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Tracking and tracing central Queensland's *Macroderma* – determining the size of the Mount Etna ghost bat population and potential threats

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Abstract. The ghost bat, *Macroderma gigas*, colony at Mount Etna was at the centre of Australia's longest-running conservation campaign. To protect the colony the Queensland Government removed recreational facilities and gated caves. The size and genetic diversity of the Mount Etna *M. gigas* population were estimated using cave searches, direct captures and molecular analysis to determine whether these actions have benefitted the species. In addition, telemetry was undertaken and red fox, *Vulpes vulpes*, scats analysed to identify possible threats. Results suggest that the population has declined by 79% since the late 1990s, has low microsatellite diversity, low effective population size (*Ne*) and is undergoing a population bottleneck. VHF- and GPS-collared animals were found to forage over agricultural land up to 11.8 km from their daytime roost, suggesting that poor land management and barbed-wire fences could be potential threats. No ghost bat remains were found in fox scats. We recommend that compliance be increased around Johansen's Cave to reduce disturbance during the maternity season and landholders be encouraged to undertake management that is sympathetic to ghost bats.

Additional keywords: *Macroderma gigas*, Megadermatidae, molecular analysis, population estimate, telemetry, threats.

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Introduction

Macroderma gigas is the largest carnivorous bat in Australia and is a predator of small mammals, reptiles, frogs, birds and insects (Richards *et al*. 2008). Prey is detected using echolocation, vision and/or hearing without echolocation (Richards *et al*. 2008). The species is listed as Endangered under the Queensland *Nature Conservation Act 1992* and Vulnerable under the Australian *Environmental Protection and Biodiversity Conservation Act 1999*.

The *M. gigas* population in the Mount Etna Caves National Park and surrounds (hereafter referred to as the Mount Etna population) in central Queensland is at the southern end of the species' range and is considered of conservation significance (Woinarski *et al*. 2014) (Fig. 1).

Genetic studies have shown that *M. gigas* is divided into genetically distinct subpopulations at both regional and local scales (Worthington Wilmer *et al*. 1994, 1999; Worthington Wilmer 1996, 2012). This is suggestive of a highly fragmented distribution with limited dispersal and interchange. The lack of migration between populations, particularly of females (Worthington Wilmer *et al*. 1994), is thought to increase the chance of localised extinction. Although *M. gigas* colonies are present throughout northern Australia, the species also once occurred in arid central Australia (Hand and York 1990) and the range is thought to be contracting northwards (Molnar *et al*. 1984).

Worthington Wilmer *et al*. (1999) found that the Mount Etna population had lower microsatellite diversity compared with Pine Creek, Northern Territory, the largest known *M. gigas* colony in Australia. They suggested that this could be due to differences in historical effective population sizes (*Ne*) particularly if the Mount Etna population had undergone sustained periods of small population size. Although the Mount Etna population was once regarded as one of the largest in Australia, (Nelson 1989) it is possible that mining and land use in the surrounding area has reduced the population size. Tidemann *et al*. (1985) reported that *M. gigas* usually foraged up to 2 km from their daytime roosts and suggested that the species was particularly vulnerable to land modification and degradation adjacent to roost sites.

At Mount Etna, semi-evergreen vine thickets dominate the vegetation surrounding the roost sites and this gradually gives way to more open eucalypt woodlands with a shrubby to grassy understorey. These vegetation communities are infested with weeds, including *Lantana camara* (lantana), *Leucaena*

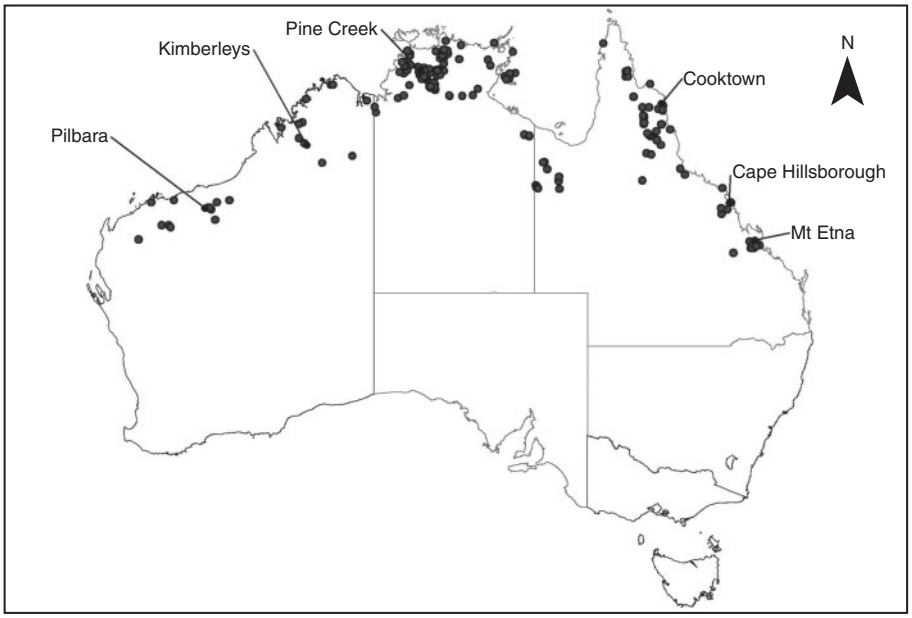

Fig. 1. Present (since 1970) distribution of *M. gigas* in Australia based on records contained in the Queensland Government's WildNet database (DSITI 2015) and the Atlas of Living Australia (www.ala.org.au, accessed 7 November 2016). The dots represent sightings of *M. gigas*.

leucocephala (leucaena), *Cryptostegia grandiflora* (rubber vine) and exotic grass species such as *Megathyrsus maximus* (guinea grass) and *Pennisetum* sp. (foxtail grass). Weeds have the potential to restrict flyways and reduce access to foraging areas (Woinarski *et al*. 2014). Further away from the caves, the vegetation has mostly been cleared for agriculture and is dominated by grass. Being carnivores, the ghost bats are at risk from a loss of prey species caused by land degradation (clearing, overgrazing and fragmentation), and competition and possibly predation from feral carnivores (foxes and cats) (Woinarski *et al*. 2014). *M. gigas* are also believed to forage close to the ground, putting them at risk of becoming entangled in barbed-wire fences (Armstrong and Anstee 2000; Woinarski *et al*. 2014). Advances in telemetry equipment may help our understanding of some of the potential threats that the species faces locally.

