Bats eat pest green vegetable stink bugs (*Nezara viridula*): Diet analyses of seven insectivorous species of bats roosting and foraging in macadamia orchards

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ABSTRACT

A growing body of literature has documented the economically significant impact of bats as predators of agricultural pest insects. This has not yet been established in macadamia agro-ecosystems where stink bugs (Hemiptera: Pentatomidae) are the major pests. The present study examined faecal pellets of seven species of bats collected from macadamia orchards in the Levubu Valley, Limpopo Province, South Africa, using both conventional microscopic as well as second generation sequencing analyses (faecal pellets from three bat species were studied by both methods). Microscopic analysis of faecal pellets and culled prey remains collected from night and day roosts and captured individuals of slit-faced bats (*Nycteris thebaica*), Angolan free-tailed bats (*Mops condylurus*), African pipistrelles (*Pipistrellus hesperidus*) and yellow house bats (*Scotophilus dinganii*) revealed important (20-50%) proportions of bugs (Order Hemiptera), higher than recorded in previous studies conducted in natural habitats. But this method was unable to provide further taxonomic resolution concerning the families or species of bugs present and whether these included the pest green vegetable stink bug (*Nezara viridula* - also known in the USA as the southern green stink bug). Preliminary results from a molecular approach, using second generation sequencing of a 157 bp fragment of the COI-barcoding region, revealed that faecal pellets of five species of bats foraging and roosting in macadamia plantations contained DNA from the pest green vegetable stink bug: slit-faced bats (*Nycteris thebaica*), Mops free-tailed bats (*Mops midas*), little free-tailed bats (*Chaerephon pumilus*), African pipistrelles (*Pipistrellus hesperidus*) and yellow house bats (*Scotophilus dinganii*). No stink bug DNA was detected in pellets of a sixth species, Sundevall's leaf-nosed bat (*Hipposideros caffer*). Based on a total of 37 faecal pellets analysed for all bat species combined, about one third (32%) of the pellets contained DNA from green vegetable stink bugs. A similar proportion of the pellets (35%) contained DNA from unidentified insects from the order Hemiptera. These data provide unequivocal evidence for predation by bats on green vegetable stink bugs, but the economic importance remains to be established although it is likely to be high.

UITTREKSEL

Meer inligting oor die ekonomies-belangrike rol wat vlermuise as predatore speel in die bekamping van insekplae in die landbou word toenemend gepubliseer. Die rol van vlermuise in die bestryding van stinkbesies (Hemiptera: Pentatomidae) as dié belangrikste plaaginsek in makadamia-boorde, is egter nog nie omskryf nie. Die onderhawige studie bevat 'n ontleding van vlermuismis van sewe vlermuisspesies wat in makadamia-boorde in die Levubu-vallei, Limpopo-provinsie, Suid-Afrika, voorkom. Ontledings is met behulp van tradisionele mikroskopiese analises, asook tweede-generasie DNS-ontleding, op die mis van drie

spesies gedoen. Mikroskoop-analises van vlermuismis en ander prooi-reste van *Nycteris thebiaca*, *Mops condylurus*, *Pipistrellus hesperidus* en *Scotophilus dinganii* wat by nagvoedingstasies en slaapskuilings (roosts) versamel is, asook die mis van individue wat in nette gevang is, het aan die lig gebring dat 'n belangrike deel (20-50%) van die vlermuise se dieet, insekte van die familie Hemiptera bevat. Hierdie syfers is hoër is as dié wat in vorige misontledings verkry is wat in natuurlike habitatte uitgevoer is. Hierdie metode kon egter nie meer taksonomiese besonderhede verskaf oor die families of spesies van Hemiptera teenwoordig nie, en of dit die groen stinkbesie (*Nezara viridula*) insluit nie. Die voorlopige resultate van tweede-generasie DNS-ontledings van die COI-geen het egter getoon dat die vlermuismis van vyf van die vlermuisspesies wat in makadamia-boorde vir prooi jag, DNS van die groen stinkbesie bevat; hierdie vyf spesies is *Nycteris thebiaca*, *Mops midas*, *Chaerephon pumilus*, *Pipistrellus hesperidus* en *Scotophilus dinganii*. Geen sodanige DNS van stinkbesies kon in die mis van 'n sesde spesie, *Hipposideros caffer*, gevind word nie. Uit al die spesies se totale ontledings van 37 mismonsters het ongeveer 'n derde (32%) DNS van die groen stinkbesiespesie bevat. 'n Soortgelyke hoeveelheid (35%) van die mismonsters het DNS van ongeïdentifiseerde Hemiptera bevat. Hierdie inligting bewys onteenseglik dat stinkbesies 'n beduidende hoeveelheid van insekvretende vlermuise se dieet uitmaak; die ekonomiese impak moet egter nog bepaal word, hoewel dit waarskynlik ook aansienlik groot is.

