmann et al., 2014; Maas et al., 2013; Puig-Montserrat et al., 2015; Russo et al., 2018; Taylor et al., 2018; Wanger et al., 2014). Most of these studies are based on exclusion experiments or avoided-cost models that use estimated levels of pest predation by bats. Moreover, evidence from exclusion experiments remain limited in so far as they fail to incorporate estimates of predation outside exclusion constructions, particularly that of high-flying *Molossidae* (McCracken et al., 2008; Voigt et al., 2018). South Africa is the world's largest producer of macadamia nuts since 2014 (DAFF, 2016), with the annual loss from insect pest damage to macadamia crop being estimated at about 15.23 million USD, with an estimated ecosystem service of 59−139$ (USD) per hectare provided by insectivorous bats, which help suppress stinkbug pest species (Schoeman, 2009; Taylor et al., 2018). Furthermore, Weier et al. (2018) recently showed that bat activity within macadamia orchards in northern South Africa nearly doubles during the macadamia growing season, suggesting that prey availability of insect pests may be higher at this time of the year, thus attracting more insectivorous bat species. Insectivorous bats have also been shown to forage on major insect pest species in rice cultivation (Puig-Montserrat et al., 2015), pecan orchards (Brown et al., 2015) and cotton fields (McCracken et al., 2012).

To determine whether insect pest species are consumed by bats and to offer incentive to farmers for a more integrated pest management approach (IPM), it is important to provide evidence of insect pest consumption by bats across a wide scale of agricultural systems.

The nocturnal and volant hunting behaviour of insectivorous bat species makes it difficult to study their diet, especially studying predation through direct observation (Brown et al., 2015; Clare et al., 2009). The least invasive tool to gain information on the foraging preferences of these predators is the study of bat faecal pellets. A common approach is the morphological analysis of prey items in bat faecal pellets (Lee & McCracken, 2002, 2005; Leelapaibul et al., 2005; Taylor et al., 2017; Whitaker et al., 1996; Whitaker, 1988). Although this approach gives insight into the variability of prey-items consumed to order and, sometimes, family level, it usually lacks species level information (Whitaker, 1988). More recently, various molecular approaches such as metabarcoding or next generation sequencing with a universal insect-prey primer pair have been used to study trophic interactions and variability in insectivorous bat diet (Aizpurua et al., 2018; Alberdi et al., 2018; Arrizabalaga-Escudero et al., 2015; Bohmann et al., 2011; Clare et al., 2013, 2011; 2009; Galan et al., 2018; Krauel et al., 2018; Mata et al., 2016; Taylor et al., 2017; Zeale et al., 2011). Recent Next Generation Sequencing (NGS) studies suggest a high and diverse level of insect pest consumption (44 different insect pest species) by two different insectivorous bat species; *Miniopterus schreibersii* in Europe (Aizpurua et al., 2018) and *Tadarida brasiliensis* in the USA (Krauel et al., 2018).

In this study, we consider a molecular approach to insectivorous bat diet analyses from bat faecal pellets using fluorescent-labelled primers, each specifically designed to amplify a single insect pest species, in a multiplex PCR reaction. We targeted the highly polymorphic regions in the mitochondrial cytochrome oxidase I (DNA-barcoding) gene to design species-specific primers. This method makes use of capillary electrophoresis, a technique more often used in genotyping (Beja-Pereira et al., 2009; Blacket et al., 2012).

The main objectives of this study were to determine the level of predation on major insect pests by insectivorous bat species in macadamia orchards and to assess whether there is a correlation between the macadamia growing cycle, respectively season, and the prevalence of these insect pest species in the faecal pellets. Therefore, we assayed the prevalence of four of the major pest species in the South African macadamia industry choosing species which are cosmopolitan and species which are indigenous; the twin-spot stinkbug (*Bathycoelia distincta,* Distant), the green vegetable bug (*Nezara viridula,* Linnaeus), the macadamia nut borer (*Thaumatotibia batrachopa,* Meyrick) and the litchi moth (*Cryptophlebia peltastica,* Meyrick). Pest insect species can damage the macadamia kernel directly, leading to a decrease in nut quality, but they can also cause nuts to drop immaturely, germinate or mould (Linden et al., in press).

