Hamersley Subregion Ghost Bat Population and Roost Assessment: 2015 - 2016

BHP Iron Ore Pty Ltd

May 2017

DOCUMENT STATUS				
Version No.	Author	Review / Approved for Issue	Approved for Issue to	
			Name	Date
1	M. O'Connell C. Knuckey	B. Durrant	G. Watson	12 April 2017
2	M. O'Connell	B. Durrant	G. Watson	20 June 2017
3	C. Knuckey M. O'Connell	B. Durrant	G. Watson	2 August 2017

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EXECUTIVE SUMMARY

The ghost bat (*Macroderma gigas*) is the largest carnivorous bat in Australia, and is currently listed as Vulnerable by the International Union for the Conservation of Nature, Vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* and Schedule 3 (Vulnerable) under the *Wildlife Conservation Act 1950*.

Survey work within the Hamersley subregion since 2011 has documented many known and potential ghost bat caves; however, our understanding of how these caves are utilised and the extent and structure of the ghost bat population within the subregion has been poor. Obtaining information on cave use by ghost bats, and in particular if they are used as maternity roosts, is problematic due to the low number of bats present at any one time, and the fact that ghost bats use multiple roosts so may not be present within a monitoring roost at the time of sampling. Genetic and hormone analyses of ghost bat tissue and scats were determined to be the most appropriate approach for increasing knowledge of cave use and movement by bats.

This study comprised the following objectives:

- Investigate if faecal metabolites could be used to determine the presence of pregnant females within caves, and therefore the presence of a maternity roost;
- Confirm if caves within BHP's Central Pilbara tenements are being used for breeding by ghost bats, and if so, what is the relative importance of each cave to the population; and
- Investigate if DNA contained in faecal material can be used to identify individuals. If so, undertake a study of the population genetics of ghost bats within the Hamersley Range, including genetic diversity, structure and short-range spatial use.

Thirty-four caves were visited to collect fresh (one to two month-old) ghost bat scats for use in the hormone and genetic analyses. The caves identified for survey were known caves within BHP and third party tenure, and new caves identified in BHP tenure not previously surveyed for ghost bats.

Caves were visited in October 2015 and sheets were placed over ghost bat middens. The same caves were revisited to collect scats deposited on sheets in November and December 2015, and April and May 2016.

Approximately 571 scats were collected from the sheets and an additional 1100 were collected from the ground (and therefore of unknown age). The number of scats deposited within the caves during the sampling period ranged from zero to over 1000. Four caves within BHP tenure consistently recorded a high number of scats on the sheets indicating they are likely to represent important caves to local ghost bat assemblages. Some caves showed sporadic use, i.e. a high number of scats in some sampling periods and no use in others. Four

caves in BHP tenure recorded a low number of scats during each survey suggesting low but consistent use by ghost bat.

Scats collected during previous surveys during the 2014 breeding season (Biologic, 2015) and the 2015 breeding season were analysed for progesterone metabolite concentrations by enzyme-immunoassay. These scats were analysed alongside scats collected from individuals at Perth Zoo in 2015, with a known breeding status.

Progesterone metabolite levels from most of the scats collected from the Pilbara roosts were found to be similar to the baseline levels (i.e. non-pregnant) of captive female ghost bats. During 2015, scats from captive individuals showed elevated progesterone levels in the sampling period prior to giving birth. Following the birth, elevated progesterone levels could not be detected in scats. These results suggest that elevated progesterone levels in ghost bats can be used to determine pregnancy, but not lactation, and therefore cannot be used to determine if juvenile rearing is taking place within a roost.

Progesterone levels of Pilbara ghost bats showed that nine caves in 2014 and three caves in 2015 contained pregnant ghost bats, with all containing at least one scat with elevated progesterone levels.

Scats and tissue collected during November/December 2015 and March/April 2016 were used to investigate the population genetics of ghost bats within the Hamersley Range, including genetic diversity, structure and short-range spatial use. Genotyping was also used to quantify ghost bat numbers in the area, and cave usage, both spatially and temporally.

DNA extractions were carried out on 324 samples (19 tissues and 305 scats) from 21 locations between Newman and near Pannawonica. Ninety eight unique individuals were identified based on genetic variation at ten nuclear genes (microsatellite markers). The effective genetic population size was estimated to be 78.6, although this estimate needs to be viewed with some caution due to the limited sample size.

The genetic analysis suggests that there is a single, large, highly diverse genetic population of ghost bats in the Hamersley subregion, ranging from Newman to Pannawonica, including the population at Southern Flank. There is no evidence of recent or long-term population declines. Between caves, the analysis showed medium to high levels of genetic variation and clear evidence that there is some admixture. The spatial structure analysis identified a neighbour size (movement distance) of between 10 and 15 km.

1. INTRODUCTION

1.1 Background

The ghost bat is the largest carnivorous bat in Australia, and is currently listed as a Vulnerable species by the International Union for the Conservation of Nature (IUCN), and under the Federal *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act) and State *Wildlife Conservation Act 1950* (WC Act). The reasons for its listing under the Federal EPBC Act, as detailed in the conservation advice (TSSC, 2016), are:

- Habitat loss (destruction of, or disturbance to, roost sites and nearby areas) due to mining;
- Disturbance of (human visitation at) breeding sites;
- Modification to foraging habitat;
- Collision with fences, especially those with barbed wire;
- Collapse or reworking of old mine adits;
- Contamination by mining residue at roost sites;
- Disease; and
- Poisoning by cane toads (*Rhinella marina*).

BHP Iron Ore (BHP) is currently seeking approvals to develop a new mine at Southern Flank, which is located approximately 100 km north-west of Newman in the Pilbara region of Western Australia. Extensive baseline studies at Mining Area C (Biologic, 2011) and Southern Flank (Biologic, 2012) determine the presence of numerous caves suitable to support the ghost bat. Subsequent monitoring events have described the location, internal morphology and physical characteristics of these caves, and quantified scats deposited within them.

Survey work within the Hamersley subregion (Figure 1.1) since 2011 has documented many known and potential ghost bat caves; however, our understanding of how these caves are utilised and the extent and structure of the ghost bat population within the subregion has been poor. Obtaining information on cave use by ghost bats, and in particular if they are used as maternity roosts is problematic due to the low number of bats present at any one time, and the fact that ghost bats use multiple roosts so may not be present within a monitoring roost at the time of sampling. Genetic and hormone analyses of ghost bat tissue and scats were determined to be the most appropriate approach for increasing knowledge of cave use and movement by bats.

BHP commissioned Biologic Environmental Survey (Biologic) to undertake a study of ghost bats within the Hamersley subregion. The purpose of the study was to gain a better understanding of this species within the region that a majority BHP's mining operations occur (Figure 1.1), and thereby allowing BHP to better understand potential impacts from its operations and to guide management of the species.

This study comprised the following objectives:

- Investigate if faecal metabolites could be used to determine the presence of pregnant females within caves, and therefore the presence of a maternity roost;
- Confirm if caves within BHP's Central Pilbara tenements are being used for breeding by ghost bats, and if so, what is the relative importance of each cave to the population; and
- Investigate if DNA contained in faecal material can be used to identify individual bat species. If so, undertake a study of the population genetics of ghost bats within the Hamersley Range, including genetic diversity, structure and short-range spatial use.

1.2 The Ghost Bat

Conventionally accepted as *Macroderma gigas* (Dobson, 1880; TSSC, 2016), *Macroderma* is a monotypic genus endemic to Australia. They can weigh up to 150 g, with an average weight of 130 g (McKenzie and Bullen, 2009), and have an average wing span of 686 mm. Ghost bats have pale grey or light brown fur with a lighter belly and pale cream to brown wing membranes. They have large ears, measuring on average over 50 mm, which join above the head, large eyes and a long simple-shaped nose leaf extending along the muzzle (Churchill, 2008).

Fossil evidence suggests ghost bats were widely spread across most of mainland Australia, including the arid zone, but their range has contracted northwards since the Holocene (Duncan *et al.,* 1999; Hoyle *et al.,* 2001). Their range is now restricted to the Pilbara, the Kimberley, the northern part of the Northern Territory (including Groote Eylandt), coastal and near coastal Queensland from Cape York to near Rockhampton (Churchill, 2008), and Western Queensland (TSSC, 2016).

In the Pilbara region, the species occurs in all four sub-regions, and was recorded in 21 of the 24 areas surveyed by the Department of Parks and Wildlife during the Pilbara Biological Survey (2002-2007; see McKenzie and Bullen, 2009).

The largest populations occur within the Chichester sub-region, where known populations are largely restricted to disused mines. A number of these roosts have disappeared, or show evidence of collapse, flooding, human intrusion or nearby active mineral exploration (TSSC, 2016). The largest colonies occur around Bamboo Creek, Marble Bar and Nullagine, with the largest confirmed observations known from natural caves occurring in the Robe Valley near Pannawonica (15-35 individuals sighted in separate caves) (R. Bullen, *pers. comm.*).

In the Hamersley subregion, populations are more widespread but are much smaller in size. Whilst there are abandoned mines in this subregion, few have shown evidence of ghost bat presence (e.g. Hashimoto [Specialised Zoological, 2009]), while others of suitable depth show continuous use, such as those along Rhodes Ridge and Bakers South (Bullen, pers. comm.).

The ghost bat population in the Pilbara is estimated to be between 1300 – 2000 individuals (TSSC, 2016). Biologic (2014) estimated the population in the Hamersley subregion to be between 300 and 400. This estimate was based on limited field studies largely restricted to mining tenure. Numbers in Western Australia are considered likely to decline by over 30% in the future, with local extinction in areas such as the central and eastern Hamersley Range, and the extent of occupancy likely to decline by over 10,000 km^2 (TSSC, 2016). The key threats are considered to be habitat loss due to mining, disturbance of breeding sites (by human visitation), modification of foraging habitat, collision with fences, collapse or reworking of old mine adits, contamination by mining residue at roost sites, disease, poisoning of cane toads and competition for prey with foxes and feral cats (TSSC, 2016).

The distribution of ghost bats in the Pilbara is determined by the presence of suitable roosting sites, either natural caves or man-made mines and adits. Natural roosts generally comprise deep, complex caves beneath bluffs or low rounded hills composed of Marra Mamba or Brockman Iron Formation, or in granite tors (Armstrong and Anstee, 2000); although Marra Mamba was considered the geology most predisposed to forming deep caves in the Pilbara suitable for use by the ghost bat. Armstrong and Anstee (2000) further noted that most caves used by ghost bats in bluffs have narrow entrances, generally less than 0.5 m^2, that opened into larger chambers.

400000 500000 600000 700000 800000 900000 1000000

7700000 7600000 7500000 7400000

Port Operations
DAMPIER
Roebourne
Nimingarra
McLarty
SHAY GAP
Callawa
Yarrie
Mackay
MARBLE BAR
Chichester
API Leases
FMG leases
Brockman 2
Hamersley
TOM PRICE
Koodaideri
Marillana
Yandi
Fortescue
Tandanya
Mining Area C
Hope Downs
Jinidi
Mudlark
South Flank
West Angelas
Rudall
Trainor
Ashburton
OB 23/24/25
OB 18
Jimblebar (inc. Hashimoto)
Mt Whaleback
OB 29/30/35
NEWMAN
Augustus

Legend

- Pilbara Towns
- Pilbara Rail
- Great Northern Hwy
- Karijini National Park
- Proposed Mining Area C Development Envelope

IBRA sub-region

- Ashburton
- Augustus
- Chichester
- Fortescue
- Hamersley
- Mackay
- McLarty
- Roebourne
- Rudall
- Trainor

Mining/ Exploration Locations

- BHP: Care & Maintenance
- BHP: Exploration
- BHP: Operations
- Third party locations

biologic Environmental Survey

N

1:1,600,000

0 10 20 40 60 80 km

BHP Hamersley Subregion Ghost Bat Assessment

Fig. 1.1: Regional location and IBRA regionalisation

Coordinate System: GDA 1994 MGA Zone 50
Projection: Transverse Mercator
Datum: GDA 1994

Size A3. Created 27/06/2017

2. METHODS

2.1 Survey Locations and Sample Collection

A total of 35 caves were visited across six surveys (October 2015 to May 2016) to collect fresh scat material (Figure 2.1A). Caves sampled were:

1. Eight caves within BHP's Southern Flank project area that had been previously identified as suitable for use as a day roost (cave prefix SF-);
2. Fourteen caves which have had consistent ghost bat presence during previous monitoring events within the BHP's Central Pilbara tenements (Area C, Area C West and Marillana; cave prefix's AC, ACW and MAR and M, respectively);
3. One cave from one of BHP's Eastern Pilbara tenement (cave prefix OB35);
4. Eight caves located on third party tenure within the Hamersley subregion, known to contain ghost bats records (based on grey literature or government database records) (cave prefixes API, FMG); and
5. Four caves not previously assessed for ghost bat presence but identified to potentially contain suitable roosting habitat. Caves located on BHP tenements Gurinbiddy and Pineapple Hill (cave prefix's GU and NT, respectively).

Caves categorised under items 1 and 2 above were visited in October 2015 (Table 2.1) and sheets were placed over ghost bat scat middens. These caves were revisited for scat collection in November and December 2015, and April and May 2016 (with the exception of cave MARXX1 which was not revisited in 2016) (Table 2.1). The aim was to collect fresh scat material deposited within a known time period that could be used for analysis. Scats were also collected from areas surrounding the sheets, from which the deposition period could not be determined.

Caves categorised under items 3 to 5 above were visited in April and/or May 2016, and scats were collected from the ground of the cave. During each collection period scats were counted (or an estimate made if the number exceeded 100) and comment made on the approximate age of the scats (ancient, old, recent, fresh).

All scats were incorporated in the genetic analysis; scats collected from November and December 2015, and during previous surveys in November 2014 (Biologic, 2015) were analysed for faecal metabolites (hormones) to determine the presence of pregnant females and therefore a maternity roost.

Trapping for ghost bats was conducted at caves considered likely to contain ghost bats. This was done by placing a white sheet across the entrance and capturing bats as they attempted to exit the cave. Tissue samples were taken using a 3 mm biopsy punch from the tail membrane. All bats captured were sexed, weighed, microchipped and their condition assessed.

Areas considered suitable to contain ghost bat caves (see item 5 above) were identified within Gurinbiddy, Pineapple Hill and Ministers North using aerial photography and geology maps (see Figure 2.1B for search transects). These areas were searched on foot over a period of eight days by two zoologists.

Table 2.1: Caves sampled and timing of surveys

Cave	Oct 2015	Nov 2015	Dec 2015	April 2016	Early May 2016	Late May 2016
SF01	X	X	X	X		X
SF02	X	X	X	X		X
SF03	X	X	X	X		X
SF04	X	X	X	X		X
SF08	X	X	X	X		X
SF14	X	X	X	X		X
SF15	X	X	X	X		X
SF27	X	X	X	X		X
AC01	X	X	X	X		X
AC10	X	X	X	X		X
AC13	X	X	X	X		X
AC17	X	X	X	X		X
ACW01	X	X	X	X		X
ACW06	X	X	X	X		X
ACW07	X	X	X	X		X
ACW08	X	X	X	X		X
ACW09	X	X	X	X		X
ACW10	X	X	X	X		X
ACW15	X	X	X	X		X
ACW31	X	X	X	X		X
M01	X	X	X	X		X
MARXX1		X	X			
BHPOB35 1						X
APIGBJE01					X	X
APIGBJE02					X	X
APIGBRH03					X	X
APIGBRH04					X	X
APIGBRH01					X	X
APIGBRH04					X	X
APIGBRH05					X	X
FMGGBCP05					X	X
GU01				X		
GU02				X		
NT01				X		
NT03				X		

400000 500000 600000 700000

7500000 7400000

APIGBJE01 APIGBRH01 APIGBRH03 APIGBRH04 APIGBRH05 FMGGBCP05

231 m 871 m 580 m 981 m 1209 m 977 1055 m

Fortescue River

Wittenoom Tom Price Karijini National Park MERSLEY RANGE

NT03 NT01 ACW01 AC19 AC03 AC05 AC10 AC13 Mt Meharry ACW10 ACW08 M01 Can fence MARxx1 GU01 GU02 OB35 1

Sources: Esri, HERE, DeLorme, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, MapmyIndia, © OpenStreetMap contributors, and the GIS User Community

1:160,000

1069 m 967 m 1042 m 1148 m 884 m

AC19 AC03 AC04 AC05 AC08 AC09 AC01 AC13 AC17 SF14 SF27 SF09 SF04 SF01 SF15 SF02

Coondewanna Airport

Sources: Esri, HERE, DeLorme, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, MapmyIndia, © OpenStreetMap contributors, and the GIS User Community

Legend

- Pilbara Towns
- Pilbara Rail
- Major Roads

Study Areas for targeted cave searches

- Gurinbiddy
- Ministers North
- Pineapple Hill
- Karijini National Park
- Proposed Mining Area C Development Envelope

Sampling type

- Genetics
- Genetics & Hormones
- Hormones

biologic Environmental Survey

N

1:1,000,000

0 10 20 40 60 km

BHP Hamersley Subregion Ghost Bat Assessment

Fig. 2.1A: Locations of genetic and hormone samples

Coordinate System: GDA 1994 MGA Zone 50
Projection: Transverse Mercator
Datum: GDA 1994

Size A3. Created 27/09/2017

640000 650000 660000 670000 680000 690000 700000 710000 720000 730000 740000 750000

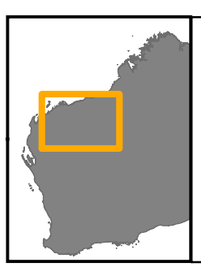
7490000 7480000 7470000 7460000 7450000 7440000 7430000

Legend

- Pilbara Rail
- Cave search transects
- Great Northern Hwy
- Karijini National Park
- Proposed Mining Area C Development Envelope

Study Areas for targeted cave searches

- Gurinbiddy
- Ministers North
- Pineapple Hill



biologic Environmental Survey

N

1:300,000

0 2 4 8 12 16 km

BHP Hamersley Subregion Ghost Bat Assessment

Fig. 2.1B: Location of Study Areas for targeted Ghost Bat cave searches

Coordinate System: GDA 1994 MGA Zone 50
Projection: Transverse Mercator
Datum: GDA 1994

Size A3. Created 27/09/2017

2.2 Survey timing

Surveys were undertaken during the following periods:

- 26th to 30th October 2015
- 16th to 18th November 2015
- 15th to 17th December 2015
- 20th to 27th April 2016
- 11th to 14th May 2016
- 26th May to 1st June 2016.

Weather conditions during the 2015 surveys were typically hot and dry with occasional showers associated with afternoon build up. Previous large rainfall events (greater than 100 mm) occurred in March and May 2015. Rainfall in late 2015 was less than the average with almost no rain falling in December (Figure 2.2). Higher than the average rainfall events occurred in January and March 2016.

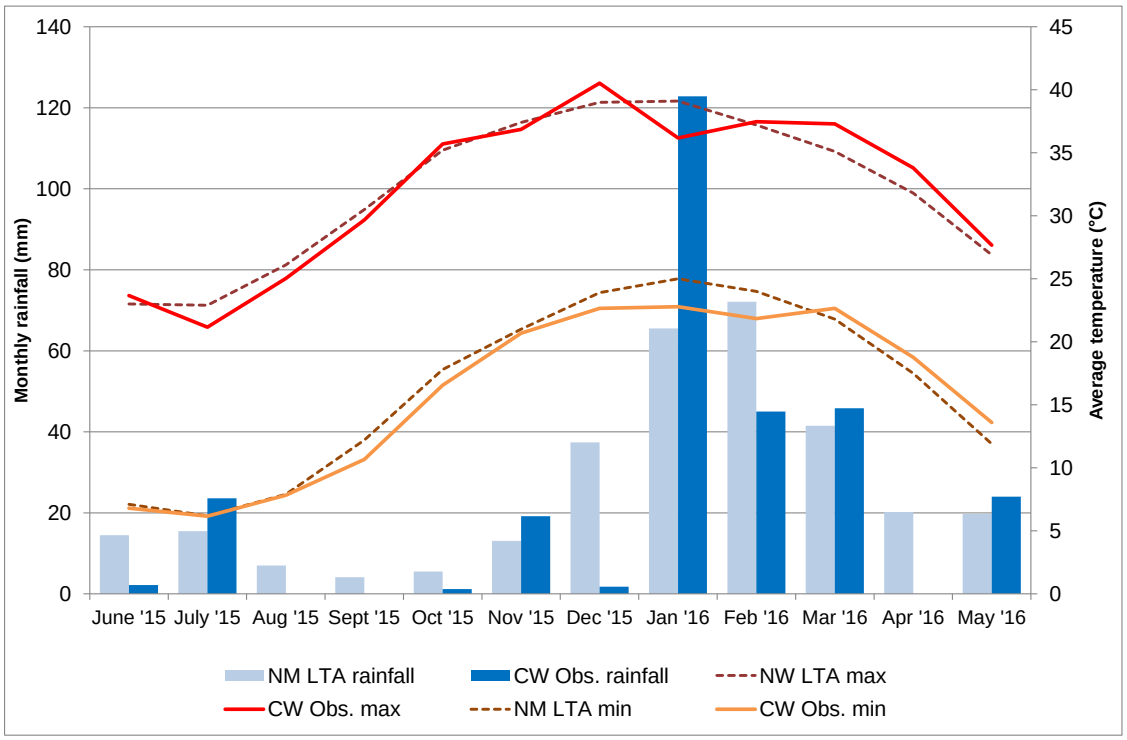


Figure 2.2: Long term average (LTA) climate data for Newman Aero (NM) compared against recent (2015-2016) observations for Coondewanna (CW) (data from BoM 2016*).