INTRODUCTION

Insect pests cause significant damage to agricultural production worldwide. Bat predation of pest insects has been shown to have a significant economic benefit to farmers (Cleveland *et al.*, 2006; Boyles *et al.*, 2011; Kunz *et al.*, 2011). In macadamia plantations in South Africa, stink bugs (Hemiptera: Pentatomidae) are the major pests of macadamias (Ironside, 1973; Jones & Caprio, 1992; Jones *et al.*, 1992; Jones & Caprio, 1994; Vincent *et al.*, 2001; Schoeman & Mohlala, 2012).

Very few African studies have examined the economic impact of bats eating agricultural pest insects. In sugarcane plantations in Swaziland, Noer *et al.* (2012) showed that two molossid bat species (*Chaerephon pumilus* and *Mops condylurus*) roosting near sugarcane plantations, selectively forage over these fields, while Bohmann *et al.* (2010 & 2011) showed that these bats feed on insect species such as boring moths (*Eldana saccharina* and *Mythimna phaea*) and stink bugs (Hemiptera, Pentatomidae).

There is an urgent need to demonstrate the economic value of bats to agriculture to promote awareness of the need for their conservation. Increasing local bat populations (*e.g.* by erecting bat houses) may prove to be an effective and less environmentally destructive means of keeping insect pest numbers down, than simply increasing chemical pesticide use which may ultimately prove counterproductive if populations of beneficial top insects predators like insectivorous spiders, bats and birds decline through poisoning (Wickramasinge *et al.*, 2003; Pocock & Jennings, 2008).

Previous studies (Taylor *et al.*, 2011, 2012a, b) demonstrated that a variety of local bat species forage in macadamia orchards and that seasonal variation of foraging activity of bats in macadamia orchards corresponds with the times of the year when major infestations of pest insects are known to occur in local macadamia orchards, *e.g.* November to January in macadamia nut borers, *Thaumatotibia batracopa*, and late austral summer to autumn in stink bugs.

The objective of this study was to establish baseline data on the diet of different bat species resident in macadamia agro-ecosystems, with a view to identifying possible insect pest families, genera and species. In particular, we were interested to establish whether bats consumed the primary pest of macadamias, green vegetable stink bugs (*Nezara viridula*) or other pest pentatomid species (such as *Bathycoelia natalicola*). To meet this objective, we examined the diet of seven bat species with very different foraging strategies, including clutter-feeders (the slit-faced bat, *Nycteris thebaica*, and Sundevall's leaf-nosed bat, *Hipposideros caffer*), clutter-edge foragers (the African pipistrelle, *Pipistrellus hesperidus*, and the yellow house bat, *Scotophilus dinganii*) and open-air foragers (the Angolan free-tailed bat, *Mops condylurus*, Mops midas bat, *Mops midas*, and the little free-tailed bat, *Chaerephon pumilus*) (see Monadjem *et al.*, 2010a for a summary of bat foraging strategies). We collected faecal pellets from known roosts of these bats in the area, as well as from captured individuals flying and presumably foraging in or near macadamia orchards. We used both conventional microscopic methods (Whitaker, 1988; Whitaker, McCracken & Siemers, 2009) as well as a second generation sequencing approach (modified from Bohmann *et al.*, 2011).

Although we would predict that clutter and clutter-edge bats would be more likely than aerial feeding bats to prey on predominantly slow-flying stink bugs which usually fly close to vegetation, Bohmann *et al.* (2011) recorded DNA of stink bugs (Pentatomidae) in eight out of 89 pellets (14%) analysed from aerial-feeding free-tailed bats (*Mops condylurus* and *Chaerephon pumilus*) in a sugarcane-dominated landscape in Swaziland. We thus predict that most of the bat species sampled would prey on locally abundant pest stink bug (and other) pest species, especially during the peak stink bug outbreak seasons of late summer and autumn (Schoeman & Mohlala, 2012; Taylor *et al.*, 2012a).