Hoyle *et al*. (2001) estimated annual adult survival rates for the Mount Etna population of 0.57–0.77 for females, 0.43–0.66 for males and 0.35–0.46 for juvenile females and 0.29–0.42 for juvenile males. Based on these low survival rates, the average age of the population being just five years, delayed sexual maturity (females commence breeding in their second year) and low fecundity, Toop and Davie (unpubl. data) suggested that the Mount Etna population only just achieved population replacement. Hence any reduction in the number of mature females or the survival rate of young would have severe effects on the persistence of the population.

Fossil evidence suggests that *M. gigas* has been present in the Mount Etna area since the mid-Pleistocene (500–280 thousand years ago) (Hocknull 2000; Martinez 2010) and has survived previous climate change events, including more arid periods when more typically desert species were present in the area (Hocknull 2000; Hocknull *et al*. 2007).

The Mount Etna population was estimated to be ~450 bats in the mid-1960s (McKean and Price 1967) and 150–180 bats in the mid-1970s to early 1990s (Toop 1985; Toop and Davie, unpubl. data). Hoyle *et al*. (2001) concluded, on the basis of capture–mark–recapture data obtained between 1975 and 1980, that the population was stable at ~150 bats.

Some of the methods used in earlier studies to estimate population size are no longer considered appropriate – in particular cave searches during the maternity season or winter because of the risk of disturbance to the species. Alternative methods that indirectly survey the population are needed. Sampling the DNA found in bat scats is one such method that offers potential but it has limitations including the rapid deterioration of the DNA (Smith *et al*. 2009), the costs of processing the samples and the high levels of PCR inhibitors jointly isolated within extracts (Morin *et al*. 2001). These limitations need to be overcome for the method to be viable.

Being at the southern end of the species' range, it is thought that *M. gigas* in the Mount Etna area requires caves with specific structural and microclimatic conditions (Toop 1979, 1985). Of the ~180 caves in the Mount Etna area, only 21 are known to be used by *M. gigas* (Toop 1979). Mating usually occurs in April to early May (Toop and Davie, unpubl. data) and pregnant females (sometimes with males) overwinter in eight of the 21 known *M. gigas* caves (Toop and Davie, unpubl. data). Birth caves (after 22 weeks gestation) are also rare (four caves) as few are within the acceptable temperature range of 23−26°C during mid-October (Toop 1985). Young are initially carried by their mothers but as they become too heavy they are left in suitable roosts at night (Douglas 1967). At approximately seven weeks, young accompany their mothers during short foraging trips (Toop and Davie, unpubl. data). Young are particularly vulnerable to human disturbance early in the maternity season as they can be

dislodged by the fleeing adults and are unable to climb back to the roost (Toop 1979, 1985). Adult males usually congregate with the females and young in Johansen's Cave towards the end of summer, as it is one of the few dry wet-season caves in the area (Toop 1979).

The Mount Etna population was at the centre of Australia's longest running (1966–92) conservation campaign to prevent the mining of limestone for cement (Scott 1989). Two caves destroyed during the mining – Elephant Hole and Speaking Tube Caves – were considered important for giving birth and/or overwintering. Their destruction is thought to have increased the species' reliance on Johansen's Cave, as the area's most important maternity and overwintering cave. As a result of the increased importance of Johansen's Cave, the Queensland Parks and Wildlife Service (QPWS) removed recreational facilities at the cave entrance and closed the cave seasonally to restrict human visitation (QPWS 2013). This study was initiated, in part, to determine whether these management actions have benefitted the *M. gigas* population and to improve our understanding of some of the threats that the species may face locally. This study also aimed to provide information that may assist with improving the feasibility of using DNA present in faecal samples to survey *M. gigas* populations in the future.

Materials and methods

Cave searches

All 21 caves known to be used by *M. gigas* within the Mount Etna area, and an additional three caves not known to be used, were searched for *M. gigas* and/or their scats. The timing of searches was based on historical cave usage data (Toop 1979). All maternity caves were searched in the later part of the maternity season (November–December) to minimise disturbance. The caves and dates of searches are listed in Table S1 (available as supplementary material to this paper). A search involved the method of Hoyle *et al*. (2001), which consisted of walking through the caves listening for calls and searching the roof with a torch for bats and the floor for scats. Known roost sites or avens within each cave were targeted in an attempt to increase the chance of species detection. These counts are considered incomplete (minimum number) due to the likelihood that not all bats were detected.

Trapping and sampling

Mist nets were erected between December and March (1830–2100 hours) at the entrances to Johansen's, Shuffle and Gigas Hall Caves to catch bats as they departed. This included a total of 45 nights at Johansen's Cave (30 nights in 2011/12 and 15 nights in 2012/13) and one night each at Shuffle Cave and Gigas Hall in 2011/12. Each captured bat was measured, weighed and microchipped (Trovan ID100 (1.25 mm × 7 mm) Nanotransponder). A tissue sample was also taken from the wing membrane using a 2–3 mm biopsy punch and stored in 70% ethanol for genetic analysis.

Molecular analysis of tissue

A total of 15 loci, four from the previous work of Worthington Wilmer *et al*. (1999) (GIGAS01, GIGAS06, GIGAS10 and GIGAS11) and nine developed during the project were used to genotype tissue samples. The marker library was developed using Ion Torrent next-generation sequencing methods. Total genomic DNA for library development was extracted using a Qiagen DNA Easy blood and tissue extraction kit (960 ng). The sequencing runs were performed on an ION PGM system using 314 chips, following the manufacturer's specifications. The resulting run returned 350 965 sequence reads. We used the QDD bioinformatics pipeline (ver. 2 β: Meglécz *et al*. 2010) to select microsatellites from the genomic dataset. QDD selected 5638 sequence reads that were longer than 80 base pairs and contained a unique microsatellite. Primer3 (Rozen and Skaletsky 2000) was used to design 2678 primers for the loci, of which 96 were tested focusing on the best available primer set and Category A and B primers (see QDD manual) (Meglécz *et al*. 2010).

