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Determination of the diet of the ghost bat (*Macroderma gigas*) in the Pilbara region of Western Australia from dried prey remains and DNA metabarcoding

Alba M. Arteaga Claramunt[A,F], *Nicole E. White*[B], *Michael Bunce*[B], *Morgan O'Connell*[C], *Robert D. Bullen*[D] *and Peter R. Mawson*[A,E]

[A]School of Animal Biology, University of Western Australia, Crawley, WA 6009, Australia.
[B]School of Molecular and Life Sciences, Curtin University, Bentley, WA 6102, Australia.
[C]Biologic, PO Box 179, Floreat, WA 6014, Australia.
[D]Bat Call WA Pty Ltd, 43 Murray Drive, Hillarys, WA 6025, Australia.
[E]Perth Zoo, Department of Biodiversity, Conservation and Attractions, PO Box 489, South Perth, WA 6151, Australia.
[F]Corresponding author. Email: albaarteaga88@gmail.com

Abstract. The ghost bat (*Macroderma gigas*) is listed as Vulnerable in Australia, and is a difficult species to study in the wild. The published literature available on even the most basic aspects of its ecology is limited. This study describes an investigation into the diet of ghost bats occupying the Pilbara region of Western Australia, using identification of dried food remains recovered from beneath roosts in the 1980s and 1990s, and DNA metabarcoding of faecal pellets collected from roost sites during 2011–12. Ghost bat diet in the Pilbara region consists primarily of small mammal and bird species, with a lesser contribution from reptiles (geckoes and skinks) and amphibians. In total, 46 vertebrate taxa were identified, with 32 taxa identified from the dried food remains, and 21 taxa by DNA metabarcoding analysis of the faecal pellets. Only seven of the taxa identified were common to both collection methods, and 32 of those taxa identified represent new prey records for ghost bats in Western Australia, and 19 prey species that had not previously been reported from any other part of Australia. Knowledge of the diet of the ghost bat will provide land managers with important information necessary to ensure the continued survival of this species across its range.

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Introduction

The ghost bat is Australia's largest microbat, weighing up to 170 g (Hudson and Wilson 1986), and is considered primarily carnivorous (Marlow 1961). This generalist microbat relies on a wide range of prey items. Its diverse diet ranges from large invertebrates such as beetles and locusts to small vertebrates such as birds and rodents (Douglas 1967; Tidemann *et al*. 1985; Toop 1985; Boles 1999; Johnston *et al*. 2015). Published data regarding diet have been collected from all three Australian states or territories currently occupied by ghost bats: Queensland (Toop 1985), the Northern Territory (Tidemann *et al*. 1985; Schulz 1986; Diete *et al*. 2016) and Western Australia (Douglas 1967; Johnston *et al*. 2015). Most of these studies classified dried prey remains dropped by ghost bats that had accumulated below their feeding perches. In all cases it was assumed that the remains belonged to prey consumed by ghost bats even when direct feeding was not observed. Few investigations were based on direct observations of prey hunting (Douglas 1967; Tidemann *et al*. 1985), and others were opportunistic (Johnston *et al*. 2015; Diete *et al*. 2016).

Studying predation events of rare species, such as the ghost bat, that live in remote locations can be challenging. In the past, stomach dissections of vouchered specimens were carried out (Douglas 1967), but this type of study is no longer considered a suitable methodology for studying the diet of a threatened species (McKenzie and Hall 2008). Ghost bats are highly sensitive to disturbance, and direct handling of bats or any human activity close to their roosting sites can increase the risk of temporary site abandonment, leading to detrimental effects on the population, especially in maternity roosts (Woinarski *et al*. 2014). Non-invasive methods such as the collection of food debris from beneath unoccupied roost sites are better tools for investigating the diet of ghost bats. They provide evidence of the presence of the ghost bats along with the prey species that they rely on, which can be important for informing conservation management decisions. Nevertheless, there is still a need to

verify the origin of the prey remains in order to avoid misinterpretations and incorrect conclusions. DNA analysis employing metabarcoding can confirm both the identity of the species that produced the faecal samples and the identity of the prey species. This study aimed to identify the dietary range of ghost bats in the Pilbara region of Western Australia.

