Woolgoolga to Ballina Pacific Highway Upgrade

Koala Population Monitoring Program Annual Report:

Year 5 2021/22

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Woolgoolga to Ballina Pacific Highway Upgrade

Koala population monitoring program

Annual report year 5 2021-2022

SANDPIPER ECOLOGICAL SURVEYS

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Cover Photo: Koala (*Phascolarctos cinereus*) at Laws Point foraging on a *Eucalyptus* spp. planted during construction of the W2B upgrade.

Executive Summary

Sandpiper Ecological Surveys (SES) was contracted by Transport for NSW (TfNSW) to implement the Woolgoolga to Ballina (W2B) Pacific Highway upgrade koala monitoring program in accordance with section 8 of the approved Koala Management Plan (KMP) (RMS version 4.4, July 2016). The broad aim of the monitoring program is to determine the effectiveness of mitigation measures implemented in Sections 1-11 of the upgrade for koalas. The following report presents results of year five (2021/22) of the monitoring program and builds upon monitoring in years one to four (Sandpiper Ecological 2019a, 2019b, 2020, 2021).

Year five population surveys were completed at 96 sites – 50 in the Broadwater focal area (sections 8-9) and 46 in the Bagotville focal area (section 10) – during spring 2021 and autumn 2022. More koalas were recorded in year 5 than year 4 in both focal areas. Bayesian estimation analyses of survey data showed increasing evidence of a negative population trend at Broadwater and a stable population at Bagotville. Density estimates for Broadwater in year five were 0.045 individuals/ha compared to a modelled baseline estimate of 0.062 individuals/ha. The year five density estimate at Bagotville was 0.08 individuals/ha compared to a modelled baseline estimate of 0.082 individuals/ha.

A prospective power analysis demonstrated that the koala population monitoring program at Bagotville was above the target level of statistical power (>0.7) whereas Broadwater was below. Measures of power and statistical certainty remain low but are improving with each successive year of monitoring. The modelling exercise confirmed the challenge of sampling populations at very low densities and drawing conclusions from sparse counts. Subsequent monitoring years should improve the precision of density estimates.

Genetic analysis of scat samples has been influenced by differences in the number and distribution of samples collected in years 1, 3 and 5. Therefore the findings of genetic analysis should be interpreted cautiously. Genetic analysis of year 5 samples confirms the moderate to strong genetic differentiation between koalas in section 10 and other regional populations and the presence of 2-3 genetic clusters within the population. Genetic cluster analysis from the three samples (i.e., 2018, 2020, 2022) showed no genetic differentiation between the east and west sides of the highway. This was supported by pairwise analysis of genetic relatedness, which revealed only weak differentiation between the east and west sides of the highway and suggests there is no genetic differentiation at this stage

In working towards achieving the key mitigation measure for section 10 to reduce koala mortality by 4-8 individuals per year, TfNSW have implemented a predator control program, installed six vehicle-activated signs at road mortality hot-spots across the broader section 10 study area, installed exclusion fence along a 2 km section of Wardell Road and a 3km section of the old Pacific Highway between Wardell and Coolgardie and installed three crossing structures on Wardell Road. These measures exceed that required by the Koala Management Plan. Since installation of fencing, no koala vehicle strike has been reported on Wardell Road or the old Pacific Highway compared to 10 in 2016/17 (FOK, unpublished data). No koala vehicle strike was recorded within the focal population areas during the 2021 road mortality surveys. One incidental koala road mortality was recorded on 8/9/2021 south of Devils Pulpit. This is the second road mortality recorded in a fenced section of highway near Devils Pulpit with an initial incident in December 2020.

Acknowledgements

We wish to thank the landholders who approved access to their properties to conduct the population surveys. Numerous monitoring sites are on private property, so landholder support is critical to achieving the goals of the monitoring program. We also extend appreciation to the Jali Local Aboriginal Land Council for their approval, support, and involvement with surveys within the Ngunya Jargoon IPA.

We would also like to extend a special note of appreciation to Friends of the Koala (FOK), particularly Maria Mathes, for her tireless work in supporting the local koala population.

The final report was improved by comments from TfNSW.