Of 96 primer sets, 42 amplified – 25 of these were monomorphic and 17 were variable. However only 11 were amplified consistently and showed no evidence of null alleles (Table S2).

The DNA extractions from wing membrane were undertaken using CTAB buffer and phenol/chloroform methods (Doyle and Doyle 1987) but modified to 100 µL. Extracted DNA was precipitated with the addition of a coprecipitant (Bioline) to increase yield. Forward primers were modified to complement a fluorescent labelled tail (Real *et al*. 2009). PCRs were performed in a 10-µL reaction consisting of 1x buffer, 1.5 mM $MgCl_2$, 0.05 µM forward primer, 0.2 µM reverse primer and labelled tail, 0.2 mM dNTPS and 0.2 U Red Taq (Astral). Reactions were amplified at 53°C for 35 cycles. Amplified product was then combined into one of two pools and run on an ABI 3130 Genetic Fragment Analyser. Alleles were scored using GeneMapper 3.1 software (ABI). The results were checked for scoring errors and null alleles with Micro-Checker (Van Oosterhout *et al*. 2004) before analysis. Heterozygosity measures were estimated with Genalex 6.41 (Peakall and Smouse 2006) and allelic richness was determined with HP-RARE 1.0 (Kalinowski 2005). This was done for all 15 loci including the four previously analysed by Worthington Wilmer *et al*. (1999). Heterozygosity measures were compared between the 1993 work and this study using a Bayesian *t*-test (Kruschke 2013). The effective population size was estimated with Colony 2 ($n = 19$) (Jones and Wang 2010). All samples had data for nine or more loci.

The program Bottleneck 1.2.02 (Piry *et al*. 1999) was used to investigate whether a recent population bottleneck had occurred in the Mount Etna population. The analysis used the 19 tissue samples collected in 2011/12. Given the anecdotal historical evidence that large numbers of individuals were killed in the early 20th century we also ran demographic simulations in BottleSim (Kuo and Janzen 2003), which has proved useful to confirm bottlenecks in fish stocks (Pinsky and Palumbi 2014). The simulations were used to estimate the size of the bottleneck, as a percentage of total population size, and the time since bottleneck that would be required to reduce diversity from that of a known large population (Pine Creek, Northern Territory: Worthington Wilmer *et al*. 1999) to that observed in this study. The input data for this simulation was produced with EasyPop 1.7 (Balloux 2001). The following parameters were used in BottleSim: diploid multilocus, constant population size, 100% generation overlap, dioecy with random mating, life span of five years (for which we relied on the data of Toop and Davie (unpubl. data)), sexual

maturity at two years, population size in bottleneck of 0, 25, 50, 75, 90 and 95% of initial population size of 1000, with 50% females and run for 200 years. Trial runs demonstrated that the results were insensitive to the initial population size, only the relative size of the bottleneck.

To investigate changes in the connectivity between populations at Cape Hillsborough and Mount Etna, we compared two alternative migration models (the two sites were modelled as two separate populations or as a panmictic system) conducting coalescent-based analysis as implemented in the software Migrate-n (Beerli 2006) ($n = 19$ Mount Etna, and $n = 12$ Cape Hillsborough). Model comparison was carried out using the log Bayes Factor (LBF) calculated with the Bezier marginal likelihoods obtained by thermodynamic integration (Beerli and Palczewski 2010). Furthermore, once the migration model was selected, we calculated the extent of the bottleneck by estimating demographic changes over time with the Bayesian Skyline Plot. For these analyses, we used a truncated exponential prior (Theta (Theta $= 4 \times Ne \times \mu$, where Ne is the effective population size and μ is the mutation rate): mean = 150, maximum = 500) and ran four replicates with a MCMC of 200 million steps using the slice sampler. Convergence and adequate effective sample size were assessed with the R package *mtraceR* (https://github.com/carlopacioni/mtraceR, accessed 7 August 2017), which was also used to generate the final plot.

We calculated the percentage decline using the mean theta estimation divided by the theta estimation at $2 \times Ne$ before present. The possible range of the decline was calculated by adding (or subtracting) 1.96 × standard deviation to the mean theta estimation. While for nuclear data the time to the most common ancestor is $4 \times Ne$ (Hotz *et al.* 2013), to take into account the uncertainty and the possible variation between loci, we used $2 \times Ne$ as a conservative reference point (Pacioni *et al.* 2015) because coalescent events become sparse close to the root of the tree and therefore the parameter estimation is less accurate (Gill *et al.* 2013; Drummond and Bouckaert 2015).

Arlequin (Excoffier and Lischer 2010) was used to estimate F_{ST} values between Cape Hillsborough and the Mount Etna sample. Evidence for significant deviations from panmixia was tested by 10 000 permutations of individuals among populations. Evidence for population structure was further tested using the program STRUCTURE (Pritchard *et al.* 2000). This program groups individuals on the basis of Hardy–Weinberg Equilibrium and linkage equilibrium. The analysis was run assuming the admixture model and was run four times for each possible number of populations from K = 1 to 4. The most likely number of populations was estimated using the Evanno method in STRUCTURE HARVESTER (Evanno *et al.* 2005).

Molecular analysis of scats

To determine the feasibility of using scats rather than live sampling, 37 scats were collected from Johansen's Cave in March 2013 and 19 from Shuffle Cave in January 2015. After collection, the scats were kept frozen until the DNA could be extracted. DNA from faecal samples was extracted using a QIAamp DNA stool kit (Qiagen) and genotyped for the same 15 loci: GB18, GB20, GB33, GB39, GB42, GB44, GB45, GB46, GB57, GB81, GIGAS01, GIGAS06, GIGAS10, GIGAS11 and GB21. Faecal PCR conditions were the same as for the wing membrane DNA except for the addition of 0.1 μg μL^{-1} of bovine serum albumin in the reaction. Positive and negative controls were used for each reaction to ensure there was no contamination. Duplicates were run for a small number of samples for which there was sufficient DNA. This resulted in all loci being duplicated in at least two individuals. All loci were tested for conformance to Hardy–Weinberg expectations in GenePop 4.2 (Tallmon *et al.* 2008).