MATERIALS AND METHODS

Study area

The study area is located in the southern foothills of the Soutpansberg range, 20 km east of the town of Makhado (previously, Louis Trichardt), encompassing the Levuvhu Valley subtropical fruit-growing area in the south, which is dominated by extensive monocultures of macadamias, pecan nuts, avocados, bananas and pine and gum plantations (Fig. 1). It is bordered to the north by mountains covered predominantly by dense thickets classified by Mucina and Rutherford (1996) as "Soutpansberg Mountain Bushveld", as well as by extensive commercial plantations of gums and pines. Annual rainfall in the study area was around 930 mm in 2010 and 960 mm in 2011 and fell mostly between November and

April. Daily maximum temperatures frequently exceed 35°C in summer (October to March) but seldom exceed 40°C and minimum daily summer temperature seldom fall below 15°C, whereas winters are much colder, with minimum daily temperatures dropping to just over 0°C on a few days in June and July and maximum daily temperatures reaching 25°C (Taylor *et al.*, 2012a). The study area is known to be a species-rich hotspot for bats (Taylor *et al.*, 2012a, c).

Collection of faecal pellets

In all cases where faecal pellets were obtained from day or night roosts (for three species), they were collected onto 1 x 2 m boards covered in clingfilm placed under the position of roosting bats approximately one week prior to collection. Pellets of the remaining four species were obtained from captured individual bats, that were held individually in cloth bags until they yielded sufficient numbers of pellets. In the case of