We hypothesized that pest predation will be higher during the macadamia growing season when insect pests peak in their abundance (Weier et al., 2018) and that the prevalence of *Hemiptera* or *Lepidoptera* insect pests in faecal pellets will depend on the species or foraging group of bats (Monadjem et al., 2010). We also aim to offer incentive and recommendations to farmers such as macadamia growers to include bats in an integrated pest management approach (IPM).

2. Materials and methods

2.1. Study area

The study was conducted in the subtropical fruit growing area of Levubu, Limpopo province, South Africa between the towns Thohoyandou (22°59′03.7 S, 30°27′12.8 E) and Makhado/Louis Trichardt (23°03′03.6 S, 29°55′12.7 E). Levubu is located within the valley of the Levuvhu River and accounts for the second highest production of macadamia in South Africa. The study area is subtropical and receives its main rain in the summer season between November and April with around 1000 mm of annual rainfall. Other dominant land use types in the study area are pecan, avocado and banana orchards as well as pine and gum plantations (Taylor et al., 2017). There are natural vegetation patches remaining in and around most orchards and plantations, which are classified as ‘Soutpansberg Mountain Bushveld’ and ‘Tzaneen Sour Bushveld’ by Mucina and Rutherford (2006).

2.2. Sample collection

2.2.1. Faecal sample collection

Between July 2015 and April 2017, we collected bat faecal pellets using two different methods to determine the prevalence of insect pests in faecal pellets. Twenty-one bathouses had been mounted on poles in four orchards in the study area by the company 'EcoSolutions' (Johannesburg, South Africa) in 2014. Eighteen of these bathouses (in sets of three) on three different farms and two *Nycteris thebaica* roosts were fitted with trays to collect pellets from those occupied by bats. We noted occupancy of bathouses to species or family level to keep disturbance minimal. Additionally, we collected pellets from individuals captured with mist nets or harp traps (Permit No. 001-CPM403-00010) on the farm Schoonuitzicht. Each captured individual was identified to species level whenever possible by using the identification key of Monadjem et al. (2010). All bats were kept in a cloth bag for at least 1 h until their release, in order to collect pellets from the bag. Release calls of captured bats were taken with either the Anabat SD2 (Titley Scientific) or the Batlogger M (Elekon AG). All collected pellets were stored in 70% ethanol in microcentrifuge tubes. A total of 103 samples were used for molecular analysis, each containing between one to ten individual pellets. The number of individual pellets per sample used for analyses was recorded, although by the time of analysis some pellets had disintegrated in the storage medium. Those samples were estimated to three pellets. In total 47 samples were obtained from trays underneath bathouses and 56 samples from bats which were captured. Bathouses were monitored for a parallel study from June 2016 to July 2017, during which we observed a maximum number of five individual bats in one bat house.

2.2.2. Insect pest collection

The South African Subtropical Growers' Association (SUBTROP) provided samples for genetic analysis of the four insect pest species used for primer development and optimisation; the twin-spot stinkbug (*B. distincta*), the green vegetable bug (*N. viridula)*, the macadamia nut borer (*T. batrachopa*) and the litchi moth (*C. peltastica*). All sample insects had been collected on macadamia orchards within the study area. The two Lepidopteran species (*T. batrachopa* and *C. peltastica*) were caught with pheromone traps, while the Hemipteran species (*B. distincta* and *N. viridula*) were collected during scouting, a chemical knockdown method using dichlorvos (Schoeman, 2012). All samples were preserved individually in 70% ethanol.

2.3. Molecular methods

2.3.1. Insect DNA extraction and amplification

The insects were rinsed in distilled water (dH_2O) for 20 min and air-dried to remove alcohol traces. The whole insect was transferred to a 2 mL tube with 5 μL of Solid Tissue Buffer. A sterilized glass rod was used to homogenise the insects after which 90 μL of Solid Tissue Buffer, 100 μL dH_20 and 5 μL Proteinkinase K was added. The samples were incubated overnight at 55 °C and extracted using the ZR Genomic DNA™-Tissue MiniPrep kit for 'Whole blood serum and plasma' (Zymo Research, Irvine, USA). We deviated from the manual by preheating the Elution Buffer to 55 °C and eluting in 15 μL followed by adding another 15 μL, 5 min later. We then waited 3—5 min before centrifuging at maximum speed for 30 s. We visualized 5 μL of each sample against 2 μL of Kappa Universal ladder (1 Kb) on 2.5% agarose gels, stained with Ethidium bromide.