Samples were identified by comparing genotypes between known individuals (19 from the first sampling year and 10 from the second year) and the faecal samples using the identity analysis function in Cervus 3.0.3 (Kalinowski *et al.* 2007). Samples were scored as a match only if they matched on all loci and had at least seven loci scored for the scat sample. For this analysis, which included tissue and scats, only 10 loci were included as five had low amplification success in the scat samples. The loci used were GB42, GB81, GB57, GB33, GB45, GB39, GIGAS10, GIGAS11, GIGAS06, and GIGAS01.

Radio-telemetry and GPS tracking

VHF radio-transmitters, weighing 1.5 g, were attached to three bats (two females and a male) and their movements were tracked for three weeks commencing on 12 March 2012. The transmitters were attached, according to the method of Tidemann *et al.* (1985), using perma-type surgical cement, to the dorsal surface in such a way that they would fall off naturally after a few weeks. The location of the three bats was determined through both triangulation and biangulation (where the difference in the bearing between the two stations was greater than 90°) of bearings obtained from two or more separate but simultaneously operated tracking stations. Two of the three tracking stations were elevated on a nearby hill and one was mounted on a vehicle (so was mobile). A clock was used at each tracking station to ensure bearings were recorded at the same time. A new bearing was obtained every 10 min. The bearing information was converted to a point location using the Bearing Tool in ArcMap 10.

A GPS datalogger (Telemetry Solutions Australia), weighing 8 g, was attached to a single male bat, weighing 159 g, on 31 January 2013. This bat was recaptured six days later. The GPS datalogger was attached using a collar around the neck and glue (perma-type surgical cement) on the fur to prevent the datalogger slipping around to the ventral surface where a signal would not be detected when the bat was flying. The collar consisted of a small (2 mm diameter) clear tube with a piece of trace tied together with cotton to act as a weak link that would break if the bat could not be recaptured. A black tube with a larger diameter (3–3.5 mm) then telescoped around the ends of the clear tube so that the trace and cotton were completely enclosed in plastic tubing. One end of the black plastic tube was glued to the clear tube. The other end was left unglued so that moisture could enter the tubing and cause the cotton to disintegrate over time. The GPS was programmed to obtain a fix every hour between 1930 and 0430 hours (sunrise and sunset at this time of year was 0536 and 1846 hours, respectively: www.timeanddate.com, accessed 7 August 2017). A total of 36 fixes was obtained over six nights. ArcMap 10 was used to determine the height above ground for each GPS point by subtracting the height above sea level from the collar's altitude.

Potential predator scat hair analysis

Twenty-seven fox scats were collected from Gigas Hall Cave in October 2013 and sent to 'Scats About' (www.scatsabout.com.au, accessed 7 August 2017) for analysis. Feral cats (*Felis catus*) were observed in the vicinity of the *M. gigas* caves but no cat scats were found.

Temperature data

The daily minimum temperature for each month and the long-term monthly average daily minimum temperature for the winter months (June–August) were obtained from the Bureau of Meteorology (BOM 2017). The monthly average-minimum temperature is the average of all available daily minima for the month. The daily minimum temperature is nominally recorded at 0900 hours (local time) and is the lowest temperature for the 24 h leading up to the observation, recorded as the minimum temperature for the previous day (BOM 2017). A below-average winter was deemed to be a winter in which the average-minimum daily temperature for one of the winter months was less than the longer-term average monthly minimum daily temperature (in °C).

Results

Cave searches

A maximum of 38 *M. gigas* was observed on the same day (21 December 2011) at Johansen's (30 bats) and Gigas Hall (eight bats) Caves. Two of the Gigas Hall bats had young still attached. *M. gigas* were also recorded at other times in Shuffle, Old Timbers, Canyon and Capricorn Caves (Fig. 2) but we were unable to determine if these had already been tagged or were part of the 38 seen previously.

Trapping

A total of 64 *M. gigas* captures were made in 47 nights of trapping over several months (1.3 bats night^{-1}). This involved 35 individual *M. gigas* – 26 (10 females, 16 males) in 2011/12 and 20 (8 females, 12 male) in 2013. *M. gigas* were caught between 1910 and 2030 hours as they departed the cave to forage (~1–2.5 h after sunset). Average weights were 144.1 g (range: 103.5–170) for males and 141.5 g (range: 94–172) for females. Average tibia length for males was 49.6 mm and 52.1 mm for females. Average forearm length for males was 102 mm and 105 mm for females.

Molecular analysis of tissue

Nineteen tissue samples collected from wing membrane in 2011/12 were genotyped, as were an additional 10 tissue samples collected in 2012/13. However, the latter were used in comparisons with scat samples only, as the sample size was quite small. Twelve tissue samples collected from the Smalley's Beach *M. gigas* population, near Cape Hillsborough, between 2011 and 2012 were genotyped. The Cape Hillsborough population is the closest *M. gigas* population to the Mount Etna population. All loci conformed to Hardy–Weinberg expectations. Population diversity metrics and *Ne* are summarised in Table 1. There was no evidence for a reduction in genetic diversity in the Mount Etna population between 1993 and 2012/13 (analysed using the same four loci in Bayes *t*-test).