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1. Introduction

1.1 Background

Sandpiper Ecological Surveys (Sandpiper) was contracted by Transport for NSW (TfNSW) to implement the Woolgoolga to Ballina (W2B) Pacific Highway upgrade koala monitoring program in accordance with section 8 of the approved Koala Management Plan (KMP) (RMS version 4.4, July 2016), excluding phased resource reduction. Koalas are listed as Endangered by the NSW *Biodiversity Conservation Act 2016* (BC Act) and Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). The primary aims of the monitoring program are to: determine the effectiveness of mitigation measures implemented in Sections 1-11 of the upgrade for koalas; and monitor trends in the size of koala populations surrounding the alignment at Broadwater (Sections 8-9) and Coolgardie-Bagotville (Section 10; hereafter referred to as Bagotville). The following report focuses on population monitoring and road mortality surveys only. Monitoring of connectivity structures is addressed by Sandpiper Ecological (2023). That report includes linkages between data presented here and use of underpasses by koalas.

Both population monitoring areas (i.e., Broadwater and Bagotville) are described as focal populations that could be adversely affected by the highway upgrade (RMS 2016). The two focal areas featured the highest density of koala records along the W2B alignment during targeted surveys for the environmental assessment (RMS 2016).

Baseline data on the focal koala populations have come from a variety of sources. Population surveys of the Broadwater focal area were conducted during 2014 and 2015 (Ecosure 2014, 2015). The Bagotville koala focal population has been the subject of detailed field and laboratory studies (see Phillips and Chang 2013; Phillips *et al.* 2015), which informed the preparation of a Population Viability Analysis (PVA) (Kavanagh 2016). The PVA was conducted in accordance with the Commonwealth Conditions of Approval (CoA 5 and CoA 7) and its outcomes have been used to guide management of koalas within the Bagotville area.

The PVA for the Bagotville focal population indicated that this population is projected to decline significantly over the next 50 years (Kavanagh 2016) unless key threatening processes are controlled. Monitoring of this population is considered important to determine whether mitigation actions have been effective in slowing population decline. As such, the Bagotville focal population will be assessed against the PVA predictions. The Broadwater population, which was not subjected to a PVA, will be assessed against a statistically significant decline at year 15 compared with baseline survey values (RMS 2016).

1.2 Scope of work, program objectives and performance indicators

The monitoring program is designed to provide reliable information with which to inform management of koalas *Phascolarctos cinereus* along the highway upgrade. The objectives of the population monitoring program for sections 8-10 of the highway upgrade as stated in the KMP and expanded upon in the Ecological Services Brief (RMS 2017) are described below. Those applicable to year 5 (2021/22) are shown in italics.

1. Determine whether there is a statistically significant decline at year 15 compared with no decline in section 8-9.
2. Determine whether the corrective actions of the KMP have been triggered by estimated population trends in section 10 in accordance with predictions of the Population Viability Analysis.
3. *Provide information that supports a program review by TfNSW at years 5 and 10 in accordance with the KMP (years 5, 10 & 15).*

4. Assess effectiveness of the revegetation program in providing additional habitat for koalas.

Based on the above objectives, the success or otherwise of the monitoring program shall be determined by program performance against relevant performance indicators (PI). In addition, scat sampling will be conducted every second year in section 10 for the purposes of genetic analysis, to provide information on distribution and relatedness of individuals across the study area. Table 8-4 in the KMP details eight performance indicators and their corresponding thresholds, corrective actions, and agency responsible. Two performance indicators are relevant to year five population monitoring (Table 1).

Table 1: KMP performance indicators and corrective actions relevant to current report.

Performance indicator	Performance threshold	Corrective actions
1. Road mortality	• No injury to an individual koala as a result of vehicle strike across all upgraded sections. • Section 10: no koala road mortality within the fenced areas of the upgrade, on existing Pacific Highway or Wardell Road.	• Examine fencing for breach or obstruction within 3 days of report & repair. • Retrofit exclusion fencing, or part there-of, with additional measures to deter koalas. • Section 10: RMS would consider erecting koala-proof fencing on Bruxner Hwy (a known koala road-kill black spot), in an effort to reduce koala mortality across the region.
2. Koala population trends in Sections 10 and 8/9	• Koala population sizes at or above the minimum expected targets including rate of population change/decline at/above the minimum expected target of 195-276 at five years; 147-272 at 10 years and 103-261 at 15 years.	• Complete program review at 5, 10 and 15 years. • Identify the key threatening processes which are continuing to impact on Koala population trends (through monitoring of roadkill, connectivity structure use, and use of re-vegetation areas). Incorporate review of road-kill data from local wildlife rehabilitation groups WIRES, Friends of the Koala. • Increase efforts to control these key threatening processes. This may include implementing additional dog control, establishing additional Koala habitat, modifying existing or creating new connectivity structures on adjacent road networks, and/or implementing measures to reduce Koala roadkill.