2.3.2. Species-specific primer development

A fragment of more than 500bp of the insect cytochrome oxidase I (COI) gene was required for all four species to design species-specific primers. We followed the PCR protocol by Tembe et al. (2014) using universal insect COI primer pair LCO1490 (5′-ggtcaacaaatcataaagatattgg-3′) and HCO2198 (5′-taaacttcagggtgaccaaaaaatca-3′) (Folmer et al., 1994). PCR products were visualized on agarose gels as described above to confirm amplification. PCR products were enzymatically purified using Exonuclease I (NEB M0293) and Shrimp Alkaline Phosphatase (NEB M0371) according to manufacturer's protocol (New England Biolabs, Inc).

All sequencing reactions were performed by Inqaba Biotechnical Industries (Pty) Ltd, using the BrilliantDye™ v3.1 Terminator Cycle Sequencing Kit (NimaGen BV, Nijmenden, The Netherlands). The sequencing products were purified using the ZR-96 DNA Sequencing Clean-up Kit™ protocol (Zymo Research, Irvine, USA), and run on the ABI PRISM™ 3500xl Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA). Bioedit version 7.2.6.1 (Hall, 1999) was used to edit and align extracted sequences from five *B. distincta*, eight *C. peltastica,* six *N. viridula* (including Genebank Accessions KR044112.1, KR037758.1 and KJ642019.1) and four *T. batrachopa* (including Genebank AccessionsKP083436.1 and KP083437.1).

Sequence data and cytochrome oxidase I (COI) genebank accessions for *B. distincta*, *C. peltastica*, *T. batrachopa* and *N. viridula* were downloaded into CLC Main Workbench (QIAGEN) from NCBI. Sequence data was assembled, and polymorphic regions between each species identified. Species-specific primers were initially developed using the software Primer-BLAST (https://www.ncbi.nlm.nih.gov/) and tested with the 'Multiple Primer Analyser' (https://www.thermofisher.com/) for primer-dimer formation, amplified fragment length, melting Temperature and percentage of CG content. Four primer pairs were chosen to be at least 23bp in length, with melting temperatures over 65 °C to maintain similar annealing temperature over 60 °C. Primers were designed to amplify fragments of various sizes between ~100 and 300bp to distinguish between

Table 1
Summary of the primers used to detect the four different macadamia insect pest species in bat faecal samples, listed with the markers (dye) used and the expected size of PCR products (bold letters indication the so-called 'GC-clamp' at the 3′-end of each forward and reverse primer).

Species	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Primer length (bp)	Dye	Fragment Size (bp)
Thaumatotibia batrachopa	GGAGCTGGGACAGGAT**GAACAG**	CCTCCTCCAGCTGGGT**CAAAA**	22/21	ATTO-532	319
Cryptophlebia peltastica	TGGAGCAGGTACTGGATGA**ACAGT**	AAAGAGCTGTAATACCAACAGCTC**AGACA**	24/29	ATTO-550	222
Nezara viridula	CCCTTTTAATAGTAAGAAGATTAGCAGAATCTG**GAGCA**	CTGCGCCTAGGATTGATGATAC**TCCTG**	38/27	ATTO-565	158
Bathycoelia distincta	GTTTATCCACCTCTATCAAGTAATTTATCACATAGAG**GAGCA**	AGGTAATGATAATAATAGAAGTAGGGCTGTAATTCC**AACGG**	42/41	6-FAM	216

each target using fragment analysis. Primers were tested for species specificity by PCR and gel electrophoresis and confirmed by fragment analysis, carried out at Inqaba Biotechnical Industries (Pty) Ltd. The forward primers were labelled with fluorophores (Table 1). Optimisation of the multiplex assay was performed using all four primer sets and template DNA from each insect species (see Supplement 1).