A recent population bottleneck was inferred in the Bottleneck (Piry *et al*. 1999) analysis under the infinite allele and a two-phase mutation model but not under the stepwise mutation model. The BottleSim analysis infers that for a population starting with

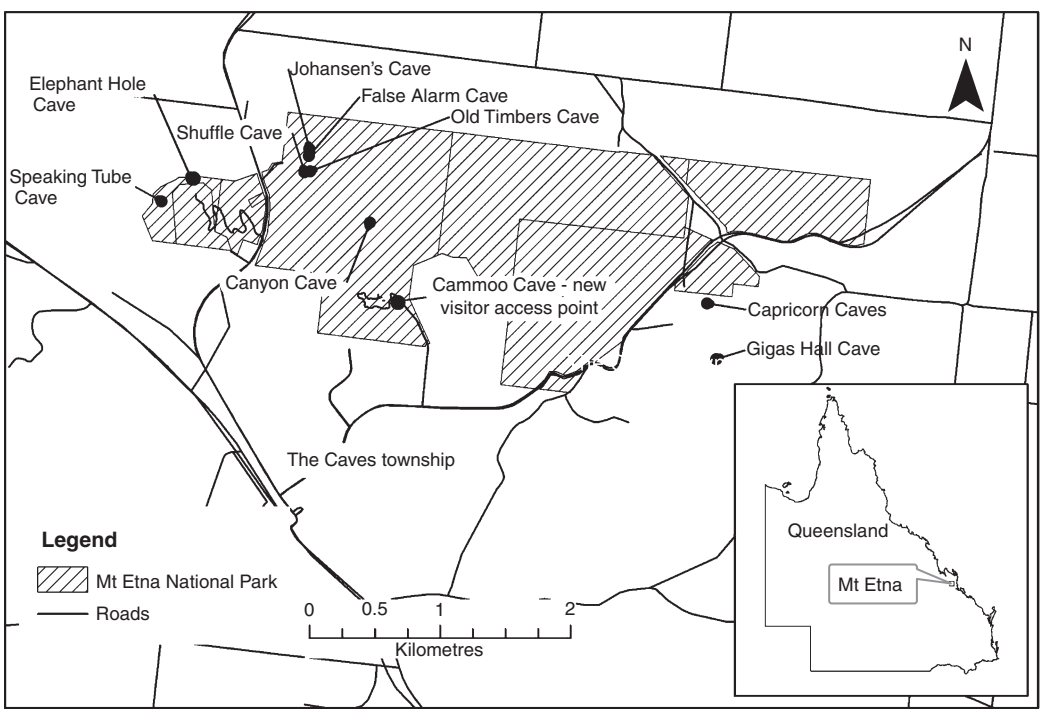

Fig. 2. Location of Mount Etna Caves National Park in central Queensland, showing significant *M. gigas* caves.

the levels of diversity found in Pine Creek to reach those measured at Mount Etna ~80 years later would require a 95% reduction in the initial population size at the start of the simulation (Fig. 3). The time taken to reach the current diversity increases as the bottleneck size decreases. The loss of the mean number of alleles per locus is more rapid than the observed heterozygosity, as expected theoretically.

Comparison of migration models performed using Migrate-n strongly supported a historical panmictic system between the Cape Hillsborough and Mount Etna populations (LBF >18 000) and the demographic analysis using the Bayesian skyline plot analysis with the combined data from the two sites indicated a decline of 76.2% (range: 75.1–77.2%) (Fig. 4). This result is similar to that of Worthington Wilmer *et al*. (1999), whose phylogenetic analysis showed that the southern Queensland sites grouped together, despite identifying highly significant differences in MtDNA, RFLP haplotype and microsatellite allele frequencies between the two populations.

The F_{ST} value between Mount Etna and the Cape Hillsborough area was 0.35 and highly significant ($P < 0.001$). The STRUCTURE analysis identified the most likely number of populations as two and indicated that the Mount Etna and Cape Hillsborough populations were genetically distinct (Fig. S1).

Molecular analysis of scats

Success rates for amplification of microsatellites from scats were quite low, with many samples producing results for only three or four loci. We obtained data for 25 of 37 scat samples from 2014 and 11 from 19 samples from 2015. Results for the first batch of samples were particularly poor, with fewer loci amplifying successfully. We therefore omitted these from further analysis. Of the remaining 11 samples, three were omitted due to having too few loci genotyped (Table S3). Although not all individuals could be reanalysed due to lack of sufficient DNA, wherever it was done the result was the same on each occasion. No scat to scat, or scat to tissue, matches were found, suggesting that we had identified 29 (tissue) + 8 (scat) = 37 unique individuals over the period 2011 to 2015.

Radio-telemetry and GPS tracking

In total, 29 fixes were obtained over a three-week period. The two female collared (VHF) bats were tracked across agricultural land to the north of Mount Etna Caves National Park within a few kilometres of their daytime roost (Fig. 5). Both of these bats were lactating at the time of capture. Only a single fix was obtained for the male, but several pulses were obtained for 14 days

Table 1. Mean effective population size (*Ne*) using 'Colony', and mean genetic diversity estimates
Numbers in parentheses are standard errors

Microsatellite diversity metrics	Cape Hillsborough (2012) $n = 12$ 15 loci	Mount Etna (2011/12) $n = 19$ 15 loci	Mount Etna (1993)[A] $n = 35$ 4 loci	Mount Etna (2011/12) $n = 19$ 4 loci
Mean *Ne* ($n = 19$)		25 (CI: 14–49)		
Mean unbiased expected heterozygosity (*He*)	0.5 (0.07)	0.5 (0.05)	0.5 (0.06)	0.4 (0.1)
Mean expected heterozygosity (*He*)	0.5 (0.06)	0.5 (0.05)	0.5 (0.05)	0.4 (0.1)
Mean observed heterozygosity (*Ho*)	0.6 (0.08)	0.5 (0.05)	0.5 (0.05)	0.4 (0.1)
Mean number of alleles per locus	3.6 (0.4)	3.9 (0.4)	3.7 (0.4)	3.2 (0.6)
Allele range	1–7	1–7	2–7	1–7
Allelic richness	3.6 (0.4)	3.5 (0.4)	3.7 (1)	3.2 (0.9)

[A]From Worthington Wilmer *et al*. (1999).