The program review required in years five, 10 and 15 of the monitoring program requires the following:

1. Recommendations for changes to the monitoring program including survey effort and techniques to improve its power to detect change
2. Population estimates and trend analysis against PVA predictions
3. Consideration of any population information resulting from genetic analysis undertaken for the project.
4. A review of road-kill data obtained from rehabilitation groups.
5. Advice on whether any corrective actions are required in accordance with the KMP.

The following report describes the methods and results of the year 5 (2021/22) monitoring period and includes an assessment of statistical power of population surveys going forward. It represents year 5 of population monitoring in sections 8-10 and road mortality/exclusion fence monitoring in section 10 along Wardell Road and the existing Pacific Highway, year 4 road mortality/exclusion fence monitoring in sections 1-2 and year 1 of road mortality/exclusion fence monitoring in sections 3-11. The report also addresses the monitoring objectives and assesses monitoring outcomes against the relevant performance indicators and whether thresholds have been breached and require corrective actions.

2. Study area

The broader study area includes sections 1-11 of the W2B Pacific Highway upgrade alignment and adjoining habitat (Figure 1). The 155 km-long upgrade stretches from Woolgoolga in the south to Ballina in the north. It is wholly located within the NSW North Coast Bioregion, one of the most diverse in NSW (W2B Planning Alliance 2012). The project boundary is located within a landscape that has been either fragmented or cleared for agriculture and rural development although a substantial area of forest persists across the broader study area (W2B Planning Alliance 2012).

For the purposes of the year 5 population monitoring report, monitoring activities were conducted in sections 1-11 (road mortality monitoring) and sections 8-10 (population monitoring) (Figures 1 & 2). In sections 8-9, the Broadwater focal population area extends 3-5 km either side of an 11 km portion of the highway upgrade from Lang Hill (northern part of section 8) north to the Richmond River (including all of section 9; Figure 1). The Richmond River forms a major movement barrier to the west and north. Within section 10, the Bagotville focal population area extends 13.5 km north of the Richmond River and includes the localities of Bagotville and Coolgardie (Figure 1).