Materials and methods

Dried prey material

Prey remains discarded by ghost bats were collected from directly beneath active roost sites located in the Pilbara. Prey remains were obtained from five abandoned mines: three copper, one iron ore and one gold. The copper mines were sampled in 1986 and 1987, the iron ore mine in 1990 and the gold mine in 1994. Faecal pellets were collected from eight ghost bat roosts located in caves in 2011 and 2012 (Fig. 1).

The unsorted food remains included a wide range of skeletal fragments such as jaws and skulls, tail vertebrae, wing and foot bones, molar and incisor teeth, feathers, lower leg and feet remains.

We took measurements (to ±0.5 mm) of all the material with an NSK vernier caliper (range 9″, Hyogo, Japan), and made detailed observations with a stereo-microscope (Olympus Line-up SZX9, Magnification ×10–20; L.M.G. Scientific Services, a division of James Optronics Pty Ltd). The prey remains were compared with vouchered specimens held in the Western Australian Museum.

Dasyurid and rodent species identification was based on the size of premolars and molars and the shape of the molar cusps. Bat species were identified on the morphology of their skulls and tooth shape. The identification of avian prey remains was based on colour, size and shape of primary wing feathers, and the size, shape, colour, number and arrangement of the metatarsi. Amphibian and reptile remains were less abundant, and only the skulls were used to identify the amphibians. Skulls and retained skin sections (showing colour and scale patterns) were used to identify the reptiles. Remains from invertebrates, such as elytra, were also found but not examined further.

The taxonomy used here follows the Checklist of Terrestrial Vertebrate Fauna of Western Australian (WAM 2017).

Scat DNA faecal dietary material

Twenty-two suspected ghost bat scats were collected from caves believed to be occupied by ghost bats in the Pilbara (Fig. 1). The samples were stored at −20°C until they were used in the extraction process.