2.3 Personnel and Licences

The surveys were undertaken by the following personnel:

- Mr Morgan O'Connell – Principal Zoologist: October, November and December 2015; April and May 2016.

- Mr Patrick Cullen – Senior Zoologist: November and December 2015; April 2016.
- Mr Thomas Rasmussen – Senior Zoologist: April and May 2016.
- Mr Bob Bullen – Specialist Bat Ecologist (sub-contractor): April and May 2016.
- Dr Cameron Mounsey – BHP Senior Ecologist: October 2015.

The surveys were completed under Licence 01-000071-1 issued to Morgan O'Connell.

2.4 Hormone Analysis

Hormone analysis of the collected scats was completed by Dr Tamara Keeley from the University of Queensland as described below (Appendix A). Methods from this work are summarised here:

Initial hormone investigations were completed on scats collected during the 2014 breeding season to investigate whether metabolites could be used to determine the presence of pregnant females within caves, and therefore the presence of a maternity roost. Scats were also collected from individuals at the Perth Zoo in November 2014 for comparison; however, the zoo population did not breed that year so known pregnant samples were not available. Results from the 2014 investigations suggested that elevated progesterone levels were present in samples collected from the Pilbara and may therefore indicate breeding. The study was therefore continued using scats obtained from Pilbara roosts and captive females at the Perth Zoo.

Samples collected from inside caves were stored as individual samples and were no older than 30 days. Faecal samples were also collected from the captive population at the Perth Zoo. Scats were collected from a housed group of seven female ghost bats to assist with the validation and analysis of faecal samples from the wild roost sites. Perth Zoo confirmed that a single male offspring was born on 15 November 2015, during the scat collection period.

Faecal samples were analysed for progesterone metabolite concentrations by enzyme-immunoassay (EIA). Prior to analysis for hormone concentrations, each faecal sample was extracted using a basic hormone extraction procedure (Keeley *et al*. 2012a; Palme *et al*. 2013). Faecal samples were subsampled to a weight of either 0.1 ± 0.02 or 0.05 ± 0.002 g to which 5 ml of 80% methanol was added. Samples were rotated gently overnight, centrifuged at 1000 g for 10 min and then decanted and stored at -20°C until analysis. The supernatant was diluted 1:20 to 1:1000 (dependant on concentration) in assay buffer prior to analysis. Faecal progesterone metabolite concentrations were quantified by double antibody EIA using a goat anti-mouse IgG (Arbor Assays, USA), monoclonal progesterone antiserum (CL425), horseradish peroxidase conjugated label (both provided by C. Munro, University of California-Davis, Davis, USA) and progesterone (Sigma Aldrich Australia, Ltd.) standards as previously described with minor modifications (Keeley et al. 2012b).

The antiserum (1:80,000) was incubated on the microtitre plate overnight, horseradish peroxidise conjugate (1:400,000), standards (0.016 - 4 ng/ml) and samples were loaded (50

µl/well) onto the plate and the EIA was performed as described elsewhere (Pollock et al. 2010; Keeley et al. 2012b). Intra and inter-assay coefficients of variation were both <10%. Cross-reactivities for the EIA antibodies were as previously described (Graham et al. 2001). Hormone concentrations were expressed as nanograms of hormone metabolites per gram of faeces (ng/g).

2.5 Genetic Analysis

Genetic analysis of the collected tissue and scats was completed by Dr Peter Spencer and Dr Jamie Tedeschi from Murdoch University (Spencer and Tedeschi, 2016; see Appendix B). Methods from this work are summarised here:

DNA extractions were carried out on 324 samples (19 tissues and 305 scats) from 21 locations between Newman and near Pannawonica (Figure 2.1A). Of these samples, three failed to amplify anything, 78 failed at 5 or more loci, and 112 samples were duplicated genotypes and so were not used in any analysis. The genetic analysis examined genetic variation at 10 nuclear genes (microsatellite markers) from the final 98 unique individuals.

Tissue samples were extracted using a QIAGEN Tissue/Blood extraction kit (Cat No./ID: 69506). Faecal material was subject to more specialised extraction using the QIAGEN Stool extraction kit (Cat No./ID: 51504). All tissue samples produced amplifiable DNA. The scat (faecal pellet) samples were taken from individual ghost bats from the Pilbara region and surrounds. A subset of 98 samples were used to evaluate the genetic diversity and structure of ghost bats in the Pilbara for this report.

Ten microsatellite loci were amplified [all that were available at the time]: GB18, GB20, GB33, GB42, GB44, GB81 (J. Hughes, *unpub. data*) and gigas01, gigas06, gigas10 and gigas11 (Worthington-Wilmer et al. 1994) from ten sampling locations. PCRs were carried out in a total volume of 40 µl with ~100 ng DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.3 µM of each primer and 1 U *Taq*. Size was determined by co-running a Genescan500 standard (Applied Biosystems, Melbourne). Fragment analysis was carried out on a 3730xl DNA Analyser (ABI systems, Melbourne) and scored with the aid of GENEMARKER (SoftGenetics). Control samples were run in each PCR run to ensure compatibility between different datasets used in the analysis.

Descriptive statistics and assumptions were calculated using GenAlEx 6.4 (Peakall and Smouse, 2006) and HW-QUICKCHECK (Kalinowski, 2006). The rarefaction method, as implemented in HP-RARE (Kalinowski, 2006), was used to calculate the allelic richness based on 11 diploid individuals. This method allows a direct comparison between populations because it equalises the sampling effort. Using the allele frequencies, the arc genetic distance between localities (Cavalli-Sforza and Edwards, 1967) was computed and subjected to principal coordinates analysis (Gower, 1966) using GenAlEx 6.4 (Peakall and Smouse 2006).

Evidence of recent population bottlenecks was investigated by testing for a deficiency of heterozygosity using BOTTLENECK (Piry et al. 1999). Due to the relatively small number of polymorphic loci analysed (n=10), a Wilcoxon sign-rank test was estimated. A mixed model of microsatellite mutation was assumed with a single step mutation assumed at 90%, variance of 12, as suggested by Piry et al. (1999) and Hampton et al. (2004).

Dispersal distance was investigated by testing for a relationship between pairwise population genetic measures (Peakall and Smouse, 2006) and geographical distance (measured as decimal latitude and longitude) using genetic spatial autocorrelation analysis, performed using the program GenAlEx 6.4 (Peakall and Smouse, 2006). The spatial autocorrelation analysis implemented in GenAlEx calculates an autocorrelation coefficient (*r*) for genetic distances (Smouse and Peakall, 1999) as a function of geographical distance (km). We used distance classes of 5 km, up to 50 km. We generated the 95% confidence intervals around the expectation of no spatial genetic structure using 1000 random permutations. The geographical distance at which the mean *r* value drops below zero has been referred to as the 'neighbourhood size' or 'patch size' (Peakall et al. 2003) and represents the largest spatial scale at which genetic similarity is non-random.

The study needs to be interpreted with some caution due to the limited dataset that was generated. The study initially used 324 samples, of which 19 were tissue (e.g. wing membrane) and 305 samples extracted from scats. Samples were included in the study on the basis that at least five loci were generated, allowing a probability of >0.99 individual confidence in assigning individuals (based on probability of identify statistics).

3. RESULTS

3.1 Cave Search Results

Eight caves deemed suitable for ghost bat roosting were recorded at Gurinbiddy, Ministers North and Pineapple Hill (Figure 3.1; Appendix C). Four were considered only suitable to be used as a night roost, two were considered potential day roosts, one was considered a potential maternity roost, and two showed no evidence of use despite suitable cave characteristics (Table 3.1). Cave GU1 was classified as a potential maternity roost and is likely to be a significant cave to local individuals. It is 40 m in depth, possessing two chambers, of which the highest was 3 m. The cave contained thousands of scats of all ages suggesting the bats are currently using the cave and have been for a very long time.

Table 3.1: New caves recorded

Cave	Categorisation
Gurinbiddy	
GU01	Potential Maternity
GU02	Day Roost
GU03	No Usage
Ministers North	
MN01	Night Roost
Pineapple Hill	
NT01	Night Roost, Day Roost
NT02	Night Roost
NT03	Night Roost
NT04	Night Roost

3.2 Scat Collections

Approximately 571 scats were collected from the sheets with an additional 1100 collected from the ground (therefore of unknown age) (Table 3.2). The number of scats deposited within the caves during the sampling period ranged from zero to over 1000. The number of scats deposited during a known time period allows for a determination of when caves were used and a comparison of usage between the caves. Table 3.2 and Table 3.3 show total numbers of scats deposited on the sheets and in the cave, respectively, whilst Table 3.4 shows the deposition rate (number of scats on the sheets divided by the number of days of collection) over each of the sampling periods. Figure 3.2 to Figure 3.4 graphically display the rate of deposition of scats in caves within the Central Pilbara tenements over the period of sampling.

640000 650000 660000 670000 680000 690000 700000 710000 720000 730000 740000 750000

7490000 7480000 7470000 7460000 7450000 7440000 7430000

NT_02 NT_03 NT_04 NT_01

MN_02 MN_01

GU_02 GU_03 GU_01

Karijini National Park

1132 m 1172 m 1207 m 1242 m 1209 m 1059 m 1116m 1045 m 1164m 977 m 1077 m 747m 945 m 1055 m 1015 m 1041 m 1019 m

Great Northern Hwy 95

Legend

- Pilbara Rail
- Great Northern Hwy
- Karijini National Park
- Proposed Mining Area C Development Envelope
- Caves recorded from targeted searches 2016

Study Areas for targeted cave searches

- Gurinbiddy
- Ministers North
- Pineapple Hill

biologic Environmental Survey

N

1:300,000

0 2 4 8 12 16 km

BHP Hamersley Subregion Ghost Bat Assessment

Fig. 3.7: Location of caves recorded during targeted searches

Coordinate System: GDA 1994 MGA Zone 50
Projection: Transverse Mercator
Datum: GDA 1994

Size A3. Created 27/06/2017

Table 3.2: Scats collected (from sheets) from each cave during the collection periods

Cave	Nov 2015	Dec 2015	April 2016	Early/Late May 2016
SF01	24	5	12	46
SF02	0	13	2	3
SF03	0	0	7	0
SF04	0	0	0	1
SF08	1	0	8	48
SF14	0	0	10	34
SF15	0	0	8	0
SF27	0	0	0	7
AC01	0	0	5	49
AC10	0	0	3	0
AC13	0	0	0	0
AC17	2	1	2	0
ACW01	6	24	21	8
ACW06	0	0	0	16
ACW07	0	0	0	0
ACW08	1	0	20	4
ACW09	0	0	0	0
ACW10	0	1	10	0
ACW15	0	0	0	0
ACW31	0	0	0	0
M01	27	30	23	24
MARXX1	-	8	-	0
BHPOB35-1	-	-	-	19
APIGBJE01	-	-	-	0
APIGBRH03	-	-	-	0
APIGBRH04	-	-	-	1
APIGBRH01	-	-	-	4
APIGBRH05	-	-	-	16
APIGBJE01	-	-	-	0
GU01	-	-	6	-
FMGGBCP05				20
GU02	-	-	12	-
NT01	-	-	5	-
NT03	-	-	7	-

Note: '-' refers to caves that were not visited during this period,

Table 3.3: Total fresh scats on sheets observed from each cave during the collection periods

Cave	Oct 2015	Nov 2015	Dec 2015	April 2016	Early/Late May 2016
SF01	15	~200	5	~150	~1000
SF02	10	0	13	2	3
SF03	0	0	0	7	0
SF04	20	0	0	0	1
SF08	10	1	0	8	~120
SF14	8	0	0	~100	~350
SF15	0	0	0	8	0
SF27	14	0	0	0	7
AC01	0	0	0	5	~1500
AC10	10	0	0	3	0
AC13	30	0	0	0	0
AC17	100	2	1	2	0
ACW01	20	6	75	30	10
ACW06	10	0	0	0	~150
ACW07	0	0	0	0	0
ACW08	30	1	0	20	4
ACW09	0	0	0	0	0
ACW10	30	0	1	10	0
ACW15	0	0	0	0	0
ACW31	0	0	0	0	0
M01	~1000	~500	~500	~1500	~2000
MARXXX1	-	0	8	-	-
BHPOB35 1	-	-	-	-	19
APIGBJE01	-	-	-	-	
APIGBRH03	-	-	-	-	9
APIGBRH04	-	-	-	-	15
APIGBRH02	-	-	-	-	13
APIGBRH05	-	-	-	-	26
APIGBJE02	-	-	-	-	-
FMGGBCP05					20
GU01	-	-	-	6	-
GU02	-	-	-	12	-
NT01	-	-	-	5	-
NT03	-	-	-	7	-

Note: -' refers to caves that were not visited during this period

Table 3.4: Scat deposition rate (# scats/ days) from each cave during the collection periods

Cave	Oct – Nov 2015	Nov – Dec 2015	Dec 2015 – April 2016	April – May 2016
SF01	8.70	0.18	1.18	26.32
SF02	0.00	0.46	0.02	0.08
SF03	0.00	0.00	0.06	0.00
SF04	0.00	0.00	0.00	0.03
SF08	0.04	0.00	0.06	3.16
SF14	0.00	0.00	0.79	9.21
SF15	0.00	0.00	0.06	0.00
SF27	0.00	0.00	0.00	0.18
AC01	0.00	0.00	0.04	39.47
AC10	0.00	0.00	0.02	0.00
AC13	0.00	0.00	0.00	0.00
AC17	0.09	0.04	0.02	0.00
ACW01	0.26	2.68	0.24	0.26
ACW06	0.00	0.00	0.00	3.95
ACW07	0.00	0.00	0.00	0.00
ACW08	0.04	0.00	0.16	0.11
ACW09	0.00	0.00	0.00	0.00
ACW10	0.00	0.00	0.08	0.00
ACW15	0.00	0.00	0.00	0.00
ACW31	0.00	0.00	0.00	0.00
M01	21.74	17.86	11.81	52.63
MARXX1	-	0.29	-	0.00
APIGBJE01	-	-	-	0.00
APIGBRH03	-	-	-	0.03
APIGBRH04	-	-	-	0.11
APIGBRH01	-	-	-	0.00
APIGBRH05	-	-	-	0.42
APIGBJE02	-	-	-	0.00
FMGGBCP05				0.00

Note: '-' refers to caves that were not visited during this period; only caves visited on multiple occasions are included.

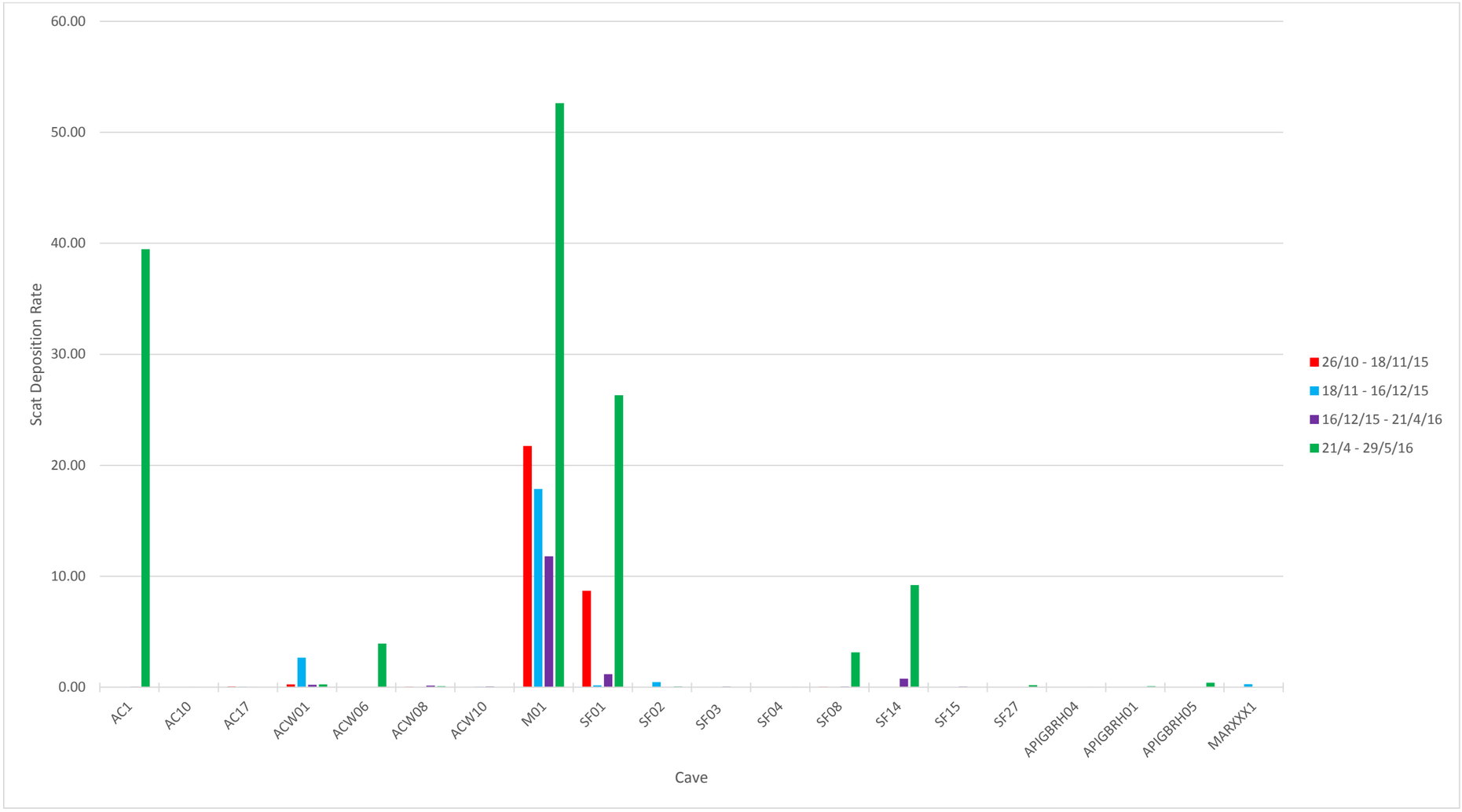


Figure 3.2: Scat Deposition Rates over the four collection periods. Note that caves from which no scats were collected are not displayed.

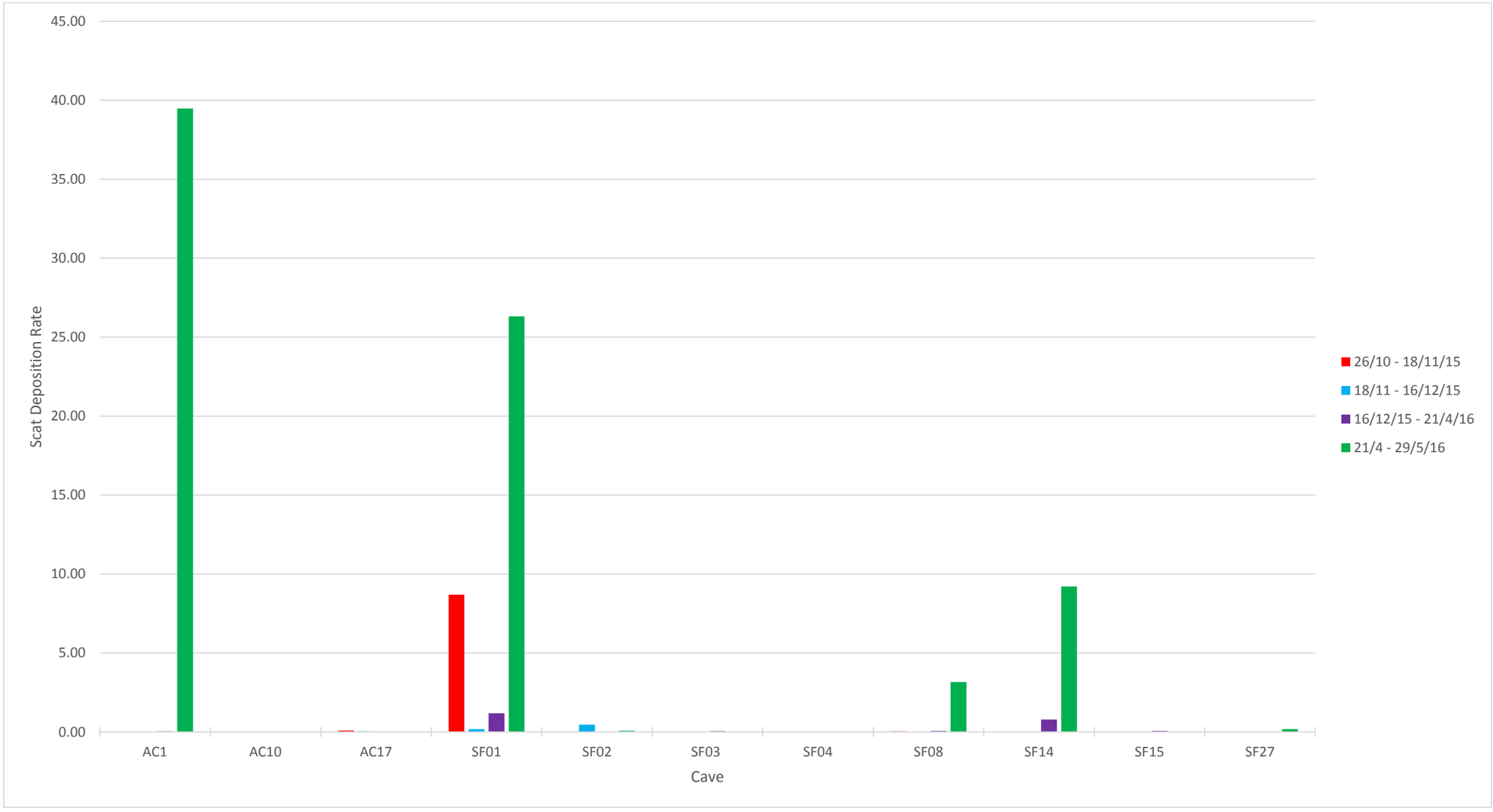


Figure 3.3: Scat Deposition Rates at Southern Flank and Mining Area C over the four collection periods. Note that caves from which no scats were collected are not displayed.