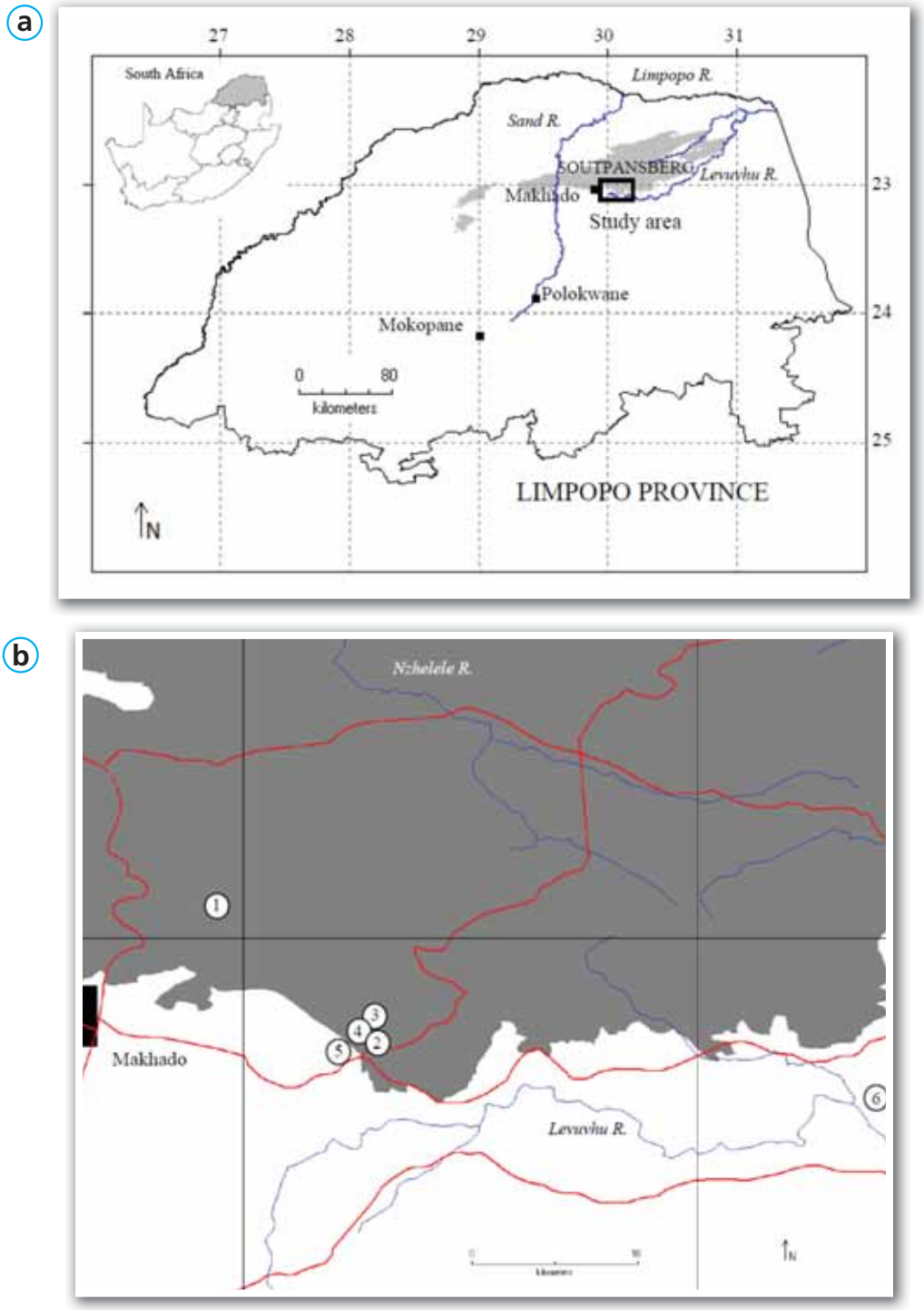

Figure 1. (a) Map of study area showing location of study area in South Africa's Limpopo Province and (b) detailed location of pellet collection sites within the study area from Farm Vlakfontein position 1, Farm Welgevonden, positions 2, 3, 4 and 5, and Farm Laatsgevonden position 6. Grey shading indicates the extent of vegetation types associated with the Soutpansberg Range.

Pipistrellus hesperidus (n=2) and *Hipposideros caffer* (n=1), these were from harp-trapped individuals, whereas in *Scotophilus dinganii* (n=3) and *Chaerephon pumilus* (n=1), these were from individuals roosting in an attic, which periodically flew accidentally into the house. Collection sites were primarily from positions 3 (day-roost of *M. condylurus*), 4 (day roost of both *M. midas* and *M. condylurus*), 2 (day roost of *S. dinganii* and *C. pumilus*) and 5 (harp trap site for collection

Table 1. Summary of faecal pellet collections used for microscopic dissection for identification of arthropod prey items in the diet of four species of bats and summary of proportions (by volume) of unidentified Hemiptera.

Species	Sample months	No. pellets analysed	No. prey orders	Hemiptera % volume	Hemiptera % occurrence
N. thebaica	July, Aug, Oct, Nov 2010	20, 4	5	19.5	25.0
S. dinganii	Feb 2011, Feb 2012, Oct 2012	24, 24	5	30.3	91.7
P. hesperidus	Sept 2011, Jan 2012	18, 4	4	50.0	75.0
M. condylurus	Apr, Nov 2010, Feb 2012	30, 30	4	38.0	76.7
Species combined		92	8	34.4	79

Table 2. Summary of results from second generation sequencing for Hemiptera prey (data for other prey insect taxa not shown).

				% occurrence (no. of pellets) of Hemiptera		
Species	**Sample months**	**No. pellets analysed**	**No. prey orders**	***N. viridula***	***M. acuta***	**Hemiptera (not identified)**
N. thebaica	May 2012, Aug 2010, Aug 2012	6	5	16.7	16.7	16.7
S. dinganii	Feb 2011, Feb 2012	2	5	100	50	100
P. hesperidus	Aug 2011, Jan 2012	4	6	66.7	0	66.7
C. pumilus	Apr 2012	1	5	100	100	100
H. caffer	Aug 2012	1	2	0	0	0
M. midas	Aug & Nov 2012	24	7	25.0	16.7	29.2
Species combined		37		32.4	18.9	35.14

of *P. hesperidus* and *H. caffer* individuals) of the farm Welgevonden, with one site located approximately 10 km to the northeast (the farm Vlakfontein, a day-roost of *M. midas*) and another 30 km to the east (the farm Laatsgevonden, a night-roost of *N. thebaica*) (Fig. 1). Pellets to be analysed microscopically were kept in ziplock bags at 4°C prior to analysis, while pellets to be analysed molecularly were stored in 90% ethanol at 4°C.