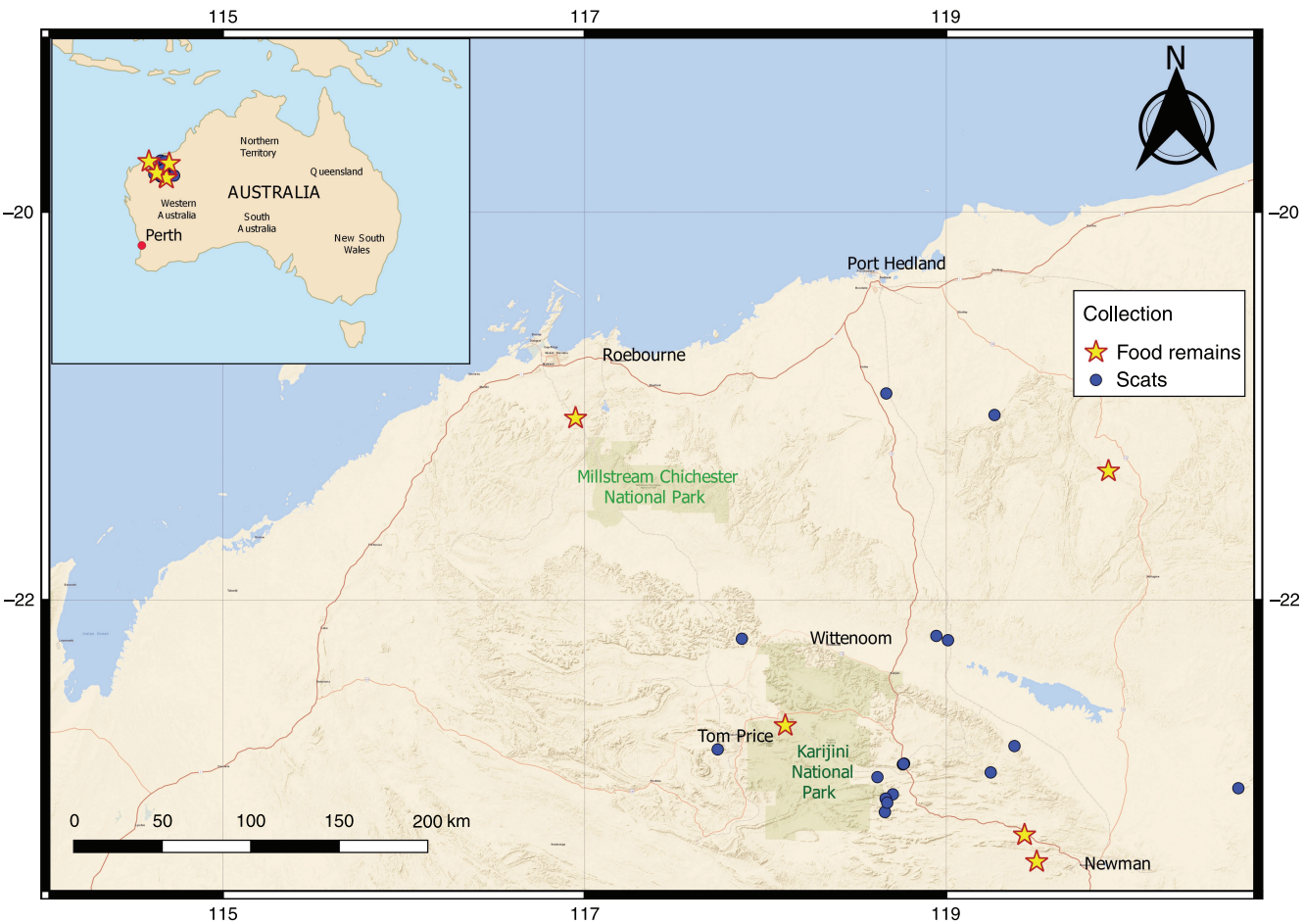

Fig. 1. Locations for the collection of dried food remains and faecal pellets.

Scat DNA extraction and quantification

Scat DNA was extracted using a QIAamp DNA stool Kit (Qiagen, CA, USA) with the addition of an overnight digest at 56°C to optimise maximum DNA yields. Genomic DNA (gDNA) and extraction controls were frozen at −20°C until further analyses.

The DNA extracts were then quantified via real-time quantitative polymerase chain reaction (qPCR) to assess the quality and quantity of gDNA, in addition to the assessment of PCR inhibition. Each gDNA extract was assessed at three DNA dilutions (undiluted, 1 : 10, 1 : 100) using five universal primer sets that target the mammal 16S rRNA gene (Taylor 1996), the bird 12S rRNA gene (Cooper 1994), the fish 16S rRNA gene (Deagle *et al*. 2007) and two regions of the vertebrate cytochrome-b gene (Kocher *et al*. 1989; Verma and Singh 2003).

From the qPCR results an optimal DNA concentration was selected for DNA sequencing if it was shown to be free of inhibition and DNA yields were of sufficient quality. It has been advocated that assessment of gDNA extracts in this way can facilitate reproducible qualitative data and the possibility of quantitative interpretations (Murray *et al*. 2011).

DNA metabarcoding

The seven gene regions targeted, from 91 bp to 450 bp in size, were amplified with modified primers and sequenced on a GS Junior (454 Life Sciences) and Ion Torrent (Thermo Fisher Scientific) high-throughput sequencing platform. The modified primers incorporated a unique 8–10-bp Multiplex Identifier tag (MID-tag) and adaptors specific for each sequencing platform used.