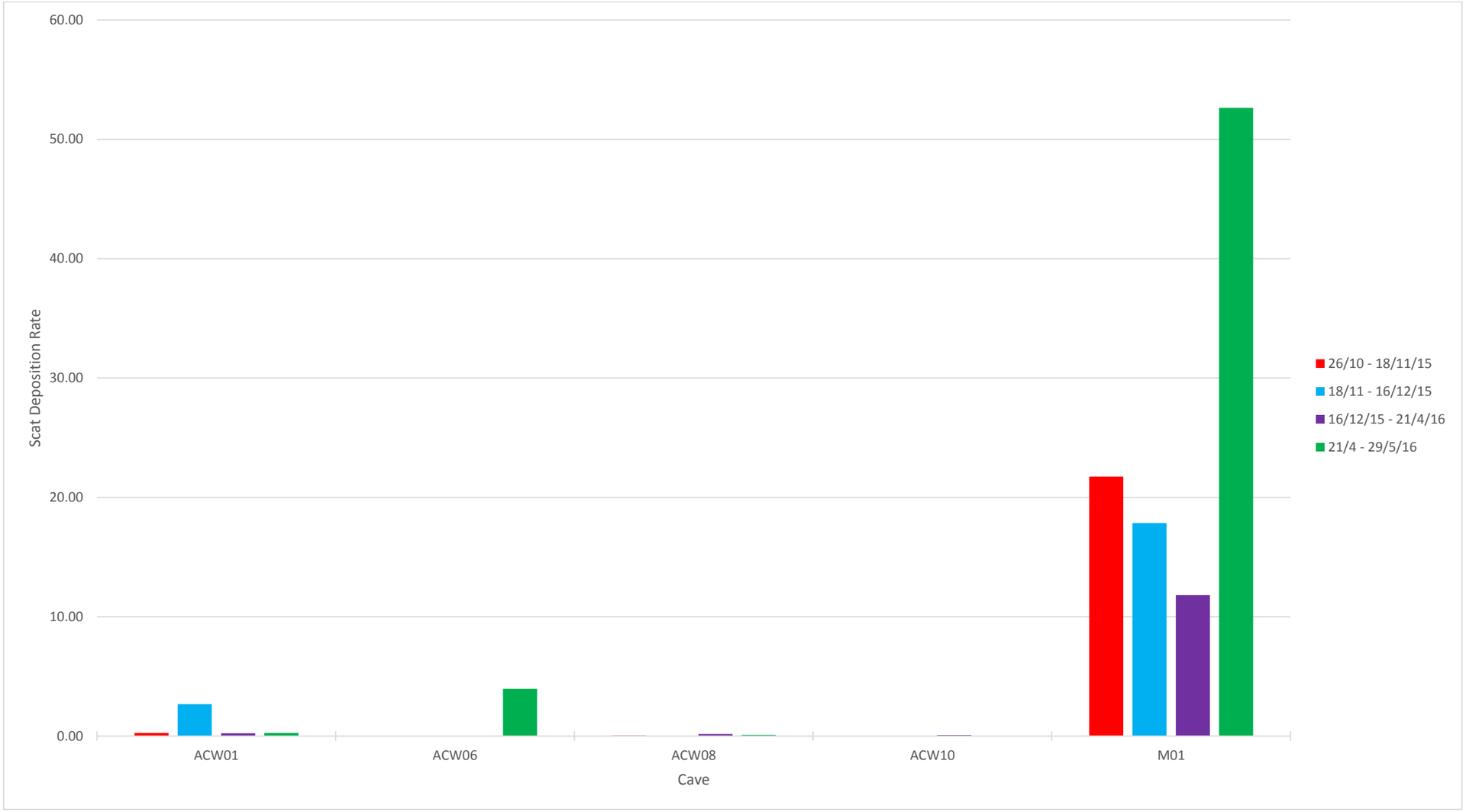


Figure 3.4: Scat Deposition Rates at Tandanya and Mudlark over the four collection periods. Note that caves from which no scats were collected are not displayed.

3.3 Ghost Bat Observations and Captures

Ghost bat individuals were observed in 14 of the 35 caves visited over the survey period (see Table 3.5). Singletons were observed from eight of the caves.

A single ghost bat was observed on four separate occasions at cave ACW01, and was captured on the fourth occasion for collection of a tissue sample, sexing and microchipping. Clear views of the individual were achieved during each encounter with no signs of pregnancy or pups observed, suggesting the same male individual may have been present over the six-month period.

Individuals were seen on two separate occasions at SF01, SF08 and MARXX1. Ghost bats were only observed at the Mudlark cave (M01) once in May 2016 (and subsequently captured) despite consistently high deposition of scats during the previous sampling periods (see Figure 3.2 and Table 3.2).

The most significant observation was made in December at SF15 where 16 individuals were recorded. On entry to the cave, a single individual was observed clinging to the side wall of the cave and two individuals, of a small size and darker in coloration, were observed on the back wall / roof. In a small cavity on the upper side wall, a cluster of individuals was observed that contained colours of white, grey and darker grey (almost black). Once disturbed the cluster split and 15 individuals flew past the observer into a side chamber near front of the cave, with a handful flying back to the original location. None of the flying individuals could be confirmed as juveniles. The second observer, positioned outside of the cave, saw approximately seven individuals flush down to an overhang approximately 70 m to the south. Only white / grey individuals flushed from the cave. It was concluded that at least 16 individuals were present within the cave at the time of observation; one on the side wall and 15 that flew past the observer. Three or four of the bats were considered to be juveniles as they were smaller and darker than other ghost bats observed.

It was also observed that approximately 70% of the general area in and around Southern Flank was burnt in the latter half of 2015, rendering a large proportion of the ghost bats home range temporarily unsuitable for foraging.

A total of 11 individuals were captured during the field work for collection of genetic material, sexing and microchipping (Table 3.6). Two were from the West Hamersley (APIJV lease), one from just east of Karijini (FMG lease), one from the very south eastern Pilbara (near Newman) and seven from east of Karijini (BHP Central Pilbara tenements). The captures consisted of eight males and three females (Table 3.6).

Table 3.5: Observations of ghost bat made during the field survey

Cave	Oct 2015	Nov 2015	Dec 2015	April 2016	Early May 2016	Late May 2016
SF01				3		1C
SF02						
SF03						
SF04						
SF08	1					1C
SF14						1C
SF15			16			
SF27						1C
AC01						
AC10						
AC13						
AC17						
ACW01	1	1	1	1C		
ACW06						
ACW07						
ACW08				4 (1C)		
ACW09						
ACW10						
ACW15						
ACW31						
M01						2 (1C)
MARXX1		2	1			
BHPOB35 1						1C
APIGBJE01						
APIGBRH03					1C	
APIGBRH04						
APIGBRH01					3 (1C)	
APIGBRH05					1C	
APIGBJE01						
FMGGBCP05					1C	
GU01						
GU02						
NT01						
NT03						

Note: *n*C = no of individuals captured as a subset of the total

Table 3.6: Captures of ghost bat during the survey work and associated details

Cave	Date captured	Microchip number	Sex
SF01	28/05/16	953010000994749	Male
SF08	28/05/16	953010000995205	Male
SF14	29/05/16	953010000994988	Female
SF27	28/05/16	953010000994231	Female
ACW01	25/04/16	000728627C	Male
ACW08	21/04/16	00074C4ED3	Female
M01	29/05/16	953010000988798	Male
BHP0B35	31/05/16	953010000992201	Male
APIGBRH01	12/05/16	953010000996923	Male
APIGBRH03	12/05/16	953010000990050	Male
FMGGBCP05	13/05/16	953010009944883	Male

3.4 Hormone Analysis

Full results from the hormone analysis study undertaken by Keeley (2016) are provided in Appendix A. Key results from the study are presented here.

In 2014, no bats at the Perth Zoo reproduced and no scats showed elevated progesterone metabolite levels (Table 3.7). In 2015, several scat samples collected from the captive population showed elevated progesterone levels in the sampling period (5th October to 9th November), and on 15th November 2015 one female gave birth. Following the birth, no samples were found with elevated progesterone levels, so it was presumed the elevated samples were sourced from the pregnant female (Table 3.7).

Table 3.7: Level of progesterone metabolite (ng/g) in captive and wild ghost bats.

Ghost Bat Group Class	2014	2015	Combined
Male Captive Bats	69.4 ± 10.8	N/A	
Female Captive Bats	95.7 ± 46.0	138.4 ± 109.0	
Presumed Pregnant Captive Bat	N/A	4485.2 ± 2898.4	
Presumed Non-Pregnant Wild Bats			201.1 ± 127.9
Presumed Pregnant Wild Bats			3330.1± 2314.9

Based on the scat progesterone levels of the captive ghost bats, it is inferred that scats were collected from pregnant (3330.1± 2314.9 ng/g) and non-pregnant (201.1 ± 127.9 ng/g) ghost bats in the Pilbara (Table 3.8). Of the 11 caves from which samples were collected during likely gestational periods, nine caves in 2014 and three caves in 2015 are considered likely to have contained pregnant ghost bats (Figure 3.5, Table 3.8).

670000 680000 690000 700000 710000 720000

7470000 7460000 7450000

1:700,000

MARXX

ACW1 ACW10 ACW8 M1

AC19 AC3 AC4 AC5 AC8 AC9 AC10 AC1 AC13 AC17

SF1 SF2 SF4 SF5 SF8 SF9 SF15

Goondewanna Airport

1001 m 1041 m 977 m 1077 m 1069 m 967 m 837 m 1179 m 1042 m 1148 m 884 m 997 m

Sources: Esri, HERE, DeLorme, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, MapmyIndia, © OpenStreetMap contributors, and the GIS User Community

Legend

- Pilbara Rail
- Proposed Mining Area C Development Envelope

Samples collected 2014

- No elevated progesterone 2014
- Elevated progesterone metabolites 2014

Samples collected 2015

- No elevated progesterone 2015
- Elevated progesterone metabolites 2015

biologic Environmental Survey

N

1:150,000

0 1.5 3 6 9 km

BHP Hamersley Subregion Ghost Bat Assessment

Fig. 3.6: Location of samples with elevated progesterone metabolites

Coordinate System: GDA 1994 MGA Zone 50
Projection: Transverse Mercator
Datum: GDA 1994

Size A3. Created 27/06/2017

Table 3.8: Roost sites with presumed pregnant ghost bats (> 1000 ng/g)

Cave	2014	2015
SF01	Yes	Yes
SF05	Yes	
SF15	Yes	
AC01	Yes	
AC04	Yes	
AC08	Yes	
AC09	Yes	
AC13	Yes	
ACW10	Yes	
M01	Yes	Yes
MARXX1	Yes	Yes

3.5 Genetic Analysis

Full results from the genetic analysis study undertaken by Spencer and Tedeschi (2016) are provided in Appendix B. Key results from the study are presented here.

In this study, DNA extractions were carried out on 321 ghost bat samples (19 tissues and 305 scats). From those, three samples failed to amplify anything, 30 samples were not used in this study, 78 samples failed at five or more loci and 112 samples were duplicated genotypes.

A total of 98 individuals were genotyped and the number of individuals identified in each cave is shown in Figure 3.6. No individual was recorded in more than one cave. Fifty four individuals were identified from Mining Area C and Southern Flank (seven caves sampled), eight individuals were identified from Tandanya (three caves sampled), one individual was identified from Orebody 35 (one cave sampled), five individuals were identified from Gurinbiddy (two caves sampled), sixteen individuals were identified from Mudlark (one cave sampled), five were identified from Pineapple Hill (two caves sampled), one was identified from Marillana (one cave sampled), two individuals were identified from API Western Pilbara tenements (two caves samples) and one individual from FMG Central Tenements.

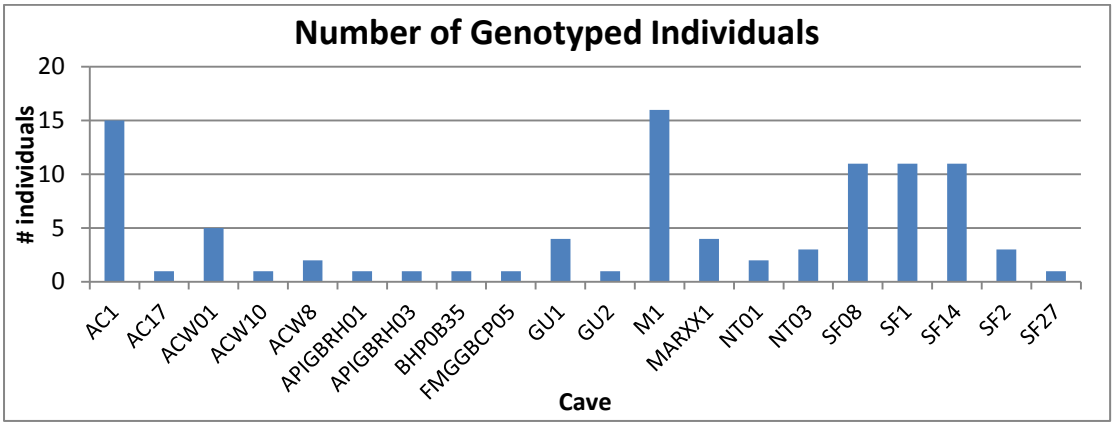


Figure 3.6: Number of genotyped individuals at each cave.

The effective genetic population size for the Hamersley subregion was estimated to be 78.6, although this estimate needs to be viewed with some caution due to the limited sample size. The effective population size is generally considered to be 10% of the census population size, therefore the census size of ghost bats within the Hamersley subregion could be as high as 700-800 individuals.

The analysis determined that there is a single, large, highly diverse genetic population of ghost bats in the Hamersley subregion, ranging from Newman to Pannawonica, including the population at Southern Flank. There is no evidence of recent or long-term population declines.

Between caves, the analysis showed medium to high levels of genetic variation and clear evidence that there is some admixture. The spatial structure analysis identified neighbour size (movement distance) as between 10 and 15 km.

3.6 Limitations

The following are limitations that may have influenced the results of this study:

Fire: Approximately 70% of the general area in and around Southern Flank was burnt in the latter half of 2015, rendering a large proportion of the ghost bats home range temporarily unsuitable for foraging. It has been suggested that fire does affect the species; Bullen and McKenzie (2011) noted that ghost bat populations would vacate an area following fire and return only as the undergrowth regenerates.

Collection Methodology: It unlikely bats only defecate on the sheets, and scats deposited outside of the cave or in areas inaccessible within the cave are not included in the analysis. Scats may get eaten by insects or degrade quickly (depending on diet) or get kicked onto or off sheets by other vertebrate species, such as wallabies or goannas.

Hormone Analysis: The control/test population for the hormone analysis was based on a limited dataset. Specifically one confirmed pregnant female from the Perth Zoo used over the two years of study.

Genetic Analysis: The study needs to be interpreted with some caution due to the limited dataset that was generated. The study initially used 324 samples, of which 19 were tissue (e.g. wing membrane) and 305 samples extracted from scats. Samples were included in the study on the basis that at least five loci were generated, allowing a probability of >0.99 individual confidence in assigning individuals (based on probability of identify statistics).

4. DISCUSSION

This study has demonstrated that the collection of ghost bat scats from caves provides the best approach to understand and monitor ghost bats in the Pilbara, and almost certainly Australia. Unlike the use of acoustic or ultrasonic recorders (SM2 or Anabat detectors) which have traditionally been used to survey for ghost bats, collection of scats over a designated time period can provide information on seasonal use, and although results from the genetic and hormone analyses are based on limited datasets, they do indicate that it can be used to determine the minimum number of bats that have used a cave during the period, whether pregnant bats were present (and therefore the cave is used as a maternity roost) and also movement of bats between different caves and over what time period.

Four of the caves sampled during the period, AC01, ACW1, M1 and SF1 (see Figure 2.1A), consistently recorded a high number of scats on the sheets suggesting they are likely to represent important caves within a local context. A high number of scats were also recorded at SF14 (see Figure 2.1A) in the latter two sampling periods while the two earlier sampling periods recorded no use. There were no noticeable disturbances within the vicinity of the cave to suggest external factors for the lack of records during the first two periods of survey. Four caves (AC17, ACW8, SF2 and SF8) recorded a low number of scats during each survey suggesting limited, but consistent use by ghost bat. There were four caves that had no signs of ghost bat use during the sampling period; however these caves have shown use by ghost bats during prior surveys.

Whilst the results from the scat counts are encouraging, and are currently considered the most effective method to sample and monitor ghost bats, the technique does have its limitations; it unlikely bats only defecate on the sheets, and scats deposited outside of the cave or in areas inaccessible within the cave are not included in the analysis. Scats may get eaten by insects or degrade quickly (depending on diet) or get kicked onto or off sheets by other vertebrate species, such as wallabies or goannas.

Previous studies have suggested that ghost bats in the Hamersley Range occur in small family groups that move from cave to cave (Armstrong and Anstee, 2000; Biologic, 2014). The scat collection data support this, with some caves having recorded no scat deposition for a number of survey periods followed by heavy use in the subsequent survey periods (e.g. SF14). The large fluctuation in cave use, such as at SF1, is likely to be a result of ghost bats moving around regularly, with presence ranging from occasional visitation to persistence over a long period. However, of the 98 individuals identified from the genetic analysis, none were recorded in more than one cave. This result was unexpected, especially in consideration of the sporadic use of some caves. There are a number of possible explanations for this result: 1) the sample size analysed was not large enough to identify such movements 2) scats aren't always deposited on sheets (as described above), so whilst bats may be present in the monitored caves, their scats weren't collected; and/or 3) there are caves in the local area that

have not been identified (where ghost bats are residing), although this seems unlikely, particularly around Southern Flank which has been extensively searched for caves; and/or 4) recent fires at Southern Flank during the survey periods may have restricted individuals to certain areas; and/or 5) individuals are moving around a much greater area than previously thought (ghost bats in Central Queensland were thought to move up to 150 km from their main breeding roosts (Toop, 1985)) and inhabit a cave for an indeterminate period of time before completely moving out of an area. Further genotyping studies undertaken over a wider area of the Pilbara and over multiple years and seasons are required to determine movement of bats between caves and if there are any seasonal patterns to this movement on a regional scale.

Approximately 70% of the general area in and around Southern Flank was burnt in the latter half of 2015, rendering a large proportion of the ghost bats home range temporarily unsuitable for foraging. It has been suggested that fire does affect the species; Bullen and McKenzie (2011) noted that ghost bat populations would vacate an area following fire and return only as the undergrowth regenerates. This fire may also be the reason why a maternity group was observed in SF15 in 2014. SF15 is a relatively small cave that has shown limited signs of use in the past, but is located a substantial distance from the fire that burnt in and around Southern Flank. This suggests that ghost bats are able to utilise less suitable roosts when foraging habitat around preferred roosts is lost due to fire or other disturbances.

The genetic study, whilst preliminary and based on a limited dataset, was considered a success and to our knowledge is the first of its kind to obtain genetic information from the scats of ghost bat. The high success rate of obtaining useable genetic material from scat samples (only a small percentage of samples failed to amplify) may be attributed to how well the scats are preserved. Scats deposited within a cave are protected from the elements such as direct light and soaking from rain which both contribute significantly to the degradation of DNA (Nicklas et al. 2011). Cave conditions are generally dry, dark and a constant 27-32 °C year-round (Biologic, 2014). Scats used in this study were between one and four months of age suggesting that scats could be significantly older and still prove useful for genetic analysis. Further studies could be undertaken to determine the relative rate of decay of DNA from scats. Increasing the collection period would reduce costs associated with sampling and also impacts to bats that may be in the cave at the time of collection.

The Hamersley Range consists of a single population of ghost bats. This appears logical given the widespread extent of records throughout their range and the large size of the bat with the ability to cover large distances. The neighbourhood size (or movement distance) for ghost bats is approximately 15 km. Previous studies in tropical northern Australia have shown that individuals generally foraged 1.9 km around a central day roost (Tidemann et al., 1985). A dispersal event could see an individual travel many times that distance, given that individuals have been recorded travelling up to 50 km, and were suspected of travelling

150 km in Central Queensland (Toop 1985). The results from this study are consistent with these observations.

Due to the limited number of individuals analysed over such a large geographic area, further work is currently being undertaken to verify the results from the preliminary study. This work includes collection of samples from the Chichester subregion to determine if there is one Pilbara population or two sub-populations and to determine if individuals can be sexed using faecal DNA. Studies undertaken by Worthington Wilmer et al. (1994) in the Northern Territory and Queensland showed that populations are genetically distinct at regional scales, with the two populations in Queensland separated by approximately 350 km. These two populations were at higher latitudes and have relatively large lowland areas between them, much of which has been cleared for agriculture. Samples from this study were collected over a distance of approximately 300 km, across an extensive mountainous range with no clearing on lowland areas for agriculture. Lowland areas are used for cattle grazing, but tree canopies and food resources are still largely intact. This study did not show the genetic separation as seen in Queensland populations. There is a potential barrier between the Chichester and Hamersley subregions where the Fortescue River and Fortescue Marsh occur. However, a ghost bat was captured (mist net) during surveys within the Fortescue Marsh (J. Turpin, pers. comm.) many kilometres from suitable roosting habitat, suggesting that this area may not provide a genetic barrier.