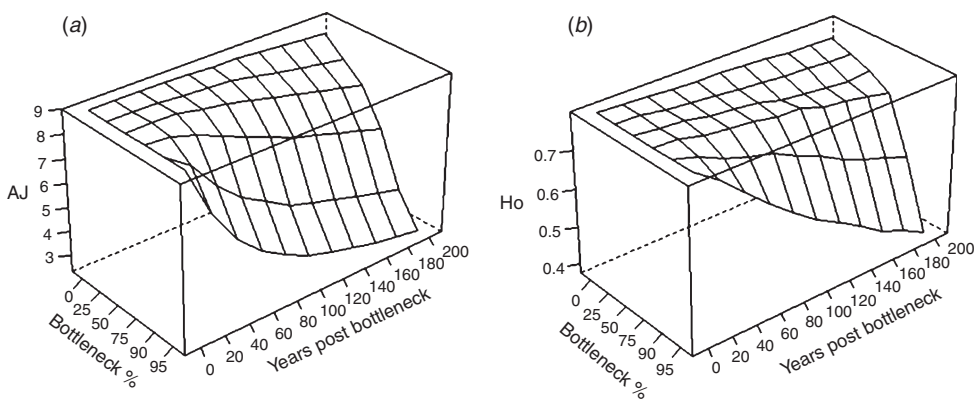

Fig. 3. BottleSim (Kuo and Janzen 2003) simulation results for (*a*) mean number of alleles per locus (*Al*) and (*b*) observed heterozygosity (*Ho*) for bottlenecks ranging from 0–95% loss of the initial population size running for 200 years after the bottleneck event.

after the bat was released. All of the single pulses were from the agricultural land to the north of the caves.

The earliest recorded time that a bat emerged from a cave was 1855 hours. Sunset was at 1807 hours on that day and twilight remained for approximately another 20 min. The bats appeared to return to the cave when large clouds or rain passed through the area but this may have been a coincidence – females may have been returning to feed young that had been left behind in the cave. The foraging areas of the two females often overlapped. Observers based at elevated tracking stations were able to locate the bats more often than observers based at the mobile ground sites.

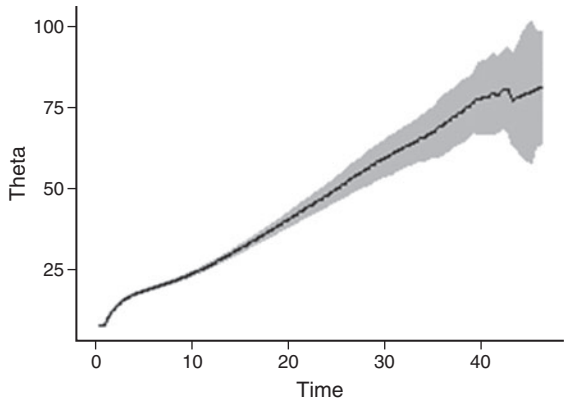

Fig. 4. Demographic changes over time detected with the combined data from the two locations, Mount Etna and Cape Hillsborough with the present being time zero. Theta is four times the effective population size multiplied by the mutation rate and time is in units of expected mutations per generation in the past.

Although the bats did appear to spend some time in the vegetation close to the caves, most of the foraging was above the cleared agricultural land. Small burrows, most likely belonging to rodents, were found in the area during incidental daytime searches.

The GPS-tagged male foraged over an area up to 11.8 km from its daytime roost, spending most of the time in cleared agricultural land rather than vine thickets surrounding the caves and also at a few sites on the edge of a few small remnants surrounded by agricultural land (Fig. S2). These remnants consist of two regional ecosystems (11.3.4 and 11.3.9: Queensland Herbarium 2015) that contained a mixture of *Eucalyptus tereticornis*, *Eucalyptus platyphylla* and/or *Eucalyptus* spp., and *Corymbia* spp. woodlands on alluvial plains. The bat appeared to favour the edge of these areas, possibly using a tree while devouring prey or to search and hunt for prey on the ground (Fig. S2). Many of the locations recorded were from the edge of an ephemeral watercourse that may have contained water at the time. The bat emerged from the cave at ~1930–2030 hours and usually returned no later than 0300 hours (sunrise was at 0536 hours and sunset was at 1846 hours on 31 January (www.timeanddate.com,

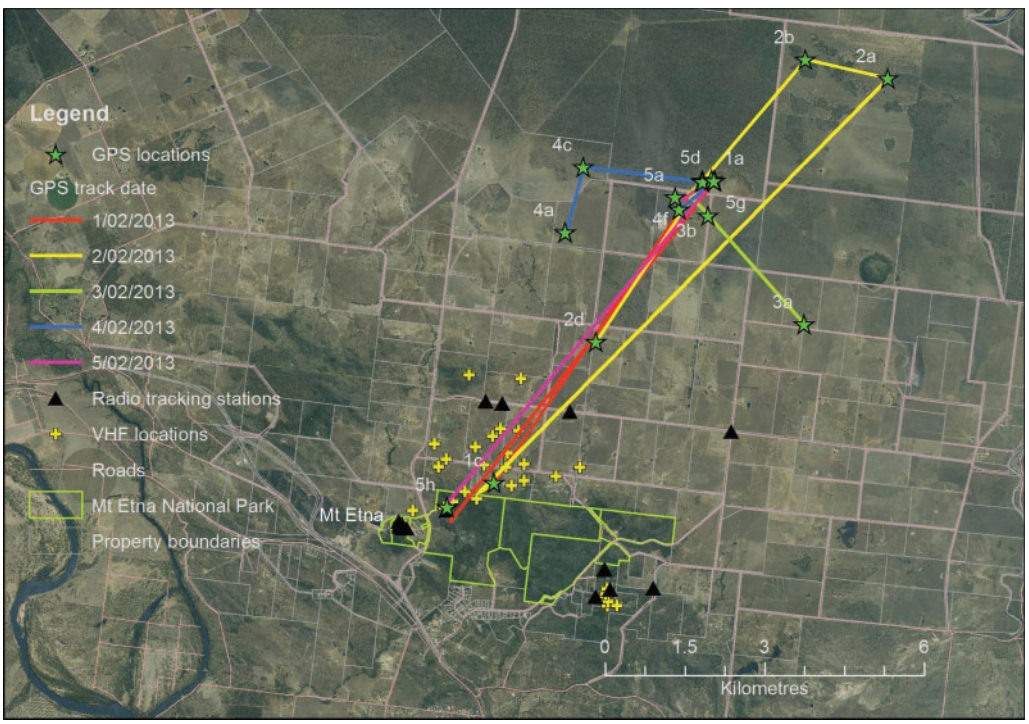

Fig. 5. Foraging locations of the GPS- (★) and VHF-collared *M. gigas* (+). The approximate daily movement of the GPS-collared bat is represented by the track line between fixes (1–5). The daily sequence (*a–h*) in which each fix was recorded is included for each of the five nights (1–5). Successive points obtained from the same location (e.g. the bat was stationary) have been removed to improve clarity on the map. Data for the first day were excluded as the bat flew only a short distance (<100 m) from where it was released.