Independent MID-tagged qPCR set-up for samples and controls was prepared in a physically separate ultraclean laboratory and was carried out using each primer set in 25-µL reactions containing 1X PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.4 mg mL^{-1} BSA, 0.25 mM of each dNTP, 0.4 µM of forward and reverse MID-tag primer, 0.25 µL AmpliTaq Gold, 0.6 µL SYBR Green and 2 µL of gDNA. The cycling conditions were as follows: initial heat denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s; 52−55°C for 30 s (annealing step); 72°C for 45 s followed by final extension at 72°C for 10 min. MID-tagged PCR amplicons were generated in duplicate for each sample and pooled together to minimise the effects of PCR stochasticity. The pooled amplicons were purified using Agencourt AMPure XP PCR Purification Kit (Beckman Coulter Genomics) following the manufacturer's protocol. Purified amplicons were electrophoresed on 2% agarose gel and pooled in approximately equimolar ratios based on ethidium-stained band intensity to form a MID-tagged DNA sequencing library. For each MID-tagged qPCR assay, extraction and PCR controls were included and, if found to contain amplifiable DNA, these reactions were incorporated into the pooled MID-tagged DNA sequencing library. GS junior and Ion Torrent sequencing were performed as per manufacturer's instruction.

Scat DNA data analysis

MID-tag PCR amplicon sequence reads obtained from GS Junior and Ion Torrent were sorted (filtered) back to the scat sample based on the MID-tags assigned to each DNA extraction using Geneious 7 (Drummond *et al*. 2011). MID-tag and primer sequences were trimmed from the PCR amplicons, allowing for no mismatch in length or base composition using Geneious. BLASTn 2.2.23 was used to search for the three genes amplified (Altschul *et al*. 1990), against the NCBI GenBank nucleotide database to enable taxonomic identification. This was automated in the internet-based bioinformatics workflow environment, YABI (Hunter *et al*. 2012). The BLAST results obtained using YABI were imported into MEtaGenome Analyzer 4 (MEGAN), where they were taxonomically assigned using the LCA-assignment algorithm (minimum bit score = 65.0, top percentage = 5%, minimum support = 5) (Huson *et al*. 2007).

Results

In total, 46 vertebrate taxa were identified from two of the investigative approaches (38 taxa to species level). The mammal DNA recovered from the 22 suspected ghost bat scats confirmed that all 22 were deposited by ghost bats. Thirty-two taxa were identified from the dried food remains collected from day roosts, and 21 taxa were identified from the DNA analysis of the faecal pellets. Only seven of the taxa identified were common to both collection methods (Table 1).

The diversity in prey species consumed was evident: two (2) frog species were recorded; four reptiles were identified to species level and two to genus level; 16 birds were identified to species level, one to genus level and four to family level; and 16 mammals were identified to species level and one to genus level. The 16 mammal taxa comprised six dasyurid, six rodent and four microchiropteran bat species. Thirty-two of the 46 taxa identified are new prey records for ghost bats in Western Australia, and 19 are prey species not previously reported from any other part of Australia. Ghost bat diet was also rich in bird species, most of which are small to medium-sized birds weighing <100 g. Surprisingly, for a region of Australia renowned for its diversity of reptile fauna, only four reptile and two amphibian species were also identified (Table 1).

Discussion

This study identified 32 prey species not previously recorded for ghost bats in Western Australia and 19 prey species not previously reported from any other parts of Australia (Table 1) (Douglas 1967; Toop 1985; Schulz 1986; Boles 1999; Johnston *et al*. 2015; Diete *et al*. 2016).

We identified prey items from 16 avian families and 11 species, all of them common. Our results agree with those of Boles (1999), who reported 50 avian species that are part of the ghost bat diet elsewhere in Australia. When arranged by body size, as was done by Boles (1999), we found that many bird species were small (<33 g body weight) and the most abundant passerine species was the singing honeyeater (*Gavicalis virescens*). Five larger (>66 g) bird taxa were identified: grey-crowned (*Pomatostomus temporalis*) and white-browed (*P. superciliosus*) babblers and magpie-lark (*Grallina cyanoleuca*) and the two taxa identified only to family level (Corvidae sp. and Laridae sp.). It has been previously documented that ghost bats are capable of lifting large (>66 g) prey items (Kulzer *et al*. 1984; Boles 1999) but given that the

Table 1. Ghost bat prey species identified from dried food remains and ghost bat faecal samples
Material was collected from disused mines and caves located in the Pilbara