2.3.3. Multiplex fragment analysis of DNA extracted from bat pellets

The Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, USA) was used to extract DNA from the faecal pellets. The Thermo Scientific™ NanoDrop™ OneC spectrophotometer was used to perform DNA quantification and quality evaluation before the multiplex PCR amplification. Samples that did not meet quality standards (280/260 between 1.4 and 1.8 and 230/260 between 1.4 and 2.0) were cleaned and concentrated using DNA Clean & Concentrator (Zymo Research, Irvine, USA). Target regions were amplified in multiplex using Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs, MA 01938, USA). PCR reactions were performed in volume of 25 μL according to manufacturer's specifications adding 10 ng of gDNA, 10 mM of each primer and nuclease free water (AMRESCO LLC, OH 44139, USA). Thermocycler conditions were set to the following program: Initial denaturation at 98 °C for 30 s. The first 20 cycles were set to 98 °C for 10 s, 65 °C for 10 s and 72 °C for 10 s followed by the second 20 cycles of 98 °C for 10 s, 68 °C for 10 s and 72 °C for 10 s. The final extension was set to 72 °C for 30 s and holding at 4 °C. Due to the suspected low concentration of target gDNA for *B. distincta*, *C. peltastica*, *T. batrachopa* and *N. viridula* in the bat pellets, fragments were prepared by adding 4 μL fluorescently labelled PCR amplicons to the LIZ500 sizing standard and Hi-Di™ Formamide (Thermo Fisher Scientific, Carlsbad, USA) mixture and denatured at 95 °C for 5 min. After denaturation, the fragments were run on the ABI PRISM™ 3500xl Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA). The data was analysed using GeneScan™ Software and interpreted using GeneMapper® v5.0. (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA).

2.4. Data analyses

2.4.1. Release call identification

AnalookW (version 0.3.8.13; Corben, 2006) or Bat Explorer (Elekon AG, Version 1.10, http://www.elekon.ch) were used to analyse the recorded calls by referring to Monadjem et al. (2010) and Taylor et al. (2013) for call identification. Because of the difficulty to distinguish small Vespertilionidae, we classified them all into one group with the exception of *Neoromicia nana*, which has a very distinct echolocation call.

2.4.2. Primer verification and sequence analyses

Samples that produced successful profiles (positives) from the fragment analysis were amplified in simplex using unlabelled species-specific primers and sequenced as described above by Inqaba Biotechnical Industries (Pty) Ltd, to verify effectiveness of the four primer sets. To test for false positive or negative fragments, all sequences were aligned in BioEdit version 7.2.6.1 (Hall, 1999) and compared against the NCBI nucleotide sequence database, using the software BLAST (ncbi.nlm.nih.gov). Since there was no *B. distincta* sequences available on GenBank, closest identity to *Bathycoelia indica* (Dallas, 1851) vouchers in BLAST was used for primer verification.

2.4.3. Statistical analyses

We split our nearly two-year data set, from July 2015 to April 2017, into a high season (December to end of May) and a low season (June to end of November) as previously used to distinguish the macadamia growing season from the off-peak season (Weier et al., 2018).

All statistical analysis was conducted with R (version 3.4, R Core Team, 2017). The R-package 'lme4' (Bates et al., 2015) was used to fit a generalized linear model (GzLM) with a binomial distribution. We analysed the relationship of the response variable 'presence or absence of insect pests' in faecal samples and the predictor variables 'number of pellets' in each sample, month of collection, season (low or high) and method by which pellets were obtained (from roosts or caught bats). We used

the R-package 'ResourceSelection' (Lele et al., 2019) to test model fit with the Hosmer Lemeshow goodness of fit test, which was not significant ($p = 0.69$) and confirmed our model is a good fit.

3. Results

3.1. Fragment analyses and sequencing

Out of the 103 samples, fragment analyses yielded 63 samples with positive profiles for one or more pest insect species (61%). We yielded a total of 92 positive fragment profiles from the 63 samples. Of the 92 positive fragments we obtained sequences data for 79 fragments. Sequencing reactions failed for 13 sequences which could not be blasted and were excluded from further analyses.

We confirmed 57 fragments as true positives, 12 fragments turned out to be amplifications of one of the other three pest insect-species and 10 fragments were amplifications of non-target species.

Primer specificity could be confirmed to 100% for the primer pairs designed for *B. distincta* (all 26 sequences obtained from fragments were confirmed by blasting) and *N. viridula* (all 12 sequences obtained were confirmed by blasting). The primer pairs for *C. peltastica* (18 confirmed out of 30 sequences) and *T. batrachopa* (one confirmed out of 14 fragments) were not sufficiently specific. From the 30 positive sequences obtained for *C. peltastica*, ten were confirmed as amplifications of *T. batrachopa*. From a total of 14 positive sequences obtained for *T. batrachopa*, two were confirmed amplifications of *B. distincta*. Additionally, we detected four false negatives within the samples. One sample showed no positive fragments but contained a positive sequence for *N. viridula*. Three samples tested positive for one pest-species but contained a positive sequence for a second species. Adding four positive sequences and one additional positive sample to the data, we obtained sequences from insect pest species from 54 of the 103 pellet samples (55.6%) with a total of 73 insect pest sequences. Looking at the individual insect pest species, we confirmed 17.5% of the samples positive for *C. peltastica*, 1% positive for *T. batrachopa,* 12.36% positive for *N. viridula* and 26.78% for *B. distincta*.