Microscopic dissection of faecal pellets

Wherever possible, a minimum of five pellets were analysed for each sample as described by Whitaker (1988) and Whitaker, McCracken & Siemers (2009). Each pellet was teased apart after adding 70% ethanol and the arthropod exoskeleton fragments were identified to order (or family if possible) using the standard key in Whitaker (1988), Whitaker, McCracken & Siemers (2009), drawings in Scholtz & Holm (1985) and a reference collection of arthropods (insects and spiders) trapped with malaise traps in macadamia orchards during two nights in December 2010 and January 2011 at Farm Welgevonden (Fig. 1, position 2). For each pellet, the percentage by volume of the different prey orders was estimated.

Identification of prey-DNA in faecal pellets

A detailed account of the molecular laboratory procedures followed is beyond the scope of this article but will be described elsewhere (Taylor *et al.*, in preparation; for similar approaches, see also Binladen *et al.*, 2007; Meyer & Kircher, 2010; Bohmann *et al.*, 2010, 2011; & Zeale *et al.*, 2011). The main steps are briefly summarised below.

Second generation sequencing of prey-insect DNA in faecal pellets

In summary, insect DNA was extracted from 44 unique bat faecal pellets. In order to identify insect-prey in the bat droppings, amplification and second generation sequencing of ca. 157 bp insect-COI fragments (Zeale *et al*., 2011) were performed on the faecal extracts following a modification of the methods described in Bohmann *et al*., 2011. The assay described here was customised for Illumina MiSeq sequencing as opposed to GS FLX sequencing used by Bohmann *et al*., 2011.

Sequencing of pest insects

Already detached legs from collected specimens were taken from each of 11 insect (Hemiptera) species collected from local macadamia plantations by JNS and identified by Dr M Stiller of the Agricultural Research Council. This list included three known macadamia pest species and an additional eight species which are not known to be pests. The pest species included *Nezara viridula* (Pentatomidae; green vegetable stink bug), *Pseudotheraptus wayi* (Coreidae; coconut bug) and *Bathycoelia rodhaini* (Pentatomidae; yellow-spotted stink bug). The non-pest species included *Dalsira costalis* (Pentatomidae; stink bug), *Caurarufi ventris* (Pentatomidae; stink bug), *Coridius nubilis* (Pentatomidae; stink bug), *Anoplecnemis curvipes* (Coreidae; giant coreid bug), *Leptoglossus australis* (Coreidae; leaf-footed plant bug), *Veterna* sp. (Pentatomidae; grass stink bugs) and *Dysdercus nigrofasciata* (Pyrrhocoridae; cotton stainer, a major pest of cotton). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Extracts were amplified with Folmer primers (LCO1490, HCO2198) (Folmer *et al.*, 1994) amplifying a 648 bp COI-fragment. Purification and Sanger sequencing of the products (both directions) were undertaken by the commercial facility offered by Macrogen (Seoul, South Korea).