Class	Family	Prey species	Detection method	
			Dried food material	DNA faecal analysis
Amphibia	Hylidae	*Cyclorana maini*[A]	✓	✓
	Limnodynastidae	*Notaden nichollsi*[A]	✓	–
Reptilia	Carphodactylidae	*Nephrurus wheeleri*[A]	✓	–
	Diplodactylidae	*Oedura fimbria*[A]	✓	✓
	Gekkonidae	*Gehyra pilbara*[A]	✓	–
	Scincidae	*Ctenotus* sp.	–	✓
		Ctenotus saxatilis[A]	✓	–
		Egernia sp.[A]	–	✓
Aves	Acanthizidae	*Smicrornis brevirostris*[A]	–	✓
	Aegothelidae	*Aegotheles cristatus*	–	✓
	Alaudidae	*Mirafra javanica*[A]	✓	–
	Columbidae	*Geopelia cuneata*	–	✓
		Geopelia striata[A]	✓	–
	Corvidae	Corvid sp.[A]	–	✓
	Estrildidae	*Emblema picta*	–	✓
		Taeniopygia guttata	–	✓
	Laridae	Larid sp.[A]	–	✓
	Maluridae	*Malurus* sp.[A]	–	✓
	Meliphagidae	Meliphagid sp.	–	✓
		Gavicalis virescens	✓	–
		Lichmera indistincta[A]	✓	–
	Monarchidae	*Grallina cyanoleuca*[A]	✓	–
	Pachycephalidae	*Pachycephala rufiventris*[A]	✓	–
	Pomatostomidae	*Pomatostomus superciliosus*[A]	✓	–
		Pomatostomus temporalis[A]	✓	–
	Psittacidae	*Melopsittacus undulatus*	✓	✓
	Rhipiduridae	*Rhipidura leucophrys*[A]	✓	–
	Zosteropidae	Zosteropid sp.[A]	–	✓
	Turnicidae	*Turnix velox*	✓	–
Mammalia	Dasyuridae	*Dasykaluta rosamondae*	✓	–
		Ningaui timealeyi[A]	✓	–
		Pseudantechinus woolleyae[A]	✓	–
		Sminthopsis sp.	–	✓
		Sminthopsis longicaudata[A]	✓	
		Sminthopsis macroura[A]	✓	–
		Sminthopsis ooldea[A]	✓	–
	Muridae	*Leggadina lakedownensis*[A]	–	✓
		Mus musculus	✓	✓
		Notomys alexis[A]	✓	–
		Pseudomys desertor[A]	✓	✓
		Pseudomys hermannsburgensis	✓	✓
		Zyzomys argurus[A]	✓	–
	Emballonuridae	*Taphozous georgianus*	✓	–
		Taphozous hilli[A]	✓	✓
	Molossidae	*Austronomus australis*[A]	✓	–
	Vespertilionidae	*Chalinolobus gouldii*[A]	✓	–

[A]Ghost bat prey species in Western Australia not previously reported in the published literature.

mass of adults of the two corvid species native to the Pilbara is 300–650 g (Johnstone and Storr 2004), it seems more likely that corvids were being hunted while small nestlings. Dried food remains of budgerigars (*Melopsittacus undulatus*) and the little buttonquail (*Turnix velox*) were the most common bird species, indicating the important role that these species have in the ghost bat diet in the Pilbara, matching the results reported by Johnston *et al*. (2015) in Karijini National Park.

We found numerous bat wings and bat skulls in our dried food samples. Previous studies have reported that ghost bats often feed upon other bat species with which they share the same roost sites (Douglas 1967; Toop 1985). We recorded four species of microbat (all with a mass <40 g) from three genera: the common sheath-tailed bat (*Taphozous georgianus*), Hill's sheath-tailed bat (*Taphozous hilli*), the white-striped free-tailed bat (*Austronomus australis*), and Gould's wattled bat

(*Chalinolobus gouldii*). Our data suggest that the ghost bat is a top-order, generalist predator in the trophic chain (White 2013) as there are few animal species such as larger owls (*Tyto* spp.), diurnal birds of prey hunting in predawn light (R. D. Bullen, pers. obs.) and snakes that prey on ghost bats (Nowak and Walker 1991).