From the samples collected, the effective genetic population size was estimated to be 78.6. The effective population size is considered to be 10% of the census population, and therefore the Hamersley Range population could comprise up to 800 individuals. Previous estimates for the area based on field observations are 1300-2000 for the Pilbara bioregion (TSSC, 2016). Worthington-Wilmer et al. (1999) estimated the population size for the Pilbara to be 120.

Measurement of faecal progesterone levels appears suitable to identify pregnant individuals within a population. Again this is based on a limited dataset, with only one confirmed pregnant female from the Perth Zoo used over the two years of study. The results from this individual suggest that high levels of progesterone metabolite quickly drops post-partum, and if further studies confirm this result, it is not possible to use this technique to determine if lactating (and hence juvenile rearing) is taking place. Nonetheless, the use of both hormone and genetic analysis can be used to identify if an individual is pregnant and if she remains in the cave for juvenile rearing. A combination of both techniques is therefore required to locate and monitor maternity roosts in the Pilbara.

Early studies on the ghost bat suggested that ghost bats are concentrated around a few highly disjunct maternity sites (Worthington Wilmer et al., 1994). Whilst this may be the case for geographically isolated populations in the Northern Territory and Queensland where populations in a single roost can be more than 1000 (e.g. Pine Creek; Pettigrew et al, 1986), and indeed in the Chichester subregion of the Pilbara, this doesn't appear to be the case in

the Hamersley subregion. Eleven of the 30 caves sampled between 2014 and 2015 for faecal metabolites were found to have elevated levels of progesterone, indicating the presence of pregnant individuals. Whilst this doesn't confirm if the individuals gave birth inside the cave, the presence of pregnant individuals and small populations observed within caves, suggest there are multiple small groups within the region, each of which has at least one cave used for breeding purposes. This is consistent with suggestions by Armstrong and Anstee (2000) that "small groups my move about within a local area, possibly in response to disturbance, microclimate or social factors." Again, longer term studies comprising both DNA and hormone studies may confirm if this is the case.

As per the genetic studies, it is unsure to what rate faecal metabolites break down and can be used for analysis. Hormone degradation trials are currently underway to determine the degradation rates of hormone levels in scats when exposed to normal cave conditions. These further studies will allow estimation of the maximum age of faecal samples collected which may increase the sampling opportunities for future studies and reduce cost and potential disturbance to bats.

Within BHP's tenements in the Central Pilbara, results from the scat collection analysis suggest that caves AC01, ACW01, M01, SF01 and SF14 are, or are periodically, important caves for the ghost bat. Three of these caves (SF01, AC01 and M01), and a further eight caves (SF05, SF15, AC04, AC08, AC009, AC13, ACW10, and MARXX1) were identified as being used by pregnant females. For one of these caves, SF15, the classification as an important cave was further supported by an observation of 16 individuals inclusive of at least 3-4 juveniles. The fact that the use of these caves seems irregular suggests that the number of caves used by pregnant individuals may differ from year to year according to surrounding environmental conditions, such as fire, and potentially includes caves not listed above. Thus, the above listed caves should be considered the minimum caves which have relative importance to the species within BHP's Central Pilbara tenements and surrounds. Further monitoring, genetic and hormone analysis of scats during and after the ghost bat breeding season will allow further conclusions to be drawn.

5. REFERENCES

Armstrong, K.N. and Anstee, S.D. (2000). The ghost bat in the Pilbara; 100 years on. *Australian Mammalogy* **22**: 93 – 101.

Biologic Environmental Survey Pty Ltd (2015) Central Pilbara Ghost Bat Population and Roost Assessment: 2014. Unpublished report prepared for BHP Billiton Iron Ore Pty Ltd.

Biologic Environmental Survey Pty Ltd and Bat Call WA (2014) Pilbara Regional ghost bat Review. Unpublished report prepared for BHP Billiton Iron Ore Pty Ltd.

BoM, Bureau of Meteorolgy (2016) Bureau of meteorology www.bom.wa.gov.au

Bullen, R. D. and McKenzie, N. L. (2011). Recent developments in studies of the community structure, foraging ecology and conservation of Western Australian bats. In: *The Biology and Conservation of Australasian Bats* (Ed. B. Law, et al.). Royal Zoological Society of NSW, Mosman, New South Wales.

Cavalli-Sforza L.L. & Edwards A.W. 1967. Phylogenetic analysis. models and estimation procedures. *American Journal of Human Genetics* **19**(3), 233-257.

Graham, L., Schwarzenberger, F., Mostl, E., Galama, W., and Savage, A. (2001) A versatile enzyme immunoassay for the determination of progestagens in feces and serum. *Zoo Biology* **20**, 227-236.

Hampton J.O., Spencer P.B.S., Alpers D.L., Twigg L.E., Woolnough A.P., Doust J., Higgs T. & Pluske J. 2004. Molecular techniques, wildlife management and the importance of genetic population structure and dispersal: a case study with feral pigs. *Journal of Applied Ecology* **41**, 735–743.

Kalinowski S.T. 2005. hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes* **5**, 187 -989.

Kalinowski, S.T. 2006. HW-QuickCheck: an easy-to-use computer program for checking genotypes for agreement with Hardy–Weinberg expectations. *Molecular Ecology Notes,* **6**, 974 -979.

Keeley, T., Goodrowe, K.L., Graham, L., Howell, C., and MacDonald, S.E. (2012a) The reproductive endocrinology and behavior of Vancouver Island marmot (Marmota vancouverensis). *Zoo Biology* **31**, 275-290.

Keeley, T., McGreevy, P.M., Fanson, B.G., Masters, K., and O'Brien, J.K. (2012b) The reproductive cycle of the Tasmanian devil (*Sarcophilus harrisii*) and factors associated with reproductive success in captivity. *General and Comparative Endocrinology* **176**, 182-191.

Nicklas, J.A, Noreault-Conti T, Buel E. (2011) Development of a Real-Time Method to Detect DNA Degradation in Forensic Samples. *Journal of Forensic Sciences* **57**, 466-471.

Palme, R., Touma, C., Arias, N., Dominchin, M.F., and Lepschy, M. (2013) Steroid extraction: Get the best out of faecal samples. *Veterinary Medicine Austria* **100**, 238-246.

Peakall R., Ruibal M. & Lindenmayer D.B. 2003 Spatial autocorrelation analysis offers new insights into gene flow in the Australian bush rat, *Rattus fuscipes*. *Evolution* **57**, 1182–1195.

Peakall, R. & Smouse, P.E. 2006. GenAlEx 6: genetic analysis in Excel. genetics software for teaching & research. *Molecular Ecology Notes* **6**, 288-295.

Pettigrew, J., Baker, G. B., Baker-Gabb, D., Baverstock, R., Coles, R., Conole, S., Churchill, S., Fitzherbert, K., Guppy, A., Hall, L., Helman, P., Nelson, J., Priddel, D., Pulsford, I., Richards, G., Schulz, M. and Tidemann, C. R. (1986). The Australian ghost bat, *Macroderma gigas*, at Pine Creek, Northern Territory. *Macroderma* **2**(1), 8-19.

Piry S., Luikart G. & Cornuet J.M. (1999). BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**, 502-503.

Pollock, K., Booth, R., R.Wilson, Keeley, T., Grogan, K., Kennerley, P., and Johnston, S.D. (2010) Oestrus in the Julia Creek dunnart (*Sminthopsis douglasi*) is associated with wheel running behaviour but not necessarily changes in body weight, food consumption or pouch morphology. *Animal Reproduction Science* **117**, 135-146.

Smouse P.E. & Peakall R. 1999. Spatial autocorrelation analysis of multi-allele and multi-locus genetic microstructure. *Heredity* **82**, 561-573.

Tidemann, C.R., Priddel, D.M., Nelson, J.E. and Pettigrew, J.D. (1985) Foraging behaviour of the Australian ghost bat, Macroderma gigas (Microchiroptera: Megardermatidae). *Australian Journal of Zoology* **33**, 705-713.

Toop, J. Habitat requirements, survival strategies and ecology of the Ghost bat *Macroderma gigas* Dobson, (Microptera, Megadermatidae) in central coastal Queensland. *Macroderma* **1**, 37-41.

TSSC, Threatened Species Scientific Committee (2016) Conservation Advice *Macroderma gigas* ghost bat. Available at www.environment.gov.au/biodiversity/threatened/species

Worthington Wilmer, J., Moritz, C., Hall, L., & Toop, J. (1994). Extreme population structuring in the threatened ghost bat, *Macroderma gigas*: evidence from mitochondrial DNA. *Proceedings of the Royal Society of London B Biological Sciences* **257**, 193-198.

Appendix A – Hormone Analysis (Keeley, 2016)

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To: Morgan O'Connell, Biologic Environmental Survey Pty Ltd
Subject: detecting hormone levels in scat material of ghost bat

22 June 2016

Introduction

A known colony of approximately 25 Ghost Bats (*Macroderma gigas*) occur in the eastern Hamersley Range (Pilbara bioregion) between Packsaddle Range to the north and Mt Robinson to the south. Currently, it is unknown if the caves used regularly by these individuals are maternity caves (i.e. used to house pregnant females and raise pups). By detecting hormone levels in scat material found inside these caves, during the breeding season, it should be possible to determine which caves are utilised as maternity roosts. This information will assist in the management of this colony to reduce impacts from any planned disturbances.

Methods - Faecal Sample Process and Hormone Analysis

Three batches of faecal samples were collected in December 2014, November 2015 and December 2015 from South Flank and surrounds. Samples were collected from inside the caves, stored as individual samples and were no older than 30 days. Faecal samples were also collected from the captive population at the Perth Zoo. Scats were collected from a housed group of seven female Ghost Bats to assist with the validation and analysis of faecal samples from the wild roost sites. Perth Zoo confirmed that a single male offspring was born on 15 November 2015, during the scat collection period.

Faecal samples were analysed for progesterone metabolite concentrations by enzyme-immunoassay (EIA). Prior to analysis for hormone concentrations, each faecal sample was extracted using a basic hormone extraction procedure (Keeley et al. 2012a; Palme et al. 2013). Briefly, faecal samples were subsampled to a weight of either 0.1 ± 0.02 or 0.05 ± 0.002 g to which 5ml of 80% methanol was added. Samples were rotated gently overnight, centrifuged at 1000 g for 10 min and then decanted and stored at -20oC until analysis. The supernatant was diluted 1:20 to 1:1000 (dependant on concentration) in assay buffer prior to analysis. Faecal progesterone metabolite concentrations were quantified by double antibody EIA using a goat anti-mouse IgG (Arbor Assays, USA), monoclonal progesterone antiserum (CL425), horseradish peroxidase conjugated label (both provided by C. Munro, University of California-Davis, Davis, USA) and progesterone (Sigma Aldrich Australia, Ltd.) standards as previously described with minor modifications (Keeley et al. 2012b). Briefly, the antiserum (1:80,000) was incubated on the microtitre plate overnight, horseradish peroxidise conjugate (1:400,000), standards (0.016 - 4 ng/ml) and samples were loaded (50 µl/well) onto the plate and the EIA was performed as described elsewhere (Pollock et al. 2010; Keeley et al. 2012b). Intra and inter-assay coefficients of variation were both <10%. Cross-reactivities for the EIA antibodies were as previously

described(Graham et al. 2001). Hormone concentrations were expressed as nanograms of hormone metabolites per gram of faeces (ng/g).

Limitations

Biological Validation. To confirm that patterns in faecal hormone levels are reflective of biologically relevant changes in physiology, such as pregnancy, sample extraction and analysis techniques require biological validation. To biologically validate our techniques we requested faecal samples from captive housed ghost bats at Perth Zoo in 2014 and 2015. Unfortunately, only a single young was born in November 2015, providing only a single pregnancy for comparison. As the female ghost bats at Perth Zoo are group housed, faecal samples were collected as a group of samples, three times per week from October to January. Individual samples (n = 2 to 4) were processed and analysed for each collection date. Elevated progesterone levels were only detected prior to the reported parturition date of 15 November, confirming these samples were likely from the pregnant female. Unfortunately, with only a single pregnant female available for validation, it is unknown how much individual hormone level variation exists between females and therefore we are unable to accurately estimate absolute cut-off points defining non-pregnant and pregnant hormone levels. Despite this, we believe data from the pregnant female and from individual samples collected from the wild suggest that levels above 900-1000 ng/g ae reflective of pregnancy. Samples from additional captive pregnant female ghost bats would further strengthen our validation process.

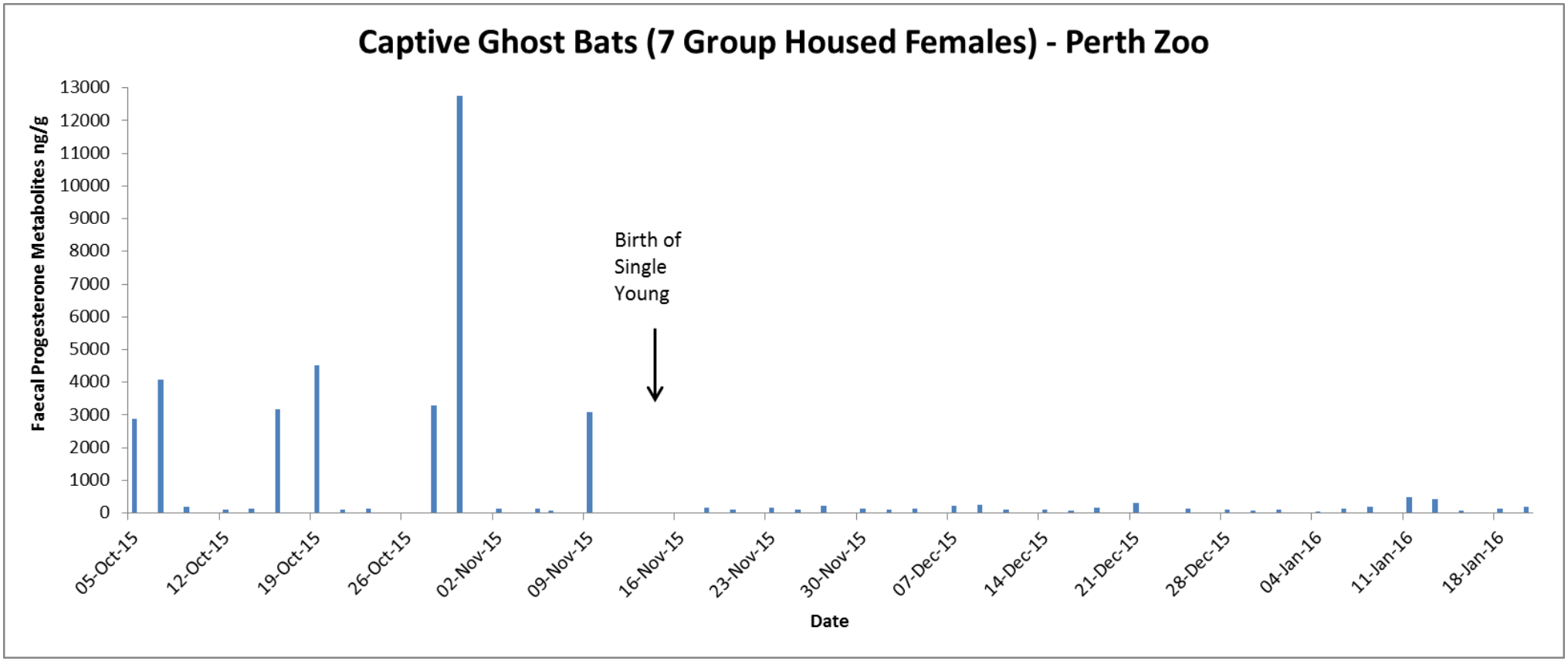
Results and Discussion

For most faecal samples analysed from the wild Ghost Bat sites, progesterone metabolite levels were similar to the baseline values of captive female Ghost Bats (see table below).

Table 1: Levels of progesterone metabolite (ng/g) in the groups of Ghost Bats.

Ghost Bat Group Class	2014	2015	combined
Male Captive Bats	69.4 ± 10.8	NA	
Female Captive Bats	95.7 ± 46.0	138.4 ± 109.0	
Presumed Pregnant Captive Bat	NA	4485.2 ± 2898.4	
Presumed Non-Pregnant Wild Bats			201.1 ± 127.9
Presumed Pregnant Wild Bats			3330.1± 2314.9

Two random samples per collection date from the captive group housed females (n = 7 females) were processed and analysed. Several samples between 5 October and 9 November had elevated progesterone levels (see table and graph below), all of which were before the birth date of the offspring, 15 November. No elevated samples were found after the birth date, confirming that these elevated samples were most likely from the pregnant female and that elevations of progesterone in early lactation may not be detectable in this species.


Captive Ghost Bats (7 Group Housed Females) - Perth Zoo
Faecal Progesterone Metabolites ng/g
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Birth of
Single
Young
05-Oct-15
12-Oct-15
19-Oct-15
26-Oct-15
02-Nov-15
09-Nov-15
16-Nov-15
23-Nov-15
30-Nov-15
07-Dec-15
14-Dec-15
21-Dec-15
28-Dec-15
04-Jan-16
11-Jan-16
18-Jan-16
Date

As these samples were usually no more than a couple days old, hormone values are representative of fresh faecal matter. Using these hormone values, we can estimate which samples from the wild population are likely to represent those collected from pregnant females. Cave sites with presumed pregnant females have been listed in Table 2, individual levels of each scat are presented in Appendix 1. Samples with hormone values suggesting pregnancy were found in both the November and December 2015 collections at the M1 site suggesting that at least one female was pregnant between 15 November and 14 December.

Table 2: Caves with scats containing high levels of progesterone metabolite.

2014 Roost Sites with Presumed Pregnant Ghost Bats (> 1000 ng/g)	2015 Roost Sites with Presumed Pregnant Ghost Bats (> 1000 ng/g)
AC Cave 8	M1 (Nov and Dec)
ACW10	SF1 (Nov)
SF15	MARXX
ML-1	
AC9	
AC13	
AC1	
SF5	
AC Cave 4	

It is unknown to what extent sample degredation effects faecal hormone concentrations in this species. As the samples collected from the wild population have the potential of being up to a month in age, there is a possiblity that hormone concentrations may decline or degrade during this time. Our results suggest that hormone degredation may be limited as we have found samples with progesterone levels similar to the baseline levels of captive non-pregnant females, or similar (or slightly lower) to the elevated levels of the captive pregnant female with progesterone levels 5+ fold greater than baseline. Some of the samples which have elevated progesterone values but remain between baseline and estimated gestation levels may represent samples from pregnant females in which hormone metabolites have degraded over time making it difficult to confirm the status of the animal of origin. Therefore the identification of cave sites with pregnant females may be an underestimate due to varying levels of sample degredation prior to collection. Regardless, the hormone results suggest that pregnant Ghost bats were present at some of the collection sites in both collection years.

Further Studies

Sample hormone degradation. Freshly collected faecal pellets from captive Ghost Bats have been used to examine degradation of hormones over time. Faecal samples (n = 24) are currently being incubated at 25^{o}C at approximately 30% humidity, in the dark to replicate environmental conditions similar to those present in the cave sites. Samples will be removed once per week (n = 6) for a period of four weeks to simulate the estimated maximum age of faecal samples collected in the wild. Once removed, each sample will be process, extracted and analysed as described.

The changes of faecal progesterone over time will be used to quantify the overall effects of sample age on the accuracy of faecal hormone levels detected from cave sites *in situ*.