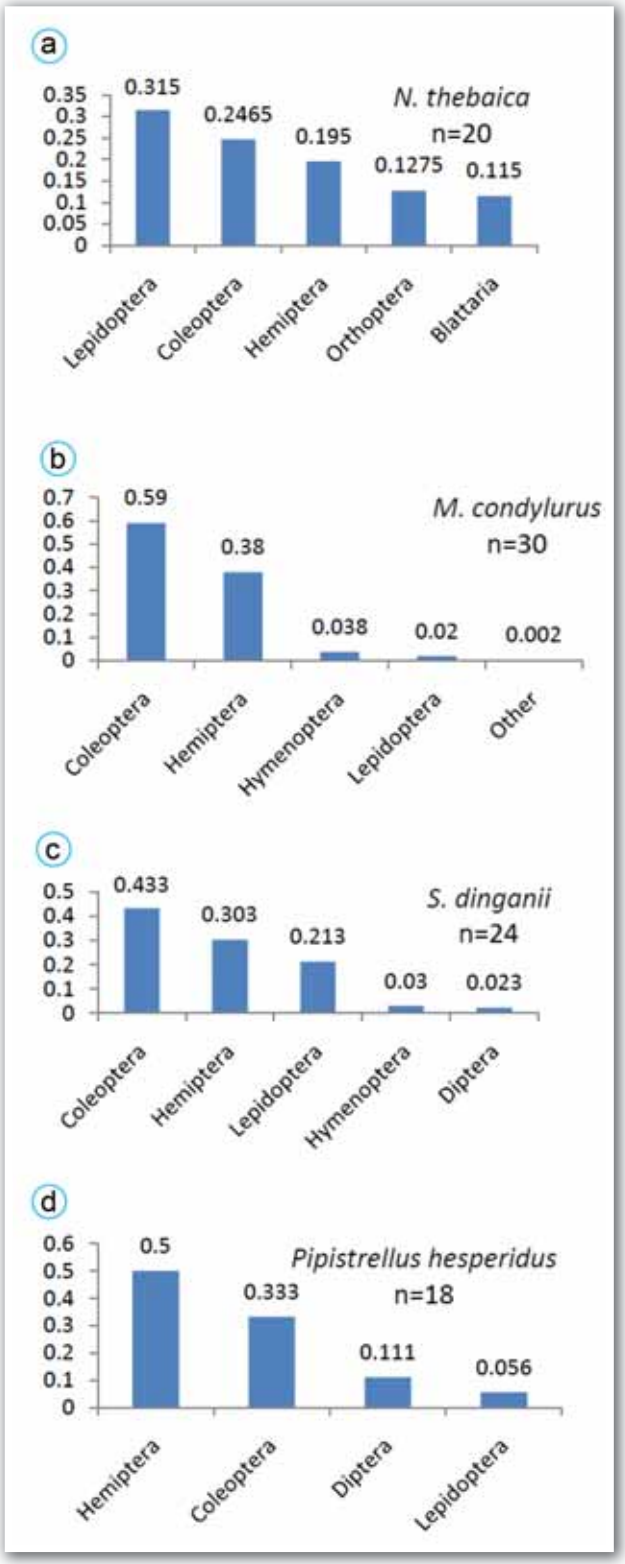

Figure 2. Bar charts showing percentage volume of different orders of prey insects in the diet of four bat species analysed using microscopic dissection of faecal pellets.

Sequence analyses

Full details are provided elsewhere (Taylor *et al.*, in preparation). COI barcode sequences from different taxonomic levels (Actinopterygii, Arachnida, Branchiopoda, Chilopoda, Insecta, Isopoda, Ostracoda and Sarcopterygii) were downloaded from the BOLD v3 (Ratnasingham & Hebert, 2007) Public Data Portal to create a database and its corresponding taxonomy map. Furthermore, the sequences of the eleven hemipteran insects that were sequenced in this study were added to the database. Species identification was made with QIIME v1.7.0 (Caporaso *et al.*, 2010).

RESULTS AND DISCUSSION

Dietary analysis by microscopic analysis of faecal pellets

Fig. 2 summarises proportions (by volume) of different orders of insects in the diet of four species of bats based on microscopic examination of 92 faecal pellets collected opportunistically between 2010 and 2012 (Table 1). All species contained an important component of unidentified Hemiptera in their diet (means of 20-50% by volume; 25-92% by occurrence) (Fig. 2, Table 1). Four to five different orders of insects (prey items) could be determined per species (eight in total). Given that this method relies on the identification of prey remains, which have been through the digestive system, further taxonomic resolution was not possible, *e.g.* we were unable to determine the presence of stink bugs (Pentatomidae or Coreidae) in general, or of individual species such as the green vegetable stink bugs (*N. viridula*).

The diet of two of these species (*N. thebaica* and *M. condylurus*) were reported in detail previously by Taylor *et al.* (2011) and Taylor *et al.* (2012c), who showed that the proportion of Hemiptera was higher than reported from previous studies conducted in natural areas. Likewise, the new data presented here for *S. dinganii* and *P. hesperidus* indicate relatively high proportions of Hemiptera (30.3 and 50.5% respectively), compared with previous studies. Based on five previous studies of *S. dinganii* conducted in natural habitats in South Africa and Zimbabwe, the proportion of Hemiptera was much lower, varying from 0 to 3.7% (Aldridge & Rautenbach, 1987; Fenton *et al.*, 1998; Schoeman, 2006; Jacobs & Barclay, 2009; Schoeman & Jacobs, 2011; Tshitande, 2011). In the above-mentioned studies, Coleoptera dominated in the diet of *S. dinganii* (42-100%), as found also in our study (43.4%). In the case of *P. hesperidus* it is known to be a bug-specialist and previous studies have shown Hemiptera to be an important component in the diet (33-40%) (Schoeman, 2006; Schoeman & Jacobs, 2011; Tshitande, 2011), only slightly lower than found in our study (50%).