Monitoring of threatened species without causing them disturbance is important from an ethical and conservation perspective. The collection of samples that are dropped naturally by animals, such as faeces, hair or food debris and their subsequent DNA analysis has allowed researchers to obtain large amounts of information on elusive endangered species, especially from carnivores (Kohn and Wayne 1997; Weiskopf *et al*. 2016). Determining prey species inventories, through faecal analyses, has the potential to contribute to a species' conservation management. DNA metabarcoding provides an in-depth window into the complexity of food chain(s), resource overlap(s), habitat associations, ecosystem health and detection of cryptic or undescribed species.

The two methodologies used in this study to determine the dietary range of the ghost bat have both advantages and disadvantages. The accumulation of food debris beneath roosts provided a bigger sample size than the 22 guano samples. This is likely due to the decomposition and/or consumption rate of faeces by invertebrates being greater than that for the skeletal material. However, our data were mainly limited to large vertebrates such as birds and mammals. Amphibian ($n = 2$ species), reptile ($n = 4$ species and 2 higher-order taxa), and invertebrate ($n = 4$, although not considered in the analysis) remains were scarce. This could be the result of several factors. The ghost bat is a generalist predator (Douglas 1967; Tidemann *et al*. 1985) and can switch its prey preference depending on what is more accessible and abundant. Due to the aridity in the Pilbara, most invertebrate species are small relative to vertebrate species. Summer temperatures in the Pilbara are extremely hot (many daytime temperatures are over 40°C) and rainfall is limited (<40%) outside of cyclone season (BOM 2016). Consequently, amphibian species could be restricted to moist refugia or remain underground for extended periods between precipitation events (Cartledge *et al*. 2008). In arid areas, like the Pilbara, the nutritional value and water content that mammal and bird prey can contribute to the ghost bat diet is higher than that derived from small insects, likely encouraging ghost bats to preferentially hunt larger prey. Such an approach to foraging contrasts with the study carried out by Tidemann *et al*. (1985), who showed that ghost bats in the Northern Territory based their diet mainly on insects (yellow-winged locust, *Gastrimargus musicus*) during the dry season, as this was the most common food source. Another possible reason why our sample size for insect remains was so small could be due to the different capacity of ghost bats to masticate and digest smaller insect prey species. Elytra of insects could be milled to small particles by the powerful jaws of ghost bats. As a result, the dried food remains might be the result of what ghost bats are not capable of ingesting, and a subset of their complete diet. The question of the full breadth of ghost bat diet in the Pilbara could be solved by DNA metabarcoding analysis but would be dependent on access to adequate DNA reference material for invertebrates. DNA metabarcoding would not be able to determine whether invertebrate DNA was ingested directly or as part of the stomach contents of a vertebrate prey item.

The small degree of overlap in diet, as determined from the two analytical methods, could be a function of the different collection times for the dried material (1986–87 and 1994) and the faecal pellets (2011–12) representing genuine changes in relative abundance of species across time. The differences could also reflect the regional and subregional affiliation of prey species to particular habitats, given the strong association of many of the small mammals inhabiting the Pilbara with particular habitat and soil types (Gibson and McKenzie 2009).

One important limit to using DNA faecal analysis is the lack of available genetic markers for the full range of the potential prey species living in the Pilbara. Another issue relevant to this technique is the fact that it does not easily provide information on prey abundance in the manner that collection of dried material can. We advocate that future dietary studies of ghost bats use a combination of the two methods to account for the individual disadvantages of each method. Finally, we suggest that ongoing monitoring of ghost bat prey is an efficient way to monitor how this vulnerable species is adapting to an environment that is affected by a variety of anthropogenic pressures.

Conflicts of interest

The authors declare no conflicts of interest.

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