References

Graham, L., Schwarzenberger, F., Mostl, E., Galama, W., and Savage, A. (2001) A versatile enzyme immunoassay for the determination of progestagens in feces and serum. *Zoo Biology* **20**, 227-236

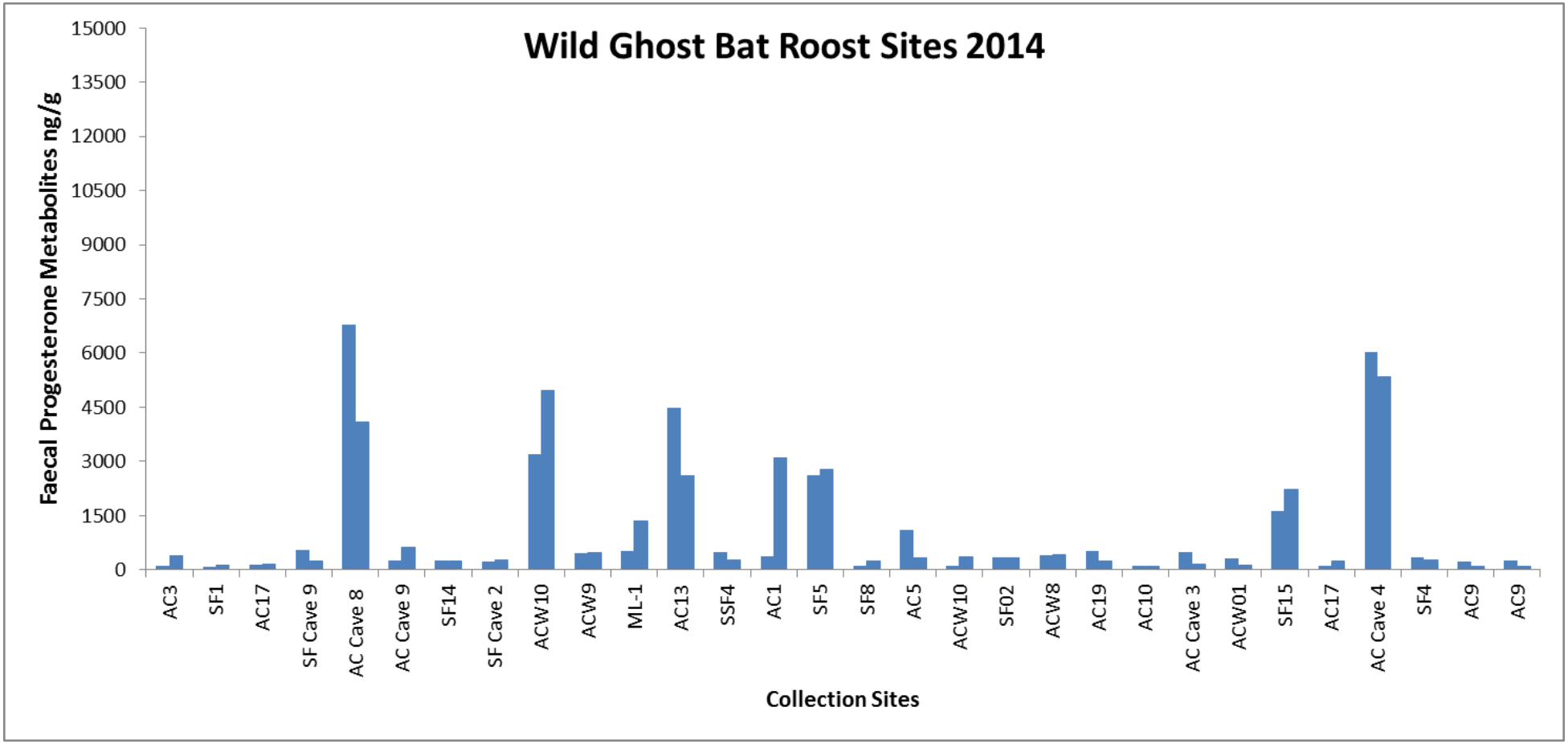
Keeley, T., Goodrowe, K.L., Graham, L., Howell, C., and MacDonald, S.E. (2012a) The reproductive endocrinology and behavior of Vancouver Island marmot (*Marmota vancouverensis*). *Zoo Biology* **31**, 275-290

Keeley, T., McGreevy, P.M., Fanson, B.G., Masters, K., and O'Brien, J.K. (2012b) The reproductive cycle of the Tasmanian devil (*Sarcophilus harrisii*) and factors associated with reproductive success in captivity. *General and Comparative Endocrinology* **176**, 182-191

Palme, R., Touma, C., Arias, N., Dominchin, M.F., and Lepschy, M. (2013) Steroid extraction: Get the best out of faecal samples. *Veterinary Medicine Austria* **100**, 238-246

Pollock, K., Booth, R., R.Wilson, Keeley, T., Grogan, K., Kennerley, P., and Johnston, S.D. (2010) Oestrus in the Julia Creek dunnart (*Sminthopsis douglasi*) is associated with wheel running behaviour but not necessarily changes in body weight, food consumption or pouch morphology. *Animal Reproduction Science* **117**, 135-146

Appendix 1


Wild Ghost Bat Roost Sites 2014
Faecal Progesterone Metabolites ng/g
15000
13500
12000
10500
9000
7500
6000
4500
3000
1500
0
AC3
SF1
AC17
SF Cave 9
AC Cave 8
AC Cave 9
SF14
SF Cave 2
ACW10
ACW9
ML-1
AC13
SSF4
AC1
SF5
SF8
AC5
ACW10
SF02
ACW8
AC19
AC10
AC Cave 3
ACW01
SF15
AC17
AC Cave 4
SF4
AC9
AC9
Collection Sites

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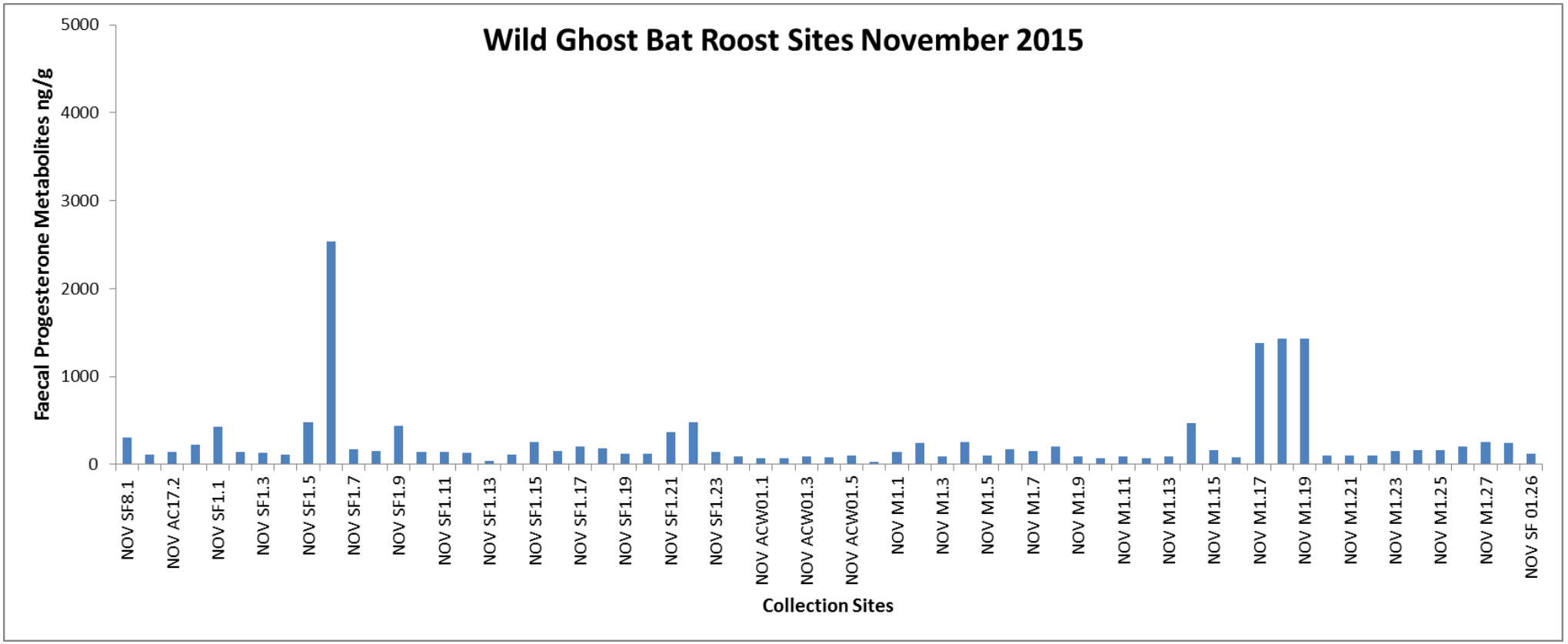
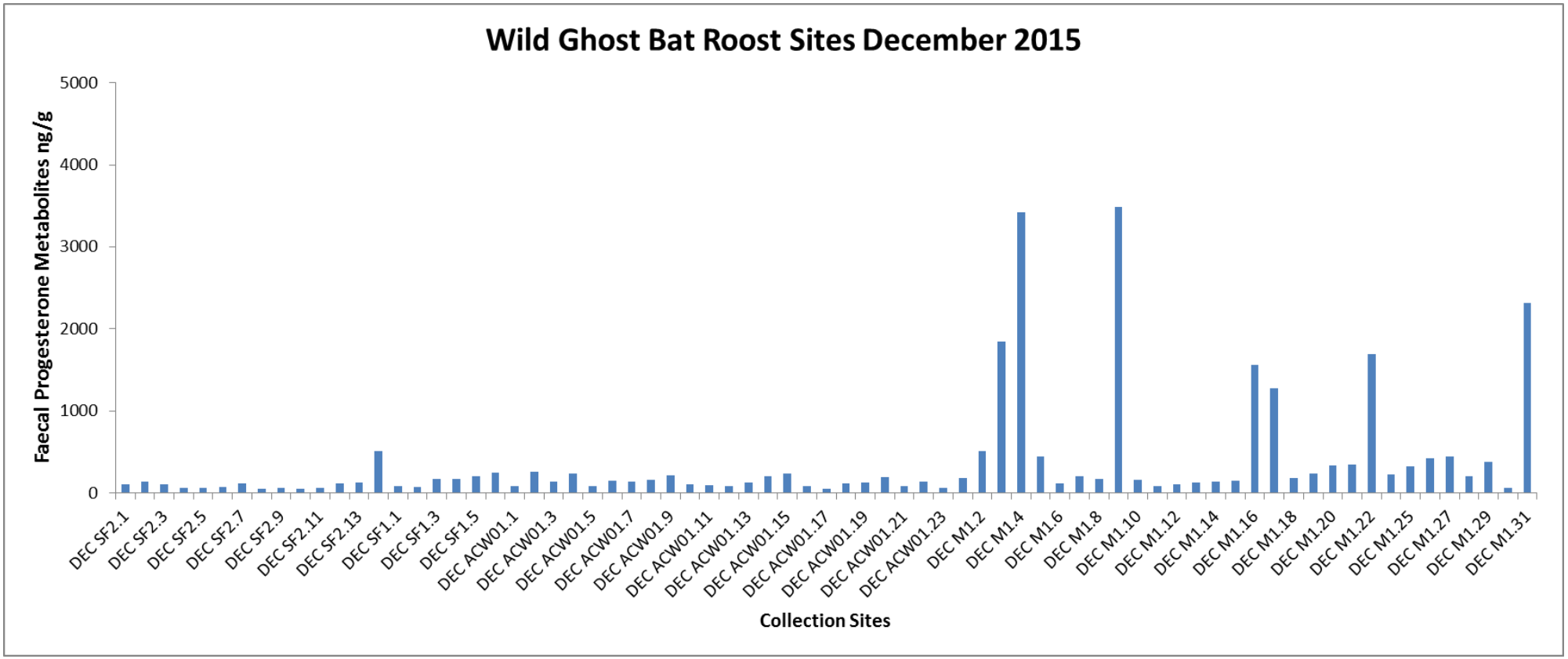
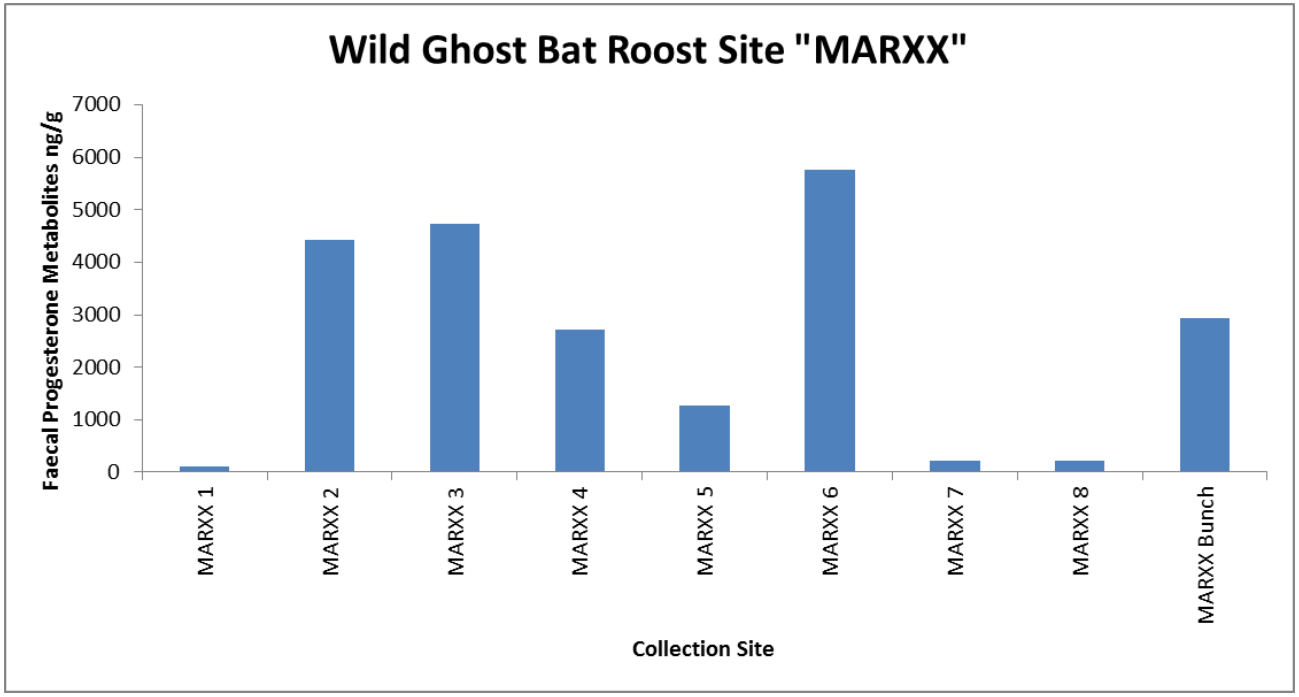



Wild Ghost Bat Roost Sites December 2015
Faecal Progesterone Metabolites ng/g
5000
4000
3000
2000
1000
0
DEC SF2.1
DEC SF2.3
DEC SF2.5
DEC SF2.7
DEC SF2.9
DEC SF2.11
DEC SF2.13
DEC SF1.1
DEC SF1.3
DEC SF1.5
DEC ACW01.1
DEC ACW01.3
DEC ACW01.5
DEC ACW01.7
DEC ACW01.9
DEC ACW01.11
DEC ACW01.13
DEC ACW01.15
DEC ACW01.17
DEC ACW01.19
DEC ACW01.21
DEC ACW01.23
DEC M1.2
DEC M1.4
DEC M1.6
DEC M1.8
DEC M1.10
DEC M1.12
DEC M1.14
DEC M1.16
DEC M1.18
DEC M1.20
DEC M1.22
DEC M1.25
DEC M1.27
DEC M1.29
DEC M1.31
Collection Sites


Wild Ghost Bat Roost Site "MARXX"
Faecal Progesterone Metabolites ng/g
7000
6000
5000
4000
3000
2000
1000
0
MARXX 1
MARXX 2
MARXX 3
MARXX 4
MARXX 5
MARXX 6
MARXX 7
MARXX 8
MARXX Bunch
Collection Site

Appendix B – Genetic Analysis (Spencer & Tedeschi, 2016)

An initial investigation into the genetic diversity, structure and short-range spatial-use by Ghost Bats in the Hamersley subregion of the Pilbara

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Prepared for:

bhpbilliton

September, 2016

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DISCOVERERS WELCOME

Summary

- This study used information from genetic profiling as an initial investigation into the genetic diversity, structure and short-range spatial-use by Ghost Bats in the Hamersley subregion of the Pilbara.

- Genetic analyses of nuclear markers are shown to provide a powerful approach to infer patterns of genetic structure and the study allowed analysis of 98 individuals from the Hamersley subregion of the Pilbara.

- In this study, DNA extractions were carried out on 324 Ghost Bat samples (19 tissues and 305 scats). From those, 3 samples failed to amplify anything, 30 samples were not used in this study, 78 samples failed at 5 or more loci, 112 samples were duplicated genotypes and 3 samples from the Kimberley (WAM specimens) were not used in any analysis.

- Genetic variation was examined at 10 nuclear genes (microsatellite markers) from the final 98 unique individuals.

- Ghost Bats showed medium to high levels of genetic variation (H_E=50-60%) when different caves were compared.

- The genetic profiles are consistent with the identity of a single genetic cluster (population) of Ghost Bats. There is clear evidence that there is some admixture between different cave sampling sites.

- The South Flank population samples clustered within the main Hamersley population. The main Hamersley population appears to be extensive, extending from Pannawonica to Newman, based on our initial analysis

- While genetic diversity is high in the Hamersley populations, none of the populations show evidence of recent or long-term population declines (detected using bottleneck analysis). This suggests that the populations have not been in recent decline.

- Spatial autocorrelation was used to define how the species is able to disperse at the local level. The spatial structure analysis identified that neighbour size (movement distance) was somewhere between 10 and 15 kilometres.

- Overall this project has been remarkably successful; however, the study needs to be interpreted with some caution due to a limited number of Ghost Bats genotyped. The low number of individuals (98) make the interpretation of the data somewhat limited and it would be desirable to increase the numbers of bats used, and use bat samples that encompass more of the Pilbara in future studies.

Table of Contents

1. Introduction

The Ghost Bat (*Macrodema gigas*) is a monotypic bat species native to the Pilbara and Kimberley regions of Western Australia (WA), the Northern Territory (NT) and eastern Australia (Fig. 1.1). In Queensland (QLD) and the NT, they are coastal and occur up to 400 km inland, throughout northern Australia and generally north of the Tropic of Capricorn. Regional populations of this species appear to have maternity roosts that are genetically isolated from each other (Worthington-Wilmer *et al.* 1994). They appear to occupy a wide range of habitats from rainforest, monsoon and vine scrub in the tropics to open woodlands and arid areas. The Ghost Bat is an obligate troglodyte, and survival is critically dependent on finding natural roosts in caves, crevices, deep overhangs and artificial roosts such as abandoned mines (Hall *et al.* 1997). Each population appears to have a regionally centralised maternity site and only 10 such sites are known to exist (Worthington-Wilmer *et al*. 1994). Populations are known to disperse in the non-breeding (dry) season (Toop 1979, 1985).

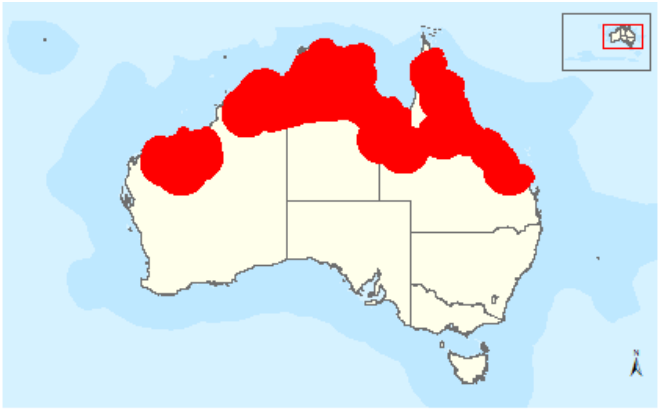


Figure 1.1 The distribution of the Ghost Bat, *Macroderma gigas*. Source: http://www.environment.gov.au/cgi-bin/sprat/public/publicspecies.pl?taxon_id=174

In Western Australia, the range appears to have contracted northwards in relatively recent times, especially in Central Australia (Churchill and Helman 1990). They persist in arid regions such as the Pilbara and are geographically isolated from extant northern Australian populations (and the historical central Australian populations) by extensive sandy deserts (Fig. 1.1). Their taxonomic status should be addressed to determine its specific status.

1. 1 Study aims

The study had three specific aims:

1. **Source and collate existing genetic material**. Tissue and specimens of Ghost Bats sourced from the Western Australian Museum (WAM) (these will act as control DNA). Additionally, optimise DNA extraction protocols for the extraction of DNA from faecal material.

2. **Optimise and generate DNA profiles** using an existing set (or subset of) the available nuclear (microsatellite) regions of the DNA.

3. **Preliminary population genetics analysis.** Use the available genetic profiles to infer;

a. the size of the population. This will be highly subjective due to the geographic sampling of Ghost Bat material.
b. Infer dispersal ability based on genetic estimators (spatial autocorrelation and neighbor-size)
c. Estimate the genetic effective population size and minimum number of bats genotyped.
d. Develop a molecular method for determining sex of Ghost Bats using faecal material.
e. Implement this method to assign sex to scat samples.
f. Determine variation in the use of caves by males and females (from genetic studies) by comparing the relatedness of individuals occurring within and between caves at the South Flank site.
g. Additional analysis will be used to investigate population-level questions including, identifying habitat with high genetic diversity and investigating whether any population(s) have been through genetic bottlenecks.

2. Study Area and Methods (Laboratory and analyses)

2.1 Trapping and sampling locations

Most samples were collected from 25 cave sites (Table 2.1a and 2.1b) to the east of Karijini National Park. Samples were also obtained from immediately west of Karijini and the most western parts of the Hamersley Range. Material was also available from tissue samples from 16 individuals from the WAM. Fewer samples were collected from around Newman (BHP0B35), on the eastern extent of the Pilbara, shown in Figure 2.1.

Table 2.1a Sample localities and numbers (*n*) of Ghost Bats used in this study. The locality names can be visualised in Figures 2.1.

Sampling location	Lat (dec.)	Long (dec.)	Number of samples
Pilbara			
SF01	-22.9997	118.9462	63
SF02	-23.0092	118.8848	13
NT03	-22.7415	118.6250	7
ACW08	-23.0366	118.6609	3
NT01	-22.7999	118.5948	5
AC01	-22.9740	119.8260	24
SF14	-22.9626	118.8866	30
MARXXX	-22.6700	119.2618	8
GU1	-23.2095	118.9666	12
GU2	-23.1908	119.0301	6
AC17	-23.0041	118.9911	2
Mt Meharry	-22.9877	118.7553	1
Fence	-23.0231	118.7586	1
ACW01	-22.8702	118.7922	30
ACW10	-23.0286	118.7206	1
APIGBRH03	-22.0863	116.2546	1
APIGBRH01	-21.9388	116.1301	1
FMGGBCP05	-22.1464	117.5440	1
SF08	-22.9930	118.8252	29
SF27	-	-	1
BHP0B35	-23.3964	119.6609	1
M1	-23.0813	118.6608	81
Kimberley mainland			
King Sound	-16.6872	123.8364	2
Bonaparte Archipelago	-15.0725	125.1853	1

Table 2.1b Cave sites sampled for scat material and the numbers of samples collected during sampling trips in the breeding season (Nov, Dec) and outside the breeding times (Apr, May).