3.2. Influence of season and environmental variables on pest detection

The step-wise selection of the GzLM for the 'presence or absence of insect pests' in faecal samples only retained the variable low season (macadamia off-growing season) which had no significant effect on the detection of insect pests in bat faecal pellets ($\beta = -0.64$, $SE = 0.42$, $p = 0.13$). Sample size was similar with 46 samples from the low season and 57 from the high season and except for April ($N = 1$) sample size per month ranged from 5 to 20 samples. For the high season, we yielded a total of 37 positive fragments for the four insect pest species and 24 negative samples and for the low season a total of 36 positive fragments and 15 negative samples (Fig. 1).

The predictor variables 'number of pellets' in each sample, month of collection and the method by which pellets were obtained (from roosts or caught bats) had no significant effect on the detection of insect pests in faecal pellets.

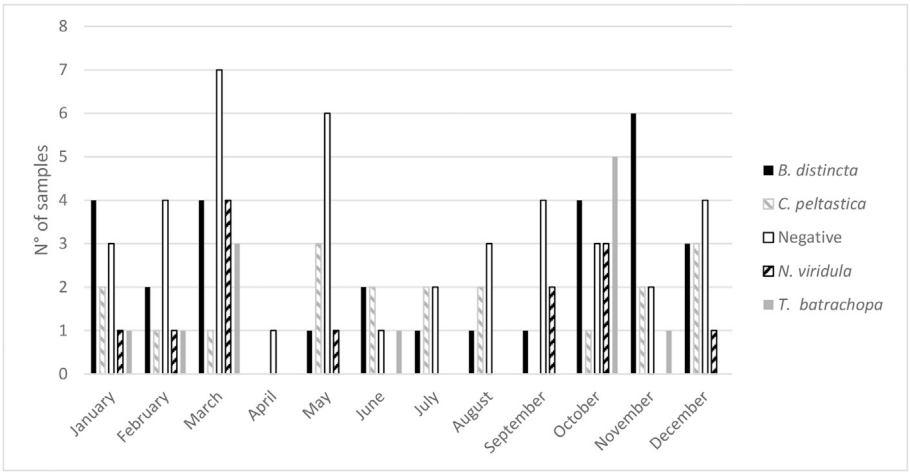

Fig. 1. Four macadamia insect pest species detected in bat faecal samples by using a PCR-sequencing approach shown over the different months. Samples in which no pest species were amplified are indicated as "Negative".

Table 2
Showing the total number of negative faecal samples as well as the positive sequences (bold) detected for each of the insect pest species in the pellets of the different species or families of bats (+indicating that some samples disintegrated in the storage medium and were estimated to three pellets).

Species/Family	N° of pellets	N° of negative samples	N° of positive samples for each insect pest			
			Cryptophlebia peltastica	*Thaumatotibia batrachopa*	*Bathycoelia distincta*	*Nezara viridula*
small Vespertilionidae	148+	18	**8**	**5**	**13**	**5**
Scotophilus dinganii	177+	14	**9**	**4**	**10**	**4**
Myotis bocagii	6	1	0	0	**1**	0
Neoromicia nana	30+	3	**1**	**1**	**3**	**2**
Rhinolophus simulator	5	0	0	0	0	**1**
Molossidae	33	3	0	**2**	**1**	**1**
Nycteris thebaica	12	0	**1**	0	**1**	0

3.3. Pest consumption of the different species/families

Our sequencing results showed that all bat species/families consumed insect pest species (Table 2). Whereas, all species and families except *Myotis bocagii* (N = 2) and *Rhinolophus simulator* (N = 1) foraged on both the Lepidopteran and Hemipteran pest species. Most of the Vespertilionidae species (except *M. bocagii*) foraged on all four insect pest species. The Molossidae consumed all but one insect pest (*C. peltastica*) and *N. thebaica* consumed one of the Hemipteran species (*B. distincta)* as well as one of the Lepidopteran species (*C. peltastica).*