Table 2. The number of GPS datalogger site records and the height above the ground

Two sites were more than 72 m below the surface; they may have been recorded from within a cave that was open to the sky thereby enabling a GPS fix

Height above ground (m)	No. of sites
<2	19
2–10	10
11–40	4
>40	1
<–72 m (possible cave)	2
Total	36

accessed 7 August 2017). After emerging from the cave the bat appeared to transit quite rapidly to the edge of the remnant vegetation/watercourse, foraged in this area without going into the remnant vegetation and then flew home without spending much time in the middle of the fields.

The GPS-tagged male flew at an altitude of up to 198 m above sea level. When these data were converted into an estimate above ground level the bat did not fly above 45 m, with 29 of the 36 fixes being less than 10 m above ground (Table 2).

Fox scat analysis

Bat remains were not found in the examined fox scats. *M. gigas* were present in the cave at the time when the fox scats were collected and the scats were fresh, suggesting that the two species would have used the cave at the same time.

Temperature data

The average daily minimum temperature at the Rockhampton Airport since 1939 in June, July and August is 11.02, 9.64 and 10.72°C respectively (BOM 2017). Between 1988 (when Speaking Tube Cave was destroyed) and 2012 there have been 25 winters where the average monthly daily mimina was below average (BOM 2017) (Fig. S3).

Discussion

Cave surveys and trapping suggest a much smaller *M. gigas* population size than expected, with possibly fewer than 40 individuals in the Mount Etna area for a given year. The estimate of *Ne* was also low (25), suggesting that there are few breeding individuals in the population. These results represent a decline of ~79% since the last surveys in 2001 (Hoyle *et al*. 2001; J. Toop, pers. comm.). There is, however, no obvious decrease in genetic diversity since samples were collected in 1992/93 (Worthington Wilmer *et al*. 1994). Nevertheless, the estimate of effective population size was lower than theoretical values required to avoid losses of genetic diversity and inbreeding (Frankham *et al*. 2014) and our results demonstrate evidence of a genetic bottleneck, indicating that the effect of genetic drift, and consequent genetic diversity loss in the studied population, is substantial. The bottleneck simulation inferred that a reduction of population size of 90–95% ~80–100 years ago would reduce genetic diversity from that of the largest extant population to that observed today in the Mount Etna population.

Reports of increased survival rates of offspring in the 1980/90s, when the population was ~150–180 (Toop and Davie, unpubl. data), clearly have not continued despite the management actions at Johansen's Cave.

In 1993, Worthington Wilmer *et al*. (1994), using the same methods, site and time of the year, caught 36 individual *M. gigas* over three nights for a capture rate of 12 bats per night while our capture rate was only 1.3 bats per night.

The DNA in scats collected in January 2015 amplified more successfully than did the samples collected in March 2013, possibly because they were sent straight to the laboratory and not stored for an extended period. While DNA was successfully extracted, thereby demonstrating the potential of the technique, more work is required to match the DNA obtained from scats with known individuals. Eight individuals that were not trapped were confirmed from DNA in scats but no tissue to scat matches were found. This could be due to the time that had elapsed between the tissue (2013) and the successfully amplified scat samples (2015) being collected.

The study was unable to determine the main cause of the population decline but we offer some reasoned speculation below. The Mount Etna *M. gigas* have an unusually short lifespan, with the average age of the population being just five years (Toop and Davie, unpubl. data). Additionally, the species has a very low fecundity (only a single young per year is produced, and females do not become sexually mature until their second year), and usually roosts communally, potentially increasing the risk of disease transmission (Toop 1985). In combination, these characteristics could predispose them to extinction. However, the fossil evidence indicates that the species has persisted in the area for at least 500 000 years (Hocknull 2000; Hocknull *et al*. 2007; Martinez 2010) despite these biological constraints. While the limitations of their reproductive system may slow the species' recovery, there must be other factors that have caused this recent population decline.

In 1988, Speaking Tube and Elephant Hole Caves were destroyed and the limestone used for cement. Toop and Davie (unpubl. data) reported that Speaking Tube was a critical overwintering roost for pregnant *M. gigas* and at times all the pregnant females from the colony were recorded using this cave. Speaking Tube was also considered to be an important dry wet-season roost and nursery cave. The location of Speaking Tube, combined with its upward-sloping chambers, created the warmest winter cave conditions in the entire area, and large open flyways inside the cave made roost access easy for pregnant females (Toop and Davie, unpubl. data). *M. gigas* cannot lower their body temperature and enter torpor (Geiser 2006) so they must find roost sites with temperatures above 23°C in order to conserve energy (Toop 1985). Below this temperature they have to shiver to keep warm (Toop 1985). As the Mount Etna population is at the cooler southern end of the species' range it is possible that mortality of pregnant females due to temperature stress is a significant issue. Since Speaking Tube Cave was destroyed there have been 25 winters where the average monthly daily minima temperature was lower than the longer-term average temperature.

The only other cave in the area that provides some relief from very cold winters is Johansen's Cave. Following the destruction of both Speaking Tube and Elephant Hole Cave, the QPWS

closed a road, relocated picnic facilities and gated cave entrances from June to February. Despite these efforts, Johansen's Cave is still subject to unauthorised visitation. The species is highly sensitive to disturbance, particularly during the maternity season when it can cause flightless young to be dislodged by fleeing adults and fall to the ground (Toop 1979; Toop and Davie, unpubl. data; Woinarski *et al*. 2014). Video surveillance, more regular park patrols and education may offer some disincentive to the illegal cavers.