	SCATS COLLECTED				
	Collection Date				
Cave	**NOV**	**DEC**	**APR**	**MAY**	**TOTAL**
AC01			5	49	**54**
AC10			3		**3**
AC17	2	1	2		**5**
ACW01	6	24	21	8	**59**
ACW06				16	**16**
ACW10		1	10		**11**
ACW08	1		20	4	**25**
APIGBRH01				4	**4**
APIGBRH04				1	**1**
APIGBRH05				16	**16**
BHP0B35				19	**19**
M1	27	30	23	24	**104**
SF02		13	2	3	**5**
SF003			7		**7**
SF04				1	**1**
SF08	1		8	48	**57**
SF01	24	5	12	46	**87**
SF14			10	34	**44**
SF15			8		**8**
SF27				7	**7**
GU2			6		**6**
GU1			12		**12**
NT03			7		**7**
NT01			5		**5**
MARXXX1		8			**8**
			TOTAL		**571**

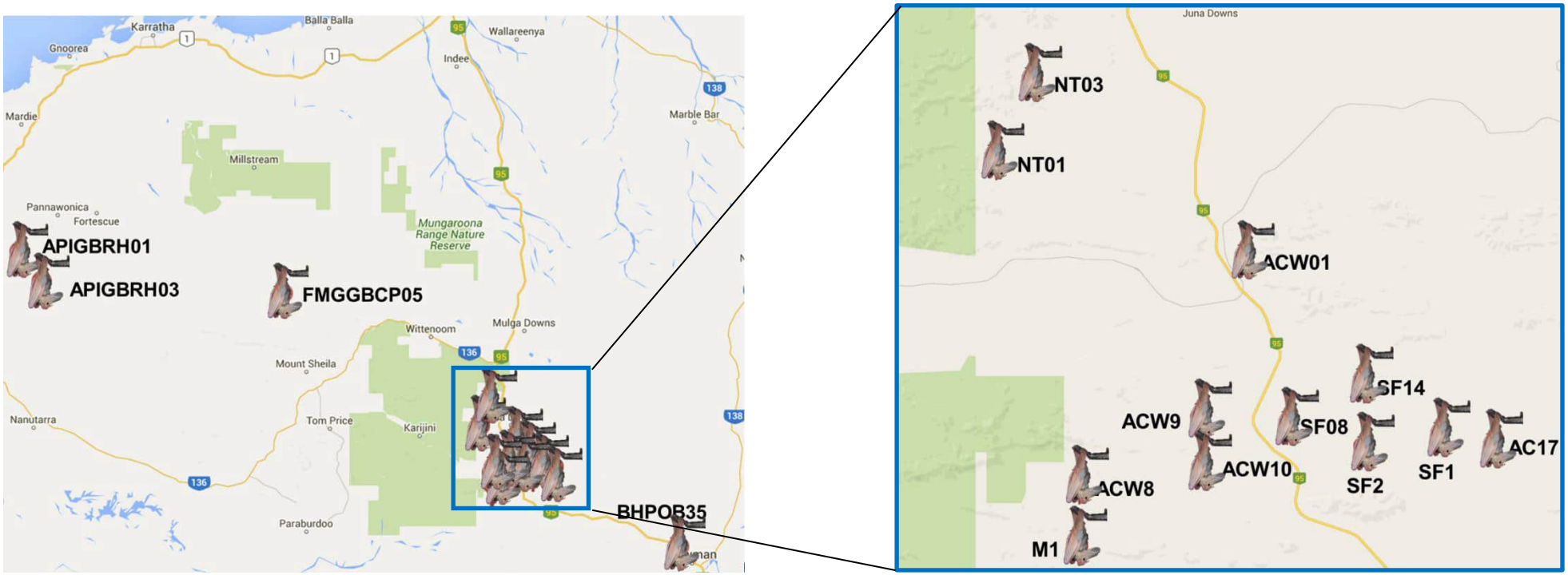


Figure 2.1. Geographic location of sampling locations the Pilbara region referred to in this report.

2.2 Material for evaluation

Tissue samples were extracted using a QIAGEN Tissue/Blood extraction kit (Cat No./ID: 69506) Faecal material was subject to more specialised extraction using the QIAGEN Stool extraction kit (Cat No./ID: 51504). All tissue samples produced amplifiable DNA (see following section). The scat (faecal pellet) samples were taken from individual Ghost Bats from the Pilbara region and surrounds (Table 2.2; Fig. 2.2). A subset of 98 samples were used to evaluate the genetic diversity and structure of Ghost Bats in the Pilbara for this report.

Table 2.2 Sample localities and numbers (*n*) of Ghost Bats used in this study. The locality names can be visualised in Figures 2.1.

Sampling location	Number of samples	Duplicates	<5 loci amplified	Failed to amplify
Pilbara				
SF01	63	29	22	1
SF02	13	9	1	0
NT03	7	0	4	0
ACW08	3	0	0	0
NT01	5	6	1	0
AC01	24	7	1	1
SF14	30	8	2	1
MARXXX	8	2	2	0
GU1	12	1	7	0
GU2	6	1	3	0
AC17	2	0	1	0
Mt Meharry	1	0	0	0
Fence	1	0	0	0
ACW01	30	2	2	0
ACW10	1	0	0	0
APIGBRH03	1	0	0	0
APIGBRH01	1	0	0	0
FMGGBCP05	1	0	0	0
SF08	29	12	5	0
SF27	1	0	0	0
BHP0B35	1	0	0	0
M1	81	40	25	0
Kimberley mainland				
King Sound	2	0	0	0
Bonaparte Archipelago	1	0	0	0

Source and collate existing material

324	- DNA extractions (19 tissues, 305 scats)
321	- 3 samples failed to amplify anything
243	- 78 samples failed at 5 or more loci (24%)
131	- 112 samples were duplicated genotypes
129	- 3 samples from the Kimberley (WAM specimens)
98	- 30 samples not used

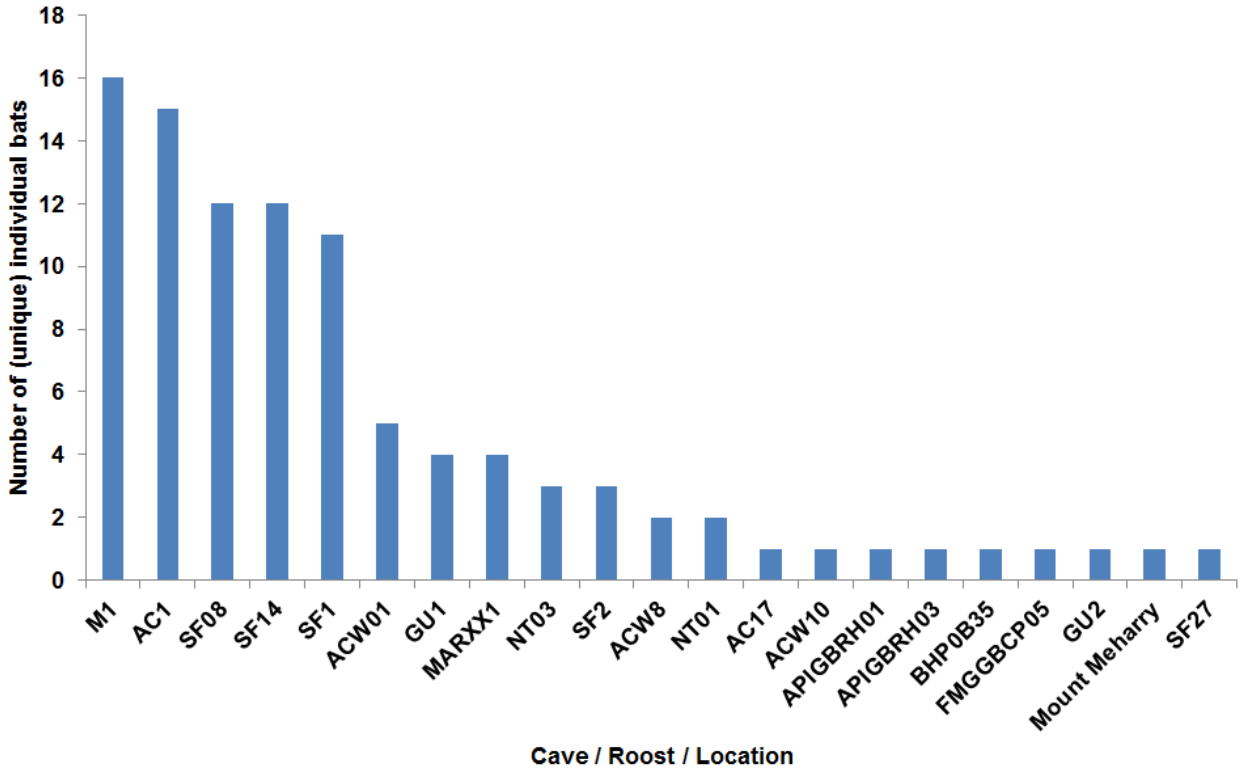


Figure 2.2 The number of unique DNA profiles generated from 21 cave sites throughout the Pilbara. The geographic location of samples is given in Fig 2.1.

2.3 Molecular Methods

2.3.1 Nuclear microsatellite amplification and analysis

We amplified 10 microsatellite loci GB18, GB20, GB33, GB42, GB44, GB81 (J. Hughes, unpubl. data) and gigas01, gigas06, gigas10 and gigas11 (Worthington-Wilmet *et al*. 1994) from 10 sampling locations (Table 2.1). Briefly, PCRs were carried out in a total volume of 40 µl with ~100 ng DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.3 µM of each primer & 1 U *Taq*. Size was determined by co-running a Genescan500 standard (Applied Biosystems, Melbourne). Fragment analysis was carried out on a 3730xl DNA Analyser (ABI systems, Melbourne) and scored with the aid of GENEMARKER (SoftGenetics). Control samples were run in each PCR run to ensure compatibility between different datasets used in the analysis.

Descriptive statistics and assumptions were calculated using GenAlEx 6.4 (Peakall & Smouse 2006) and HW-QUICKCHECK (Kalinowski 2006). The rarefaction method, as implemented in HP-RARE (Kalinowski 2006), was used to calculate the allelic richness based on 11 diploid individuals. This method allows a direct comparison between populations because it equalises the sampling effort. Using the allele frequencies, the arc genetic distance between localities (Cavalli-Sforza & Edwards 1967) was computed and subjected to principal coordinates analysis (Gower 1966) using GenAlEx 6.4 (Peakall & Smouse 2006).

Evidence of recent population bottlenecks was investigated by testing for a deficiency of heterozygosity using BOTTLENECK (Piry *et al.* 1999). Due to the relatively small number of polymorphic loci analysed (n=10), a Wilcoxon sign-rank test was estimated. A mixed model of microsatellite mutation was assumed with a single step mutation assumed at 90%, variance of 12, as suggested by Piry *et al*. (1999) and Hampton *et al*. (2004).

Dispersal distance was investigated by testing for a relationship between pairwise population genetic measures (Peakall & Smouse 2006) and geographical distance (measured as decimal latitude & longitude) using genetic spatial autocorrelation analysis, performed using the program GenAlEx 6.4 (Peakall & Smouse 2006). The spatial autocorrelation analysis implemented in GenAlEx calculates an autocorrelation coefficient (*r*) for genetic distances (Smouse and Peakall 1999) as a function of geographical distance (km). We used distance classes of 5 km, up to 50 km. We generated the 95% confidence intervals around the expectation of no spatial genetic structure

using 1000 random permutations. The geographical distance at which the mean *r* value drops below zero has been referred to as the ‘neighbourhood size’ or ‘patch size’ (Peakall *et al*. 2003) and represents the largest spatial scale at which genetic similarity is non-random.

3. Results

3.1. Molecular population genetics: microsatellite

3.1.1 Population structure

Ninety-eight percent of individuals clustered with their source population (Figure 3.1 , Table 3.1). The available samples formed a single genetic cluster within the Pilbara (Figure 3.1, 3.2, 3.3).

There appears to be a widely distributed population ("Hamersley" cluster). The samples (Figure 3.1, 3.3) indicate a single population unit. Table 3.2 details the individuals recorded from each cave. Table 3.3 and 3.4 provide a measures of microsatellite variability of Ghost bats from different caves and measures of microsatellite variability of Ghost bats from different caves, respectively.

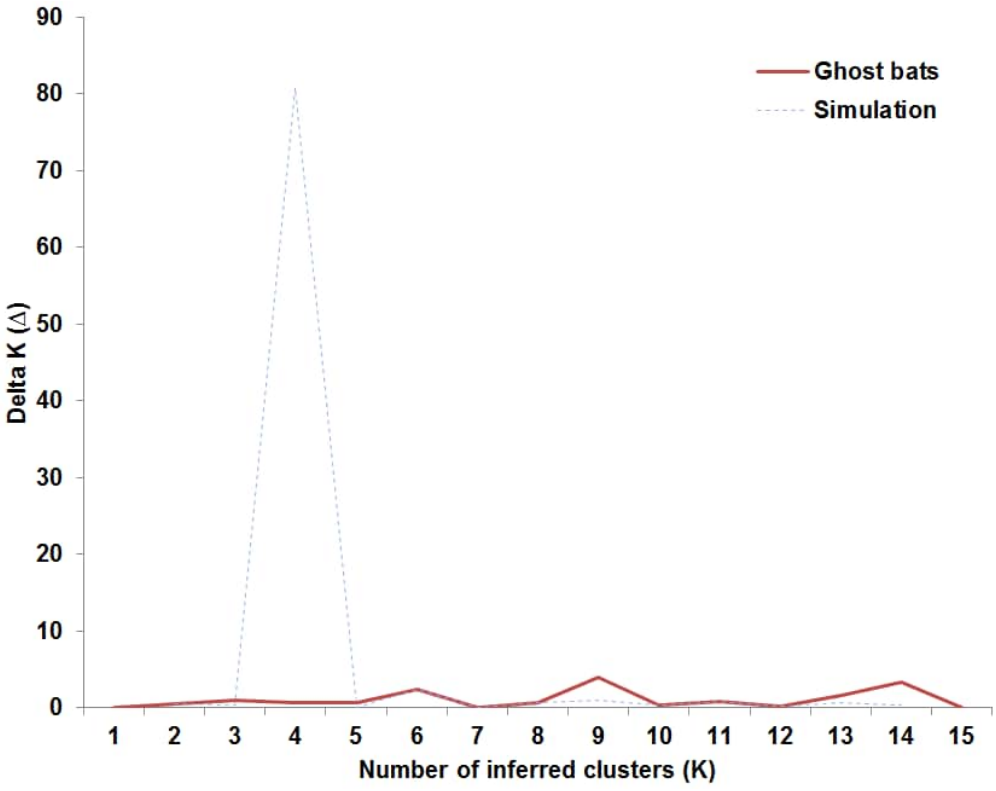


Figure 3.1 Bayesian population structure analyses. Bayesian assignment of the 98 Ghost Bats, based on 10 nuclear microsatellite loci, assuming a population number of K = 1. The plot shows the delta rate change (for identifying the number of 'true' genetic clusters). The blue line represents a hypothetical simulation, where K is most likely to be K-4 (the greatest rate of

change). The Hamersley Ghost Bats show no strong clustering (from K=1-15, with 1,100,000 simulations)

Table 3.1 The number of Ghost Bats that were assigned to unique genetic clusters and the proportion belonging to the cluster. Sample localities and numbers of Ghost Bats used in this study. The locality names can be seen in Figures 2.1.

Cave	Assigned to the cave the bat was sampled from Pop	Assigned to another cave
AC1	15	0
AC17	1	0
ACW01	5	0
ACW10	1	0
ACW8	2	0
APIGBRH01	1	0
APIGBRH03	1	0
BHP0B35	1	0
FMGGBCP05	1	0
GU1	4	0
GU2	1	0
M1	16	0
MARXX1	4	0
Mount Meharry	1	0
NT01	2	0
NT03	3	0
SF08	11	1 (Assigned to AC01)
SF1	11	0
SF14	11	1(Assigned to AC01)
SF2	3	0
SF27	1	0
Total	96	2
Percent	98%	2%

Table 3.2 Roost/locations where individual Ghost Bats were identified.

Cave / Roost / Location	Dec. latitude	Dec. longitude	Individual sample No.	Lab No.
AC1	-22.9740	119.8260	APR AC1.1	B16-179
	-22.9740	119.8260	APR AC1.4	B16-182
	-22.9740	119.8260	MAY AC1.1	B16-286
	-22.9740	119.8260	MAY AC1.2	B16-287
	-22.9740	119.8260	MAY AC1.4	B16-289
	-22.9740	119.8260	MAY AC1.6	B16-291
	-22.9740	119.8260	MAY AC1.7	B16-292
	-22.9740	119.8260	MAY AC1.8	B16-293
	-22.9740	119.8260	MAY AC1.10	B16-295
	-22.9740	119.8260	MAY AC1.11	B16-296
	-22.9740	119.8260	MAY AC1.12	B16-297
	-22.9740	119.8260	MAY AC1.14	B16-299
	-22.9740	119.8260	MAY AC1.15	B16-300
	-22.9740	119.8260	MAY AC1.18	B16-303
	-22.9740	119.8260	MAY AC1.19	B16-304
AC17	-23.0041	118.9911	NOV AC17.1	B16-082
ACW01	-22.8702	118.7922	ACW1 CHIP# 728627C	16-006_T
	-22.8702	118.7922	NOV ACW01.1	B16-117
	-22.8702	118.7922	NOV ACW01.2	B16-118
	-22.8702	118.7922	NOV ACW01.4	B16-120
	-22.8702	118.7922	DEC ACW01.1	B16-122
ACW10	-23.0286	118.7206	DEC ACW10.1	B16-116
ACW8	-23.0366	118.6609	ACW1 CHIP# 00074C4ED3	16-007_T
	-23.0366	118.6609	NOV ACW8.1	B16-084
APIGBRH01	-21.9388	116.1301	CHIP# 953010000996923	16-009_T
APIGBRH03	-22.0863	116.2546	CHIP# 953010000990050	16-008_T
BHP0B35	-23.3964	119.6609	CHIP# 953010000992201	16-188_T
FMGGBCP05	-22.1464	117.5440	CHIP# 9530100009944883	16-010_T
GU1	-23.2095	118.9666	APR GU1.1	B16-152
	-23.2095	118.9666	APR GU1.2	B16-153
	-23.2095	118.9666	APR GU1.3	B16-154
	-23.2095	118.9666	APR GU1.11	B16-162
GU2	-23.1908	119.0301	MAY GU2.3	B16-166
M1	-23.0813	118.6608	CHIP# 953010000988798	16-186_T
	-23.0813	118.6608	NOV M1.12	B16-024
	-23.0813	118.6608	NOV M1.14	B16-026
	-23.0813	118.6608	DEC M1.10	B16-048
	-23.0813	118.6608	DEC M1.14	B16-052
	-23.0813	118.6608	DEC M1.19	B16-057
	-23.0813	118.6608	DEC M1.20	B16-058
	-23.0813	118.6608	DEC M1.29	B16-066
	-23.0813	118.6608	APR M1.4	B16-265
	-23.0813	118.6608	APR M1.6	B16-267
	-23.0813	118.6608	APR M1.7	B16-268
	-23.0813	118.6608	APR M1.9	B16-270
	-23.0813	118.6608	MAY M1.1	B16-274

Cave / Roost / Location	Dec. latitude	Dec. longitude	Individual sample No.	Lab No.
	-23.0813	118.6608	MAY M1.5	B16-278
	-23.0813	118.6608	MAY M1.7	B16-280
	-23.0813	118.6608	MAY M1.11	B16-284
MARXX1	-22.6700	119.2618	DEC MARXX1.2	B16-145
	-22.6700	119.2618	DEC MARXX1.4	B16-147
	-22.6700	119.2618	DEC MARXX1.5	B16-148
	-22.6700	119.2618	DEC MARXX1.6	B16-149
Mount Meharry	-22.9877	118.7553	M48754/M48758	16-003_T
NT01	-22.7999	118.5948	APR NT01.1	B16-001
	-22.7999	118.5948	APR NT01.5	B16-005
NT03	-22.7415	118.6250	APR NT03.3	B16-008
	-22.7415	118.6250	APR NT03.4	B16-009
	-22.7415	118.6250	APR NT03.6	B16-011
SF08	-22.9930	118.8252	CHIP# 953010000995205	16-184_T
	-22.9930	118.8252	APR SF8.1	B16-214
	-22.9930	118.8252	APR SF8.4	B16-217
	-22.9930	118.8252	APR SF8.5	B16-218
	-22.9930	118.8252	APR SF8.6	B16-219
	-22.9930	118.8252	MAY SF8.4	B16-225
	-22.9930	118.8252	MAY SF8.10	B16-231
	-22.9930	118.8252	MAY SF8.11	B16-232
	-22.9930	118.8252	MAY SF8.13	B16-234
	-22.9930	118.8252	MAY SF8.14	B16-235
	-22.9930	118.8252	MAY SF8.16	B16-237
	-22.9930	118.8252	MAY SF8.20	B16-241
SF1	-22.9997	118.9462	CHIP# 953010000994749	16-187_T
	-22.9997	118.9462	NOV SF1.1	B16-085
	-22.9997	118.9462	NOV SF1.2	B16-086
	-22.9997	118.9462	NOV SF1.4	B16-088
	-22.9997	118.9462	NOV SF1.8	B16-092
	-22.9997	118.9462	NOV SF1.10	B16-094
	-22.9997	118.9462	APR SF1.3	B16-185
	-22.9997	118.9462	APR SF1.6	B16-188
	-22.9997	118.9462	MAY SF1.7	B16-200
	-22.9997	118.9462	MAY SF1.12	B16-205
	-22.9997	118.9462	MAY SF1.15	B16-208
SF14	-22.9626	118.8866	CHIP# 953010000994988	16-189_T
	-22.9626	118.8866	MAY SF14.1	B16-242
	-22.9626	118.8866	MAY SF14.2	B16-243
	-22.9626	118.8866	MAY SF14.3	B16-244
	-22.9626	118.8866	MAY SF14.5	B16-246
	-22.9626	118.8866	MAY SF14.6	B16-247
	-22.9626	118.8866	MAY SF14.7	B16-248
	-22.9626	118.8866	MAY SF14.8	B16-249
	-22.9626	118.8866	MAY SF14.13	B16-254
	-22.9626	118.8866	MAY SF14.16	B16-257
	-22.9626	118.8866	MAY SF14.19	B16-260
	-22.9626	118.8866	MAY SF14.20	B16-261
SF2	-23.0092	118.8848	DEC SF2.1	B16-069
	-23.0092	118.8848	DEC SF2.11	B16-079

Cave / Roost / Location	Dec. latitude	Dec. longitude	Individual sample No.	Lab No.
SF27	-23.0092	118.8848	DEC SF2.12 CHIP# 953010000994231	B16-080 16-185_T

Figure 3.2 Ghost Bats were assigned to a single genetic cluster, based on 98 samples analysed from the Pilbara (blue). The locality names can be seen in Figures 2.1.