4. Discussion

All families of bats for which we collected faecal pellets (Molossidae, Nycteridae, Rhinolophidae and Vespertilionidae) have been confirmed to forage on at least one of the four pest insects. Our results provide some interesting new insights into the foraging ecology of the different species (Monadjem et al., 2010, see Supplement 2). The limited number of pest species detected in *M. bocagii* (N = 2) and *R. simulator* (N = 1) faecal samples is most likely a result of the small sample size collected for those species as well as for *N. thebaica* (N = 3). The Vespertilionidae species, for which a large sample size was collected, appear to be highly generalist predators with most species consuming both Hemiptera and Lepidoptera insect pests. Hemiptera species are listed as one of the major families of prey insects included in the diet of two out of the small Vespertilionidae recorded in the study area and as a possible prey of *S. dinganii* but not at all for *N. nana* (Supplement 2). However, we found a high prevalence of Hemiptera pest species in the faecal samples of *N. nana, S. dinganii* and the small Vespertilionidae (Table 2). Likewise, Lepidopteran species are not known to be a major prey item in the diet of *S. dinganii* but were quite prevalent in our analyses of faecal pellets. *Nycteris thebaica* was also confirmed to forage on moth (*C. peltastica*) as well as stinkbug (*B. distincta*) species.

Generally, our results suggest that all the insectivorous species and families recorded in the study area are much more generalist and presumably opportunistic feeders than previously assumed (Monadjem et al., 2010) with more than half of the samples analysed (55.6%) containing sequences of at least one of the four insect pests.

Our results for *C. peltastica* (found in 17.5% of faecal samples) are similar to the results of McCracken et al. (2012) looking at consumption of corn earworm moths (*Helicoverpa zea*) by bats in Texas, United Stated. The study found between 34.4 and 17.3% of samples in triplicate qPCR reactions positive for corn earworm (McCracken et al., 2012). The rice borer moth (*Chilo supressalis*) was found in 20% and 50% of bat faecal samples in Iberia, during two peaks in abundance of this moth (Puig-Montserrat et al., 2015). The results of another study (Brown et al., 2015) focusing on the consumption of insect pests by bats in pecan orchards, in Georgia and Texas, found 1.4% of samples positive for pecan nut casebearer moths (*Acrobasis nuxvorella*), 3.8% for hickory shuckworm moths (*Cydia caryana*) and 5.4% for corn earworm moths (*Helicoverpa zea*). The same study also directly sequenced 22 insect pieces from bat faecal pellets, of which seven were confirmed to be *N. viridula* (Brown et al., 2015). Our results confirm a wide range of pest insect consumption by species such as *S. dinganii* and are in line with previous studies looking at insect pest consumption by individual *Miniopterus schreibersii* in Europe and *Tadarida brasiliensis* in the USA (Aizpurua et al., 2018; Krauel et al., 2018). *Miniopterus schreibersii* was confirmed to forage on 44 different insect pest species and 94% of faecal samples contained insect pest species (Aizpurua et al., 2018). Likewise, faecal samples of *Tadarida brasiliensis* also contained a range of 44 insect pest species (Krauel et al., 2018).

Our study shows that multiplex fluorescent-fragment analyses can be an efficient tool to screen bat faecal samples for specific insect species. However, our results also tell a cautionary tale about primer specificity. Even though, optimisation assays of the primer sets showed target specificity, the primer sets for the Lepidopteran species (*T. batrachopa* and *C. peltastica*) were not sufficiently specific and sequencing of target products showed cross amplification as well as amplification of non-target species. Therefore, it is imperative to sequence PCR products for the initial validation of primers. However, we assume that the two stinkbug primers developed and verified for specificity in this study, can now be used quickly and cost-effectively to expand faecal diet analyses to other agricultural systems or to study pest predation by other vertebrate species. The green vegetable stinkbug (*N. viridula*) is considered a pest across many agricultural systems worldwide including soybean

and cotton (Prado et al., 2009; Tillman, 2006). Whereas, the two-spotted stinkbug (*B. distincta*) is indigenous to Southeast Africa but also a major (or minor) pest to crops other than macadamia such as avocado (Schoeman, 2016; Schoeman, 2013).

The sample size (number of pellets in each sample) had no influence on the probability of detecting one or more of the four insect pest species in our study. We frequently detected pest insects in small samples ($N < 3$ pellets). Nonetheless, studies focusing on the overall foraging behaviour of bats suggest a sample size of 20 pellets per bat species (for each location and collection event) or five pellets per individual bat, and confidence does evidently increase with sample size (Whitaker et al., 2009; Whitaker et al., 1996).