Dietary analysis by second generation sequencing

Of the 44 pellets initially extracted, 37 were successfully amplified with insect generic minibarcode primers (Zeale *et al.*, 2011) and sequenced. Here, given the relevance to biological control of stink bugs, we report only on the Hemiptera component of the diet. More detailed and broader taxonomic analysis of the diet of the six species investigated will be reported elsewhere (Taylor *et al.*, in preparation). The molecular analyses identified the green vegetable stink bug (*N. viridula*) and the predatory pentatomid, *Macrorhaphis acuta*, among the Hemiptera sequences. Combining data for all species, 32% of pellets contained *N. viridula*, 19% contained *M. acuta* and 35% contained unidentified Hemiptera (Table 2). Apart from *N. viridula*, no DNA matches were obtained for the other 10 hemipteran species sequenced by this study (see Methods) and which are known to occur on macadamia farms. Very little is known about the life cycle of these bug species, which included three pest species. Wider sampling of faecal pellets throughout the year may reveal the presence of these species. The majority of our faecal samples, including those of *M. midas*, were collected from August to November (austral spring and early summer), whereas most pests of macadamia become prevalent from late austral summer onwards (Taylor *et al.*, 2012b).

Five out of six bat species analysed (all except for *H. caffer*) contained *N. viridula* DNA in their faecal pellets (Table 2). The prevalence varied from 16.7% (*N. thebaica*, n=6 pellets) to 100% in *S. dinganii* (n=2 pellets) and *C. pumilus* (n=1 pellet). In the species with the largest sample of pellets (*M. midas*; n=24 pellets), one quarter of all pellets from this bat species contained *N. viridula* DNA. The bats shown to consume *N. viridula* represent a wide range of different feeding groups, from a gleaning clutter-feeder (*N. thebaica*) to clutter-edge, aerial feeders (*S. dinganii*, *P. hesperidus*) to open-air feeders (*C. pumilis* and *M. midas*). Thus, our prediction that green vegetable stink bugs would be disproportionately represented in the diet of gleaning clutter foragers is not supported; instead, our data suggest that a wide range of bats are opportunistic foragers taking advantage of the local abundance of pest species such as the green vegetable stink bug.

CONCLUSIONS AND FUTURE PROSPECTS

Conventional microscopic analyses of faecal pellets from four bat species occurring in the study area revealed that all species contained an important component of Hemiptera in their diet (20-50% by volume and 25-92% by occurrence). Although this method was not able to positively identify the presence of pest stink bugs of the Pentatomidae, second generation sequencing of 157 bp mini-barcode fragment (COI) revealed DNA from green vegetable stink bugs (*N. viridula*) in five of six bat species examined (three of these which have also been analysed by conventional analysis). Although second generation sequencing allows for much greater precision and accuracy in determining the diet of bats and determining the presence of particular pest species, there might be a bias in terms of *e.g.* unequal amplification of target DNA from different orders of insects which at present limits the opportunity to quantify accurately the intake of pest insects (see for example review by Pompanon *et al.*, 2012). Hence, we can currently refer accurately only to % occurrence of analysed pellets. Equally, the microscropic method of analysing faecal pellets may also be biased and may not reflect actual intake of different orders. Feeding experiments where captive bats are fed known proportions of different insect orders should help to clarify the nature of these quantitative biases in both the microscopic and molecular methods. Until then, estimates of percentage occurrence of analysed pellets are the best way to present the results. Indeed, when we consider percentage occurrence for the microscopic results, we found similar occurrence of Hemiptera to the values obtained for unidentified

Hemiptera and for *N. viridula* in the molecular results, when we compare results for the same bat species (Tables 1 and 2). Thus, microscopic and DNA methods respectively showed percentage occurrence of Hemiptera or *N. viridula* as 25% and 17% for *N. thebaica*, 75% and 67% for *P. hesperidus* and 92% and 100% for *S. dinganii*.

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