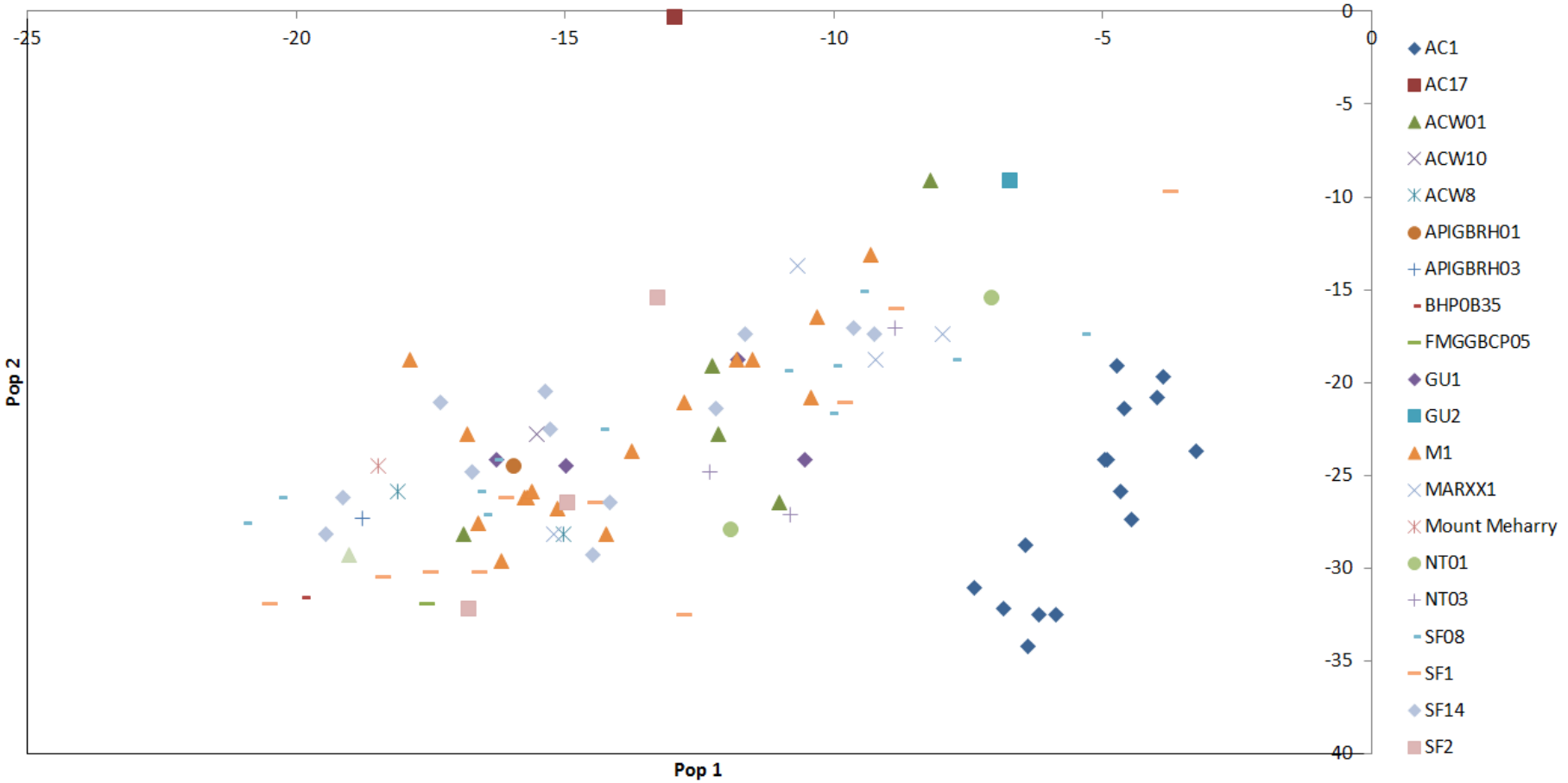


Figure 3.3 Principle components (PCA) plot of the 89 Ghost Bat samples, based on the results generated from 10 nuclear microsatellite loci. The axes are arbitrary. The analysis shows no distinct populations for the Hamersley samples.

Table 3.3 Measures of microsatellite variability of Ghost bats from different caves (and containing more than five unique individuals). n, number of individuals genotypes; NA, number of alleles; NAR, number of allelic richness (standardised for sample size); NE, Effective number of alleles; Ho, observed heterozygosity; HE, expected heterozygosity; SE, standard error;

Inferred population	**n**	**N_A (SE)**	**N_{AR} (SD)**	**H_E (SE)**	**H_O (SE)**	**F**
AC1	12.4 ± 1.0	3.7 ± 0.6	2.3 ± 0.3	0.549 ± 0.101	0.521 ± 0.078	-0.107 ± 0.110
ACW01	3.9 ± 0.3	2.2 ± 0.2	2.0 ± 0.2	0.655 ± 0.146	0.506 ± 0.087	-0.527 ± 0.215
M1	13.4 ± 0.8	4.5 ± 0.6	3.1 ± 0.5	0.616 ± 0.090	0.612 ± 0.080	-0.034 ± 0.048
SF08	9.5 ± 0.8	3.1 ± 0.5	2.2 ± 0.3	0.572 ± 0.093	0.505 ± 0.078	-0.220 ± 0.102
SF1	9.3 ± 0.4	3.7 ± 0.3	2.2 ± 0.2	0.645 ± 0.113	0.531 ± 0.063	-0.158 ± 0.140
SF14	9.7 ± 0.9	3.7 ± 0.3	2.1 ± 0.2	0.619 ± 0.119	0.498 ± 0.073	-0.235 ± 0.140
Pilbara	3.8 ± 0.3	2.2 ± 0.1	1.8 ± 0.1	0.600 ± 0.029	0.565 ± 0.027	-0.558 ± 0.034

Table 3.4 Allelic variability for different nuclear markers for a subset of 49 Pilbara Ghost Bats.

	N	Na	Ne	Ho	He	F
GB18	16.5 ± 5.5	4 ± 1	2.45 ± 0.4	0.568 ± 0.159	0.6 ± 0.064	-0.02 ± 0.39
GB20	19.5 ± 2.5	5.5 ± 1.5	3.04 ± 1.21	0.658 ± 0.07	0.626 ± 0.157	-0.12 ± 0.17
GB33	20.5 ± 10.5	7.5 ± 4.5	4.35 ± 2.15	0.839 ± 0.161	0.717 ± 0.143	-0.32 ± 0.52
GB42	25 ± 7	5.5 ± 3.5	3.57 ± 1.57	0.764 ± 0.014	0.666 ± 0.152	-0.24 ± 0.31
GB44	24.5 ± 6.5	3.5 ± 2.5	1.34 ± 0.34	0.161 ± 0.161	0.206 ± 0.206	0.2 ± 0.21
GB81	19 ± 11	3 ± 1	2.48 ± 0.48	0.625 ± 0.125	0.603 ± 0.07	-0.13 ± 0.37
gigas01	20.5 ± 10.5	7 ± 5	3.78 ± 2.18	0.605 ± 0.105	0.62 ± 0.226	-0.09 ± 0.24
gigas06	13.5 ± 4.5	4 ± 2	2.84 ± 1.16	0.722 ± 0.167	0.598 ± 0.173	-0.28 ± 0.1
gigas10	24 ± 6	4.5 ± 1.5	2.49 ± 1.37	0.472 ± 0.361	0.432 ± 0.322	-0.08 ± 0.04
gigas11	22.5 ± 8.5	6 ± 3	3.59 ± 1.31	0.776 ± 0.062	0.696 ± 0.114	-0.16 ± 0.11

n, number of individuals genotypes; $N_{A,}$ number of alleles (standardised for sample size); H_o, observed heterozygosity; H_E, expected heterozygosity; given as mean ± standard error.

3.1.2 Descriptive statistics and population genetic 'health'

A total of 98 Ghost Bat samples were scored at 10 highly variable microsatellite loci. All the sample groups were polymorphic at all loci with moderate variation, containing between 4 and 7 alleles per locus (5.10 ± 0.7 S.E.) with heterozygosity (H_E) ranging from 20 to 70% (mean = 0.57 ± 0.05; Table 3.2).

3.1.3 Detection of recent and long-term bottlenecks

The Hamersley population showed no evidence of either a recent (P > 0.9965; Table 3.5) or a long-term bottleneck (e.g. allelic diversity, heterozygosity; Table 3.5), suggesting that the population does not appear to have experienced reductions in numbers (i.e. bottlenecks). This is in contrast to findings from many small populations, particularly those that are isolates, such as those on islands.

Table 3.5 The detection of a genetic bottleneck was not found in the Ghost Bat population from the Pilbara.

Inferred population	**Significance (*P*-value)**	**Shifted mode**	**Genetic bottleneck**
Pilbara	0.9965	Normal	No

3.1.4 How unique are the Ghost Bat Caves?

No particular cave was identified as unique, as all had nearly identical levels of diversity with heterozygosity, for instance, varying between 55-65% (Table 3.3) , the average for the Hamersley being 60.0 ± 2.9%.

3.1.5 Localised dispersal: Spatial autocorrelation

Ghost Bats showed a significant spatial structure (p=0.01, Fig 4). Nevertheless, no statistical difference could be assessed between the two genders as we currently do not have the information on individual sex. The *r* value intercepts zero between 10 and 15 km (Figure 3.4), suggesting this is their neighbour size (or dispersal distance). In support of this, the mean area-span (based on sampling sites) was approximately the same distance.

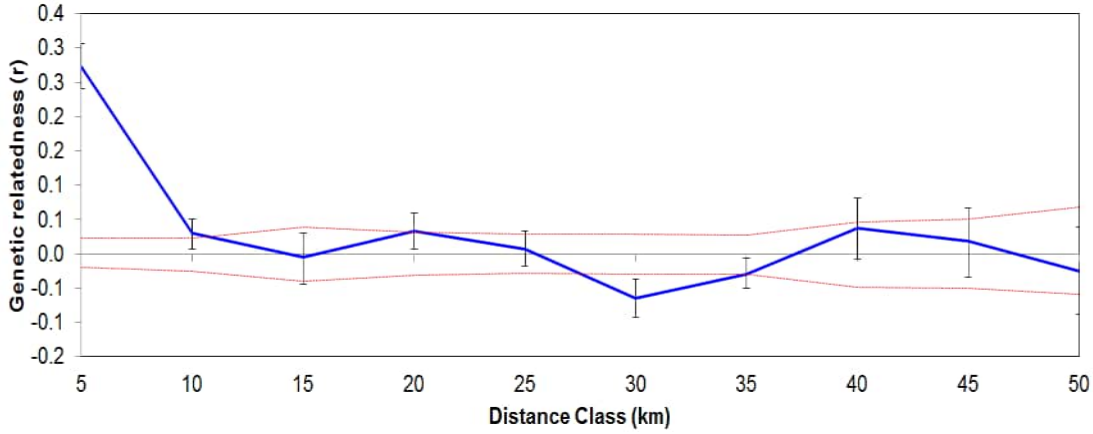


Figure 3.4. Multilocus spatial autocorrelation analyses for Ghost Bats showing that the relatedness decrease with increasing distance up to 50 km. Data points are correlation coefficient values (*r*) of the genetic distance between northern Ghost Bats within each distance class (0-5 km, 5-10 km, 10-15 km etc.).

4 Discussion

4.1 Summary

This study is the first to document any population genetic information from the Hamersley Ghost Bats, and has provided valuable information on the genetic structure and performance of this species in this part of the Pilbara. The Ghost Bats from the Hamersley subregion (and surrounds) represent a large and genetically diverse population of bats. Genetically, Ghost Bats in the Hamersley subregion are from a single large genetic population. In the main population, Ghost Bats retain high levels of genetic diversity, show no indication of exhibiting a genetic bottleneck, and suggest substantial gene flow amongst the sites. The initial analysis suggests dispersal of Ghost Bats (determined from neighbor size) of somewhere between 10 and 15 km. This result is consistent with the finding of a single large genetic population.

Summary of outcomes.

Source and collate existing genetic material.

Tissue and specimens of Ghost Bats were sourced from field site collections by Biologic and the WAM (4 only, and all in poor condition). Additionally, we have optimised DNA extraction protocols for the extraction of DNA from faecal material. Overall, this project has been remarkably successful as previous studies have only amplified a fraction of the faecal material (<40% success).

Optimise and generate DNA profiles

We optimised 10 polymorphic nuclear (microsatellite) regions of the DNA.

Preliminary population genetics analysis. Use the available genetic profiles to infer;

a. The size of the population.

The study identified a single genetic population from the 98 samples available. The population occupies a vast area that ranged from Pannawonica to Newman, covering

much of the Hamersley subregion, and essentially included all the samples. See point C for estimates.

b. Infer dispersal ability based on genetic estimators (spatial autocorrelation and neighbor-size)

The spatial autocorrelation analysis identified a positive relationship between genetic relatedness and distance. The analysis identified a neighborhood size, or dispersal distance of somewhere between 10 and 15 km. This suggests that bats would be capable of moving distances of up to 15 km. The analysis was not able to identify if there were any sex-bias in movement, but this will be considered as more information becomes available.

c. Estimate the genetic effective population size, and minimum number of bats genotyped.

Two estimates were generated. The minimum number of individuals from the main population was 98, from 21 sites. The second estimate was defined as the effective population size. The Hamersley population was estimated to be 78.6; however, these estimates need to be viewed with some caution because of the limited sample size. The effective population size is generally considered to be only 10% of the census population size, therefore the census size of Ghost Bats over the study area would be in the range of 700-800.

d. Develop a molecular method for determining sex of Ghost Bats using faecal material.

We attempted to identify sex using a molecular method, but have so far been unsuccessful. This is being pursued using alternate primer/molecular combinations.

e. Implement this method to assign sex to scat samples.

See above. This analysis will be done when more information is available.

f. Determine variation in the use of caves by males and females (from genetic studies), by comparing the relatedness of individuals occurring within and between caves at the South Flank site

See above. This analysis will be done when more information is available.

g. Additional analysis will be used to investigate population-level questions including; identifying habitat with high genetic diversity and investigating whether any population(s) have been through genetic bottlenecks.

Initial analysis identified that the Hamersley population retains high levels of genetic diversity. The caves within the study area showed remarkably similar levels of diversity (Table 3.5). There was no detectible genetic bottlenecks (P>0.99) in any populations.

Table 3.5 Measures of microsatellite variability of Ghost Bats from different caves (and containing more than five unique individuals). $N_{A,}$ number of alleles; H_E, expected heterozygosity; SE, standard error;

Roost / Cave	No. of Alleles (± SE)	Heterozygosity (± SE)	F(± SE)
AC1	3.7 ± 0.6	0.549 ± 0.101	-0.107 ± 0.110
ACW01	2.2 ± 0.2	0.655 ± 0.146	-0.527 ± 0.215
M1	4.5 ± 0.6	0.616 ± 0.090	-0.034 ± 0.048
SF08	3.1 ± 0.5	0.572 ± 0.093	-0.220 ± 0.102
SF1	3.7 ± 0.3	0.645 ± 0.113	-0.158 ± 0.140
SF14	3.7 ± 0.3	0.619 ± 0.119	-0.235 ± 0.140
Pilbara	2.2 ± 0.1	0.600 ± 0.029	-0.558 ± 0.034

Limitations

The study needs to be interpreted with some caution due to the limited dataset that was generated. The study initially used 324 samples (see figure 4.1), of which 19 were tissue (e.g. wing membrane) and 305 samples extracted from scats. Samples were included in the study

on the basis that at least five loci were generated, allowing a probability of >0.99 individual confidence in assigning individuals (based on probability of identify statistics).

Samples were removed from the dataset due to a number of reasons, including three samples that failed to amplify anything, 78 samples failed at five or more loci (24%) and 112 samples that were identified as originating from an already genotyped sample (i.e. duplicated genotypes). The three samples from the Kimberley (WAM specimens) were also not used in any analysis. Tissue samples remain the preferred starting template; however, the study, should it continue, could use faecal material, although the costs are higher for this approach.

Overall, this project has been remarkably successful, as previous studies have only amplified a fraction of the faecal material (<40% success).

The generation of only 98 individuals makes the interpretation of the data somewhat limited, particularly at the scale used (the Hamersley subregion).

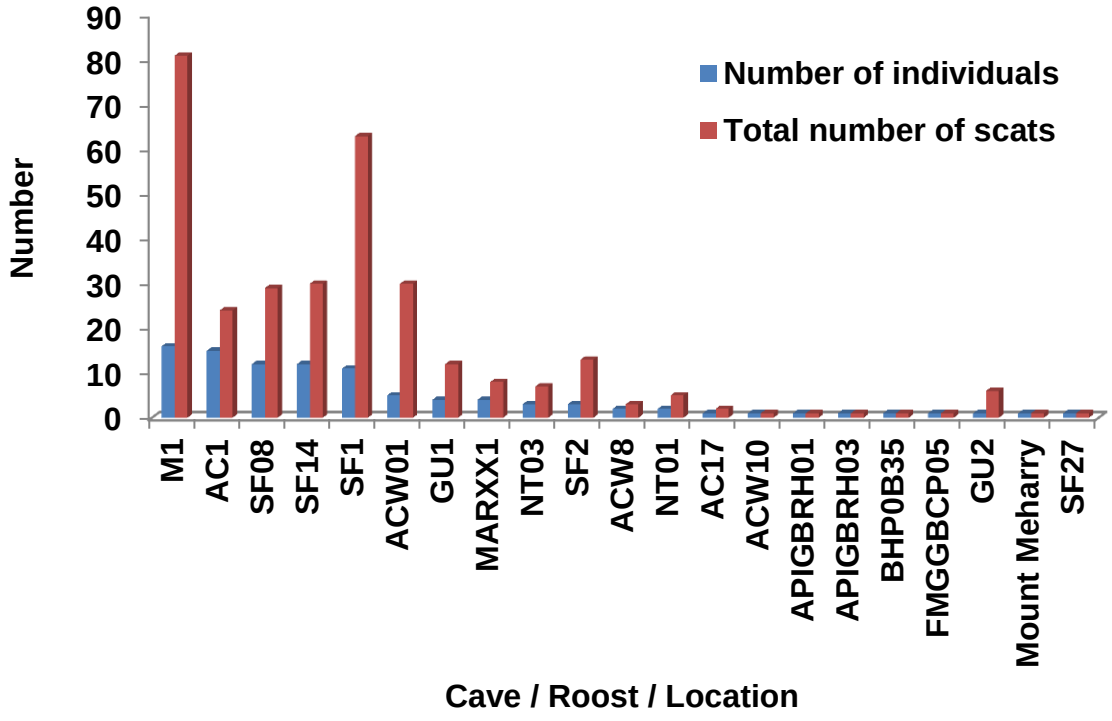


Figure 4.1: Number of samples analysed and individuals identified from each cave.

5. References

Cavalli-Sforza L.L. & Edwards A.W. 1967. Phylogenetic analysis. models and estimation procedures. *American Journal of Human Genetics* **19(3),** 233-257.

Churchill S.K. and Helman P.M. 1990. Distribution of the ghost bat, *Macroderma gigas*, (Chiroptera: Megadermatidae) in central and south Australia. Australian Mammalogy 13, 149–156.

Hall L., Richards G.C., McKenzie N. L. and Dunlop N. 1997. The importance of abandoned mines as habitat for bats. pp. 326–334 in P. Hale and D. Lamb (Eds) Conservation Outside Nature Reserves. Centre for Conservation Biology, University of Queensland, Brisbane.

Hampton J.O., Spencer P.B.S., Alpers D.L., Twigg L.E., Woolnough A.P., Doust J., Higgs T. & Pluske J. 2004. Molecular techniques, wildlife management and the importance of genetic population structure and dispersal: a case study with feral pigs. *Journal of Applied Ecology* **41,** 735–743.

Kalinowski, S.T. 2006. HW-QuickCheck: an easy-to-use computer program for checking genotypes for agreement with Hardy–Weinberg expectations. *Molecular Ecology Notes*, **6**, 974 -979.