Bat guano was mined in Johansen's Cave in the early 1900s and anecdotal reports from miners claim that sulfur dioxide was released in the cave to remove the bats. They claim that the 'flying-foxes were carried out in wheelbarrow loads' (N. Sands, pers. comm. oral history from Ossie Foster, a deceased guano miner). Flying-foxes are not known to live in caves in central Queensland and these bats would most likely have been *M. gigas*. If such an event was the beginning of the decline of this population, further destruction of caves in 1988 would have impeded population recovery and contributed to the genetic bottleneck currently observed.

Habitat destruction and/or reduced prey abundance as a result of poor land management could be another factor contributing to the population decline. Tidemann *et al*. (1985) found that *M. gigas* in the Northern Territory generally foraged within 2 km of their daytime roost. In our study we found that the collared females usually foraged within 3 km of their roost but both males (one radio-collared and one fitted with a GPS collar) appeared to range up to 11.8 km from their daytime roost. If the bats' habitat is either cleared or in poor health then the bats may be forced to fly further from their roost in order to obtain sufficient food. Both females were lactating at the time of capture and they may not have ranged far from the cave if the young were either following or had been left behind in a cave to which they had to return in order to feed. It was also quite stormy at the time of the survey and the bats may have simply preferred to stay close to their daytime roost in case they needed to seek shelter from the rain. The presence of rodents might also have attracted the females to the area.

Semi-evergreen vine thicket (Regional Ecosystem 11.5.5) (Queensland Herbarium 2015) dominates the area immediately adjacent to the karst/caves. Weed invasion, particularly lantana, Madeira vine and rubber vine, restricts flyways and makes it more difficult to hunt prey. Further from the caves the semi-evergreen vine thickets give way to more open eucalypt woodlands with a shrubby or grassy understorey. These areas are also affected by weeds, including lantana, leucaena, rubber vine and exotic grass species such as Guinea grass and fox-tail grass. These invasive species often form a dense understorey and this also reduces foraging opportunities.

The GPS-tagged bat favoured the edges of small isolated remnant vegetation patches in an otherwise cleared landscape, particularly where the edge was along an ephemeral watercourse. Kulzer *et al*. (1984) and Tidemann *et al*. (1985) found that while *M. gigas* can hover and catch prey, they often hang from a perch from which they search for prey and mount attacking raids. This would certainly accord with our GPS data and suggests that bats may need trees or other roosts from which to hunt efficiently.

Land management strategies (fire, pest, grazing and agriculture) that negatively affect the prey base are likely to be a significant issue for *M. gigas* and it is recommended that landholders be encouraged to undertake practices that are conducive to promoting healthy small fauna populations.

Armstrong and Anstee (2000) demonstrated that barbed-wire fences are a significant issue for *M. gigas* in the Kimberley and Pilbara. Barbed-wire fences are very common in the agricultural land to the north of the study area where the collared bats were tracked. The GPS-collared bat was recorded flying at a height of less than 45 m above the ground with most of the tracking points being less than 10 m above the ground. Our radio-tracking data also suggest that the bats were flying low due to the difficulty we had picking up individuals from the ground, even when they were relatively close to the mobile ground stations in comparison to the elevated sites. Some of the fixes were either directly above or close to a boundary fence, suggesting that they may even use the fences for foraging or to consume recently caught prey. While we did not locate any bats snared on barbed-wire fences during the study, these may be an issue for the Mount Etna population.

Foxes and feral cats are prevalent in the area and are likely to compete with *M. gigas* for food, particularly small mammals and birds. Although we found no direct evidence that either species was preying on *M. gigas*, the entrance to Gigas Hall Cave is small and the flight path would easily be within reach of a fox or cat.

Cane toads have been blamed for the recent declines in the *M. gigas* populations in the Kimberley, Kakadu and Riversleigh areas in northern Australia (White and Madani 2013; White *et al*. 2016; B. Bullen, pers. comm.). We found no evidence of cane toad-related mortality and Toop (1985), who conducted a comprehensive dietary analysis in the 1980s, found only a few native amphibian remains. Toads have been present in the Mount Etna area for a considerable time, including during the 1970–90s, when the *M. gigas* population was thought to be stable (Hoyle *et al*. 2001). It therefore seems unlikely that toads have played any significant role in the recent declines at Mount Etna unless there has been a change in the prey availability which has then caused *M. gigas* to shift to toads.

Both the current study and data from Worthington Wilmer *et al*. (1999) indicate some historical level of connectivity between the two *M. gigas* populations at Cape Hillsborough and Mount Etna. Our analysis with a structure coalescent sampler demonstrated that individuals at these two sites belonged to the same panmictic population. This historical high-level connectivity has clearly been altered, most likely due to habitat fragmentation and small population sizes, as demonstrated by the identification of two separate genetic clusters in the Bayesian assignment test and *F*st statistics. This lack of connectivity further heightens the conservation concerns for this species, as it will be difficult for the two populations to retain or improve their current, already relatively low, genetic diversity. The current levels of genetic diversity are likely to further limit this population's ability to recover.

Without a holistic approach to the management of the Mount Etna *M. gigas* population that includes ensuring their winter and maternity caves are protected from human disturbance, their habitat is free of barbed-wire fences and is managed appropriately, and their current genetic diversity is conserved, the population in central Queensland is at considerable risk of extinction. It is critical to monitor reproduction and mortality rates to clearly identify which is limiting population growth.

Without this knowledge, the chances of adequate management are greatly reduced (Caughley and Gunn 1996). The sampling of DNA contained in scats is likely to be adequate to monitor the population but further testing is required.

Should a reduction of fitness be identified, possibly associated with the lack of genetic diversity, assisted migration or a captive breeding program may offer some remedy to restore part of the genetic diversity that was lost from the Mount Etna population over the last century or so. To this end, an accurate identification of the species' evolutionarily important units (Moritz 1999), extending our comparisons of migration models to other sites within the state, could facilitate the selection of possible source populations.

Conflicts of interest

The authors declare no conflicts of interest in relation to this paper.

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