Further insight could be gained by extending this molecular approach to other agroecosystems and by comparing pest predation in extensive and lower intensity or organic systems (Park, 2015). The majority of research, including this study, is currently focusing on common insectivorous bat species, presumably providing ecosystem services such as pest control for the most part. However, as suggested by Russo et al. (2018), rare species such as the *Rhinolophus* sp., which are 'gleaners', might also play a key role in suppressing certain pest insect species and will be affected considerably more by ongoing land-use change and, possibly, displacement by common species. The low sample size for *Rhinolophus* sp. ($N = 1$) but also for the *M. bocagii* ($N = 2$) and *N. thebaica* ($N = 3$) in our study provides very limited insight into the foraging prevalence of insect pests for those species. However, insect pests have been detected in samples from both bat species and future studies should aim to compare the prevalence of pest insects in the diet of both common and rare bat species with a similar sampling effort.

Apart from information about the foraging ecology of bats regarding insect pest consumption, our study also provides an indication on the presence and absence of insect pest species in macadamia orchards throughout the year over a 22 months period (July 2015 to April 2017). Our results suggest that the indigenous *B. distincta* was present in macadamia orchards year-round, whereas *N. viridula* was absent from bat faecal pellets from June to September (Fig. 1). *T. batrachopa* was absent in May and from July to October, whereas *C. peltastica* was only absent from bat faecal pellets in September (Fig. 1). While monitoring of insect pest species in macadamia orchards is usually limited to the macadamia growing season (De Villiers & Joubert, 2003) our results are in line with the previous suggestion of immigration (with nutset in October) and emigration (with the end of the harvest) of *B. distincta* populations to and from macadamia orchards (Schoeman, 2013). However, it also shows that at least some of the *B. distincta* population remains in or around macadamia orchards after macadamia harvest. Therefore, molecular approaches such as fragment analyses of bat faecal pellets could be used for the detection and monitoring of dispersal of new insect pest species through bat faecal pellets (Maslo et al., 2017; Russo et al., 2018).

In conclusion, the results of this study may incentivize farmers such as macadamia growers to consider bats in their integrated pest management approach (IPM) in order to enhance or maintain natural pest control. Reviewing the findings of other studies (Crisol-Martínez et al., 2016; Fuentes-Montemayor et al., 2011; Park, 2015; Weier et al., 2018) natural and semi-natural vegetation promote bat activity and potentially biological control of major crop pests in agricultural landscapes circumglobally. The study of Weier et al. (2018) highlights that the conservation of bat species and the promotion of their ecosystem services requires macadamia growers to keep natural and semi-natural vegetation patches intact. Apart from conserving natural vegetation, it is therefore recommended to maintain fallow periods by practicing extensive and rotational agriculture in the vicinity of orchards for example in cattle farming (Starik, 2016). Previous studies have suggested that bat activity is positively influenced by the presence of natural and artificial waterbodies (Park, 2015; Sirami et al., 2013; Stahlschmidt et al., 2012). Adding artificial roosting opportunities to orchards might further improve pest control provided by bats (Puig-Montserrat et al., 2015). It is also essential to monitor the effects of implementing new agroecosystem management approaches and extend management beyond farm boundaries. The implementation of 'Agri-environment schemes' on 18 farms in Scotland, which each followed at least three schemes of managing 'field margins or beetlebanks'; 'hedgerows'; 'water margins; and 'species-rich grasslands'; did not improve bat activity, however, the presence of periphery woodlands had a positive influence on bat activity (Fuentes-Montemayor et al., 2011).

We, therefore, suggest farmers should maintain and restore natural and semi-natural (fallow) vegetation, provide roosting opportunities in and around macadamia orchards and add water sources such as ponds and dams to promote bat activity. Furthermore, we strongly advise against scheduled sprays and recommend keeping pesticide treatments to a minimum possible threshold. While it remains largely uncertain what direct effects pesticides have on bats, it becomes increasingly clear that pesticides are one of the major factors leading to the current mass insect species extinction and, therefore, a threat to vertebrates such as bats depending on an abundance of insect prey (Mineau and Callaghan, 2018; Sánchez-Bayo and Wyckhuys, 2019).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gecco.2019.e00626.

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