McKenzie, N. L., S. van Leeuwen, and A. M. Pinder. 2009. Introduction to the Pilbara Biodiversity Survey, 2002-2007 A Biodiversity Survey of the Pilbara region of Western Australia, 2002-2007. Records of the Western Australian Museum Supplement 78. p 3-89. Western Australian Museum, Perth.

Peakall R., Ruibal M. & Lindenmayer D.B. 2003 Spatial autocorrelation analysis offers new insights into gene flow in the Australian bush rat, *Rattus fuscipes*. *Evolution* **57,** 1182–1195.

Peakall, R. & Smouse, P.E. 2006. GenAlEx 6: genetic analysis in Excel. genetics software for teaching & research. *Molecular Ecology Notes*, **6**, 288-295.

Piry, S., Luikart, G. & Cornuet, J-.M. 1999. Bottleneck: A computer programme for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **90,** 502-503.

Smouse P.E. & Peakall R. 1999. Spatial autocorrelation analysis of multi-allele and multi-locus genetic microstructure. *Heredity* **82,** 561-573.

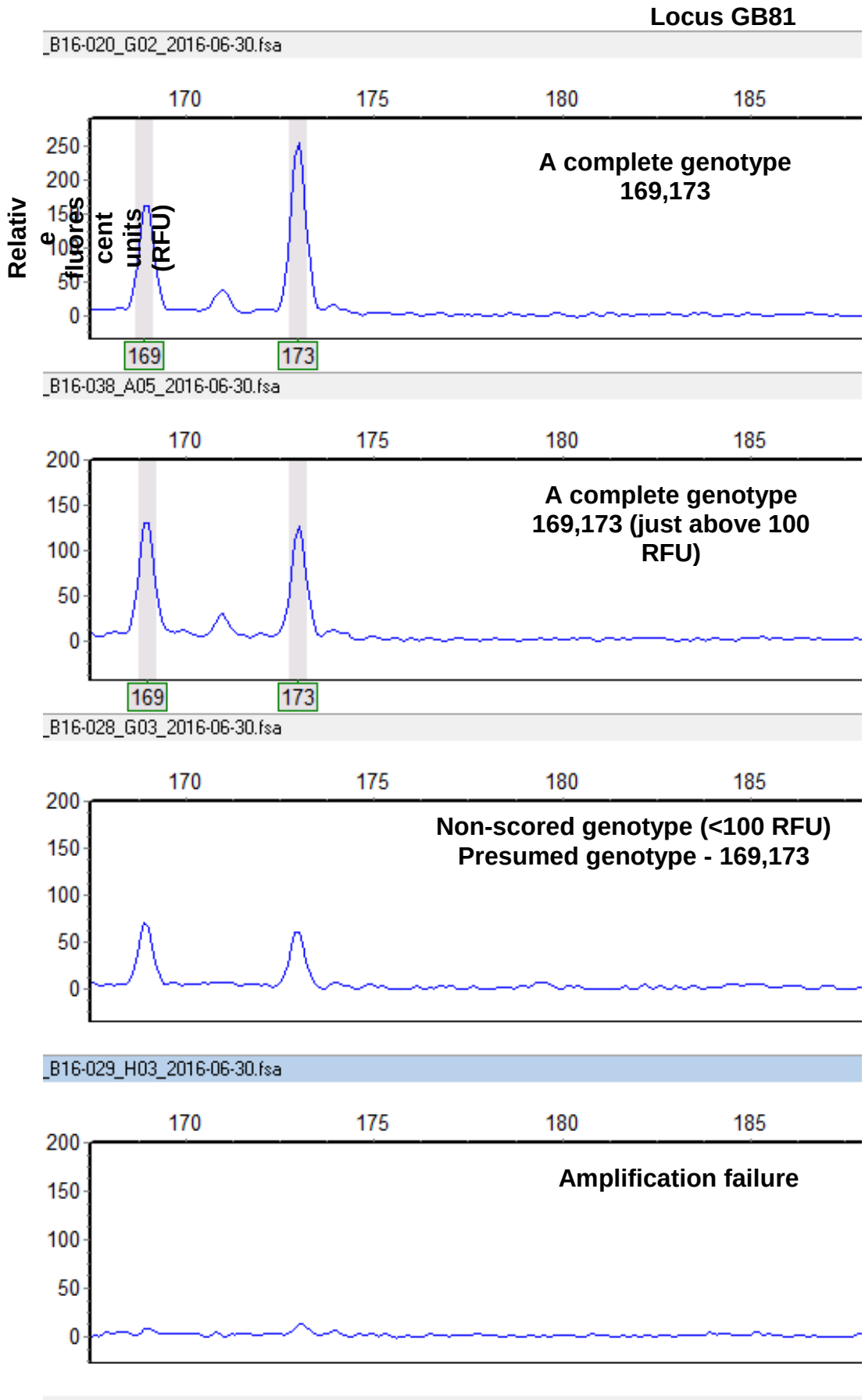
Toop G.J. 1979. Ghost bat studies: compiled reports to Australian National Parks and Wildlife Service, 1975–79. Australian National Parks and Wildlife Service, Canberra (unpublished).

Toop G.J. 1985. Habitat requirements, survival strategies and ecology of the ghost bat, *Macroderma gigas* Dobson, (Microchiroptera, Megadermatidae) in central coastal Queensland. Macroderma 1, 37–41.

Worthington-Wilmer J., Moritz C., Hall L. and Toop J. 1994. Extreme population structuring in the threatened Ghost Bat, *Macroderma gigas*: evidence from mitochondrial DNA. Proceedings of the Royal Society, London (1974) 257, 193–198.

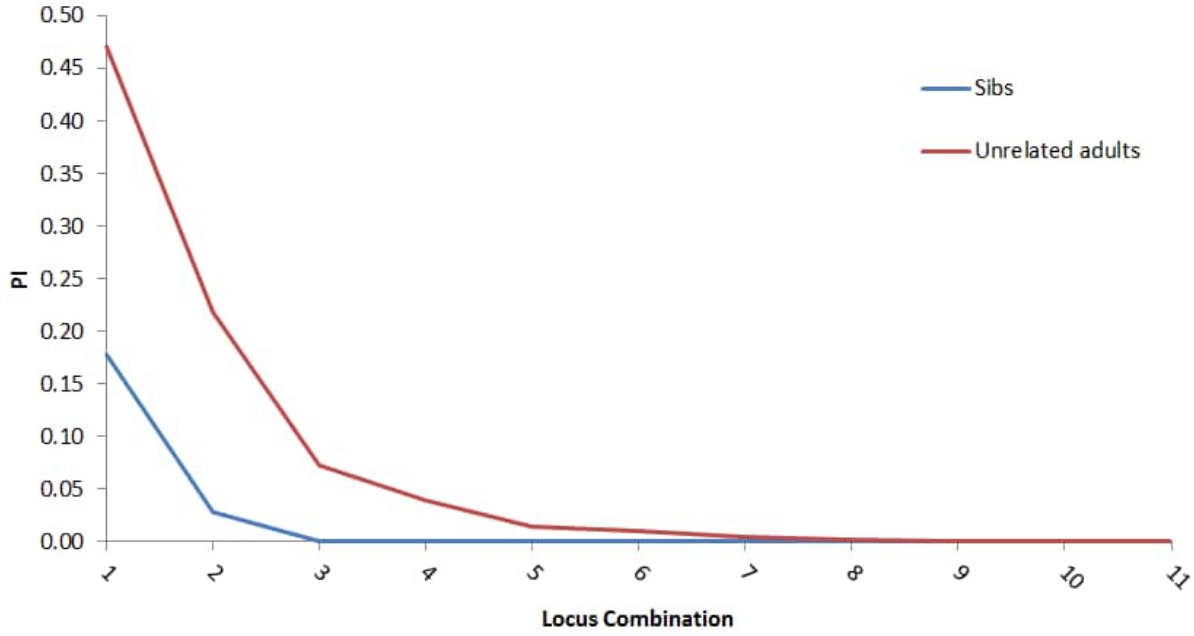
Criteria for excluding individuals / genotypes

1. If RFU on electropherogram was <100.



2. <5 loci amplified.

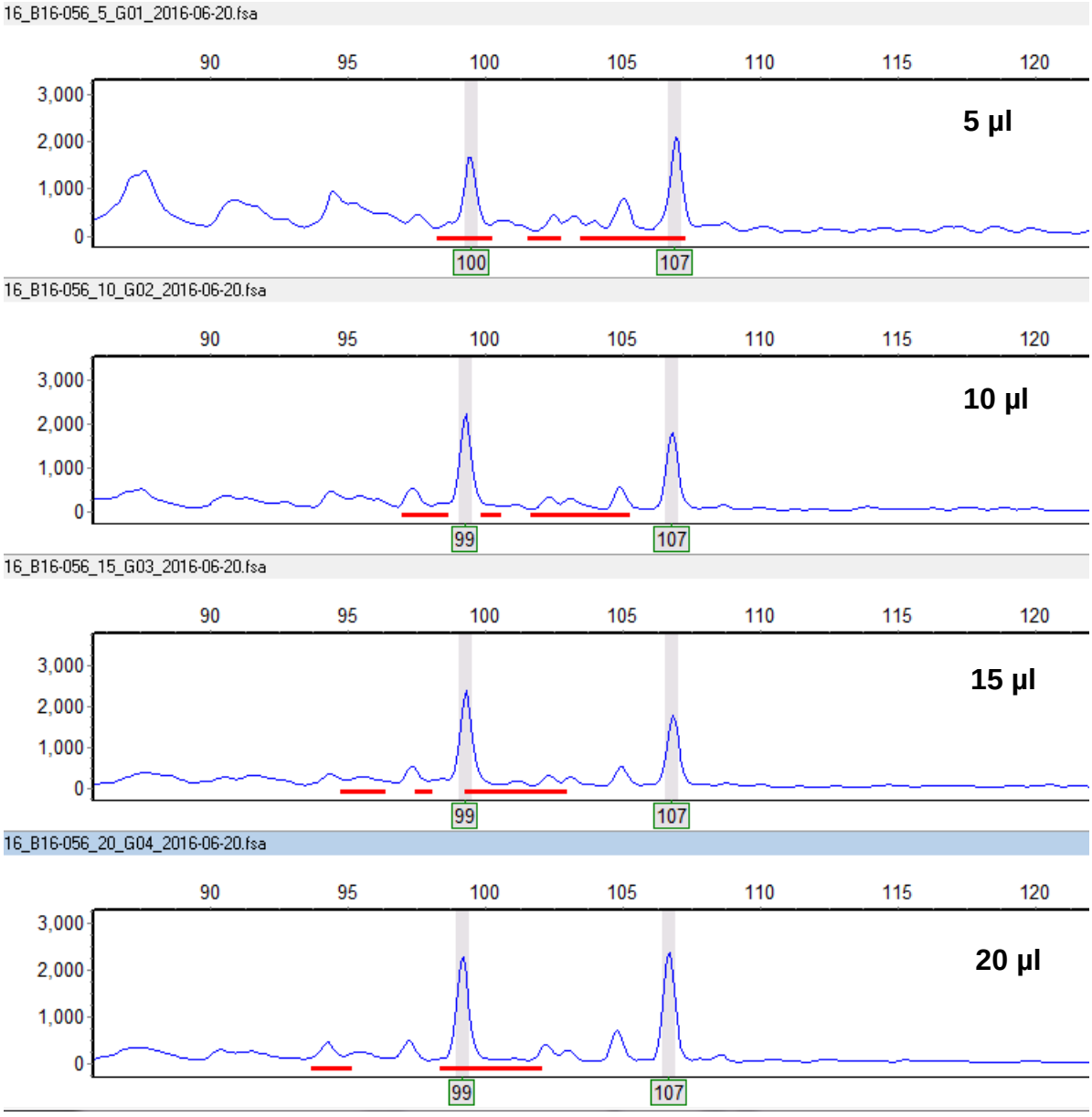
This was based on P_{ID} estimates. At 5 or more loci, there is a 99.999% chance of an individual sharing an identical genotype



# loci	Unrelated	No. of bats	Sibs	No. of bats
1	1.8E-01	6	4.7E-01	2
2	2.8E-02	35	2.2E-01	5
3	1.1E-03	900	7.3E-02	14
4	2.8E-04	3,589	3.9E-02	26
5	2.2E-05	45,586	1.5E-02	68
6	1.1E-05	90,250	1.1E-02	93
7	2.2E-06	448,049	5.2E-03	193
8	1.4E-07	7,084,933	1.9E-03	527
9	1.5E-08	66,892,161	7.7E-04	1,291
10	1.7E-09	604,581,690	3.2E-04	3,138
11	1.7E-10	5,870,283,219	1.3E-04	7,724

3. DNA concentration below 10 ng/µl

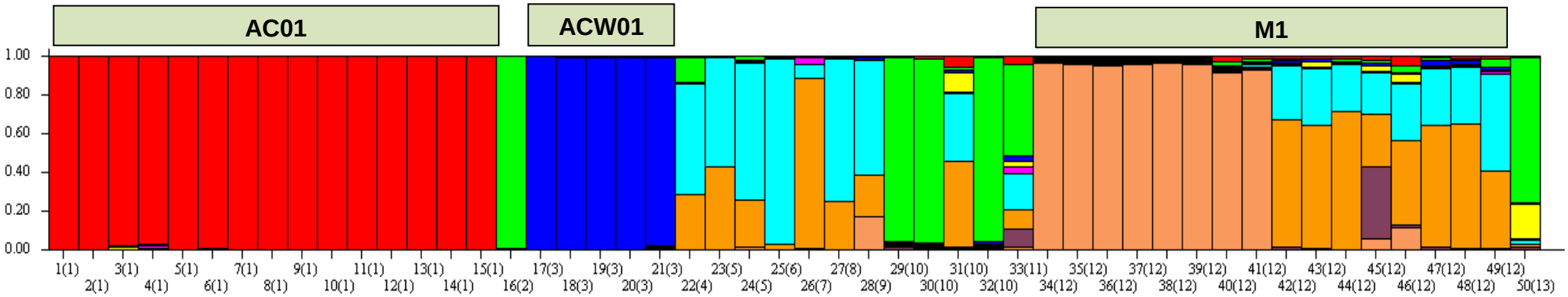
DNA was estimated using Nano drop technology to estimate concentration of DNA (ng/µl). A titration experiment was carried out on multiple samples (n=8) using 5, 10, 15 and 20 µl of starting template DNA in a PCR. We determined that a volume of 10 µl was a required minimum of template DNA required for amplification. We chose this particular volume (over 10 or 20 µl) because the elution volumes, from the extractions, were small, usually < 100 µl and most of the markers would not work in multiplex reactions together. A total of 9 PCRs were needed to amplify the 9 microsatellite loci, and as a result, we required DNA for all 9 of the reactions. Simply, 15 µl of DNA or more would yield better genotype scoring, but would prevent all markers being amplified in the 9 required PCRs.

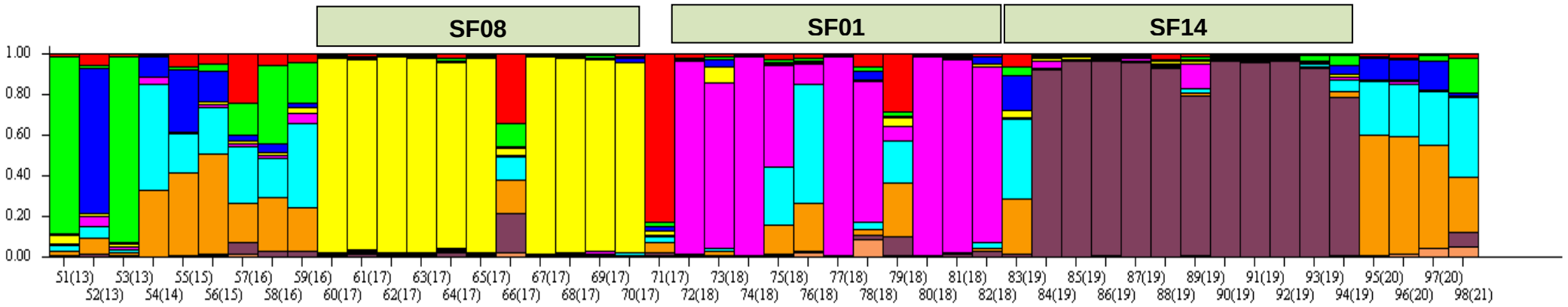


Figure, on following page

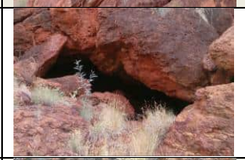
An assignment plot for each individual Ghost Bat, sorted according to cave that it was sampled in. The plot show the number of clusters to be K=9, generated from the STRUCTURE runs of 1,100,000 replications.

Cave Number	CAVE	No. bats	Individual numbers
1	AC1	**15**	**1 – 15**
2	AC17	**1**	**16**
3	ACW01	**5**	**17- 21**
4	ACW10	**1**	**22**
5	ACW8	**2**	**23, 24**
6	APIGBRH01	**1**	**25**
7	APIGBRH03	**1**	**26**
8	BHP0B35	**1**	**27**
9	FMGGBCP05	**1**	**28**
10	GU1	**4**	**29 – 31**
11	GU2	**2**	**32, 33**
12	M1	**16**	**34 – 49**
13	MARXX1	**4**	**50 – 53**
14	Mount Meharry	**1**	**54**
15	NT01	**2**	**55, 56**
16	NT03	**3**	**57 – 59**
17	SF08	**12**	**60 – 71**
18	SF1	**11**	**72 – 82**
19	SF14	**12**	**83 – 94**
20	SF2	**3**	**95 – 97**
21	SF27	**1**	**98**
	Grand Total	**98**	


AC01
ACW01
M1
1.00
0.80
0.60
0.40
0.20
0.00
1(1) 2(1) 3(1) 4(1) 5(1) 6(1) 7(1) 8(1) 9(1) 10(1) 11(1) 12(1) 13(1) 14(1) 15(1) 16(2) 17(3) 18(3) 19(3) 20(3) 21(3) 22(4) 23(5) 24(5) 25(6) 26(7) 27(8) 28(9) 29(10) 30(10) 31(10) 32(10) 33(11) 34(12) 35(12) 36(12) 37(12) 38(12) 39(12) 40(12) 41(12) 42(12) 43(12) 44(12) 45(12) 46(12) 47(12) 48(12) 49(12) 50(13)


SF08
SF01
SF14
1.00
0.80
0.60
0.40
0.20
0.00
51(13) 52(13) 53(13) 54(14) 55(15) 56(15) 57(16) 58(16) 59(16) 60(17) 61(17) 62(17) 63(17) 64(17) 65(17) 66(17) 67(17) 68(17) 69(17) 70(17) 71(17) 72(18) 73(18) 74(18) 75(18) 76(18) 77(18) 78(18) 79(18) 80(18) 81(18) 82(18) 83(19) 84(19) 85(19) 86(19) 87(19) 88(19) 89(19) 90(19) 91(19) 92(19) 93(19) 94(19) 95(20) 96(20) 97(20) 98(21)

Appendix C – Caves recorded during the targeted searches

X	Y	Cave ID	Date	Cave Type	Cave position	Floor slope	Quadrant B	Cave exposure	Water	Entrance	Entrance width	Entrance height	Cave depth	Number chambers	Height chamber	Ghost bat scats	Ghost bat scat age	significance	Photo
119.1465	22.8309	MN01	21/04/2016	Cavern, Cavity	Upper Slope	Flat	West	Semi Exposed	None	Round/Oval	4	2	40	3	1.3	No Scats	No Scats	Night Roost	
118.5948	22.7999	NT01	23/04/2016	Cavern	Mid Slope	Flat	South	Semi Exposed	None	Round/Oval	20	5	40	1	4	1 to 5	Recent (1 to 6mths),Old (6mths to 3yrs)	Night Roost, Day Roost	
118.5928	22.7388	NT02	24/04/2016	Cavity	Upper Slope	Flat	South	Sheltered	None	Horizontal	5	2	30	1	1	No Scats	No Scats	Night Roost	
118.625	22.7415	NT03	24/04/2016	Cavity	Mid Slope	Incline	North/East	Sheltered	None	Horizontal	4	2	25	1	2.5	6 to 20	Old (6mths to 3yrs)	Night Roost	
118.626	22.7464	NT04	24/04/2016	Cavity	Mid Slope	Flat	South/East	Sheltered	None	Round/Oval, Horizontal	3	1	15	1	2	No Scats	No Scats	Night Roost	
119.0301	23.1908	GU02	25/04/2016	Cavity	Mid Slope	Incline	North/ East	Sheltered	None	Vertical	0.3	2	30	1	3	6 to 20	Recent (1 to 6mths),Old (6mths to 3yrs)	Day Roost	
118.9666	23.2095	GU01	25/04/2016	Cavity	Mid Slope	Incline	North/ East	Sheltered	None	Vertical	2	1	40	2	3	1001 plus	Fresh (<1mth),Recent (1 to 6mths),Old (6mths to 3yrs),Very Old (3 to 10yrs),Ancient (>10yrs)	Potential Maternity	
118.9505	23.2156	GU03	26/04/2016	Cavern	Mid Slope	Incline	East	Sheltered	None	Round/Oval	2	1.2	25	1	1.2	No Scats	No Scats	No Usage	Unavailable
119.1164	22.8145	MN02	20/10/2016	Cavity, Cavern	Lower Slope	Incline	East	Sheltered	None	Round/Oval	2	1.5	35	2	3.5	No Scats	No Scats	Unknown