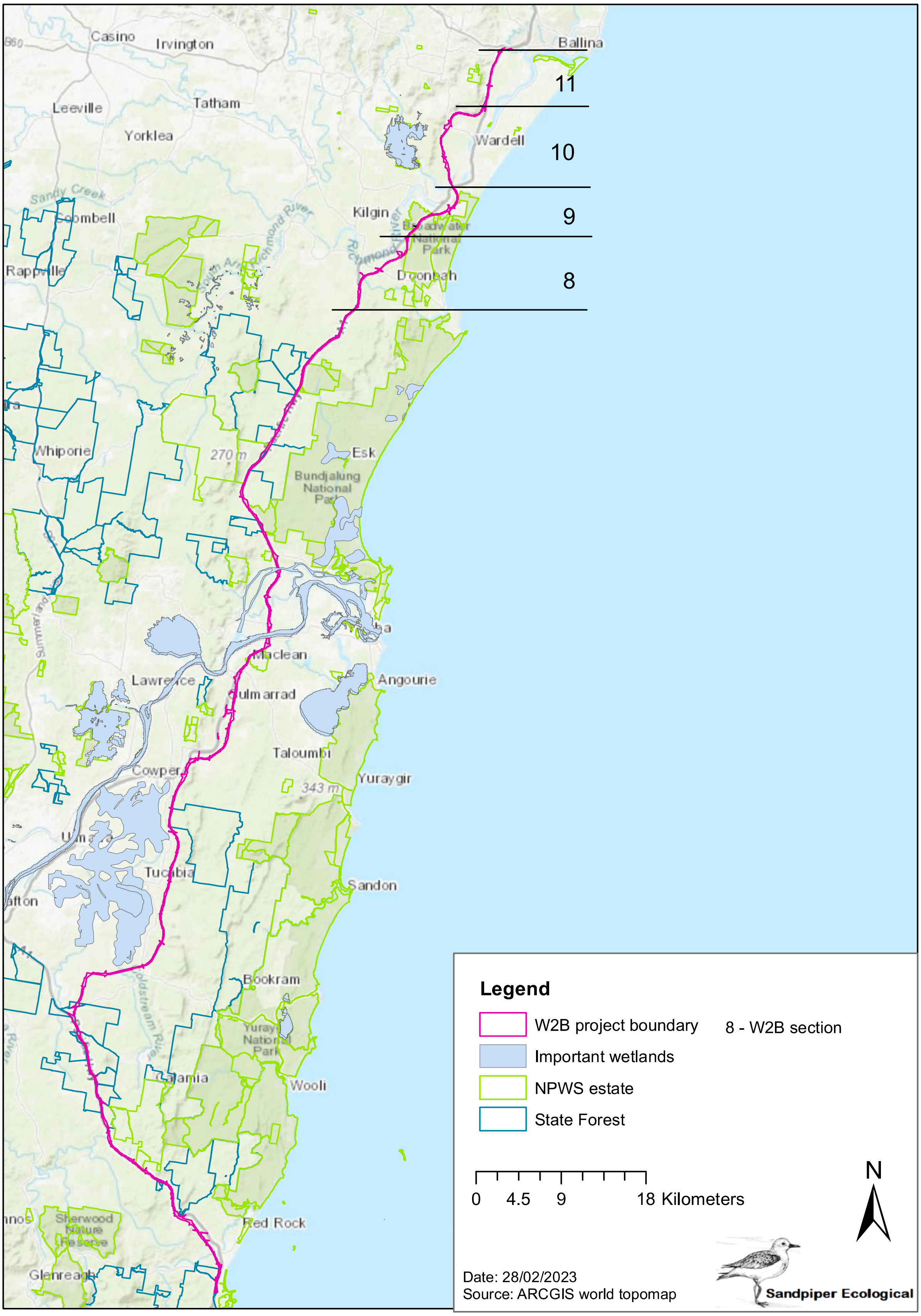

Figure 1: Sections 1-11 of the W2B Pacific Highway Upgrade.

3. Methods

3.1 Population surveys

Diurnal and nocturnal population surveys were conducted during spring/summer 2021 and autumn/winter 2022. Surveys during both periods were delayed due to wet weather and flooding of sample sites. Surveys covered 96 sites (50 in Broadwater and 46 in Bagotville) and were completed by teams of three ecologists experienced in koala surveys (Figures 3 and 4). Four sample sites were unable to be sampled in the Bagotville study area (Figure 4). This was due to refusal of entry by new owners (1 site), or the inability to contact owners (3 sites). At least two sites were unoccupied following flooding in early autumn 2022.

At each site two direct count methods were used:

1. **Transect searches**

   Direct counts on 250 m x 40 m transect (approximately 1 ha) involved three observers walking 20 m apart – one on the centerline and one on each side. Observers were equipped with binoculars (& spotlight) and searched trees for koalas.

2. **Radial searches**

   Direct counts within a radial area involved three observers slowly searching all trees within a 25m radius of the mid-point of the belt transect (approximately 0.196 ha). Radial areas and transects were conducted concurrently.

During year 1 and year 2 surveys, the same team completed diurnal followed by nocturnal surveys on the same day. To address concerns about inadequate survey independence, year 3, 4 and 5 diurnal and nocturnal surveys were completed on non-consecutive days.

All koala observations were recorded with a handheld GPS unit and data collected on individual characteristics (e.g., sex, age class, health status, behaviour, identifying features), tree species and diameter at breast height of tree. Handheld spotlights were used to assist with nocturnal surveys.

Spring 2021 surveys were completed between 14 October and 7 December 2021. Diurnal surveys were generally completed between 0800 hours and 1830 hours and nocturnal surveys between 1900 hours and 2400 hours. Weather conditions on sample days were mostly fine, with the occasional shower. Temperatures ranged from 17°C to 25°C and winds were variable.

Autumn 2022 population surveys were completed between 19 May and 20 July 2022. Diurnal surveys were generally completed between 0900 hours and 1700 hours and nocturnal surveys between 1730 hours and 2430 hours. Weather conditions on sample days were generally good for surveying koalas, with clear days and nights and occasional light wind. Light rain/shower was recorded on three sample days and temperatures ranged from 9°C to 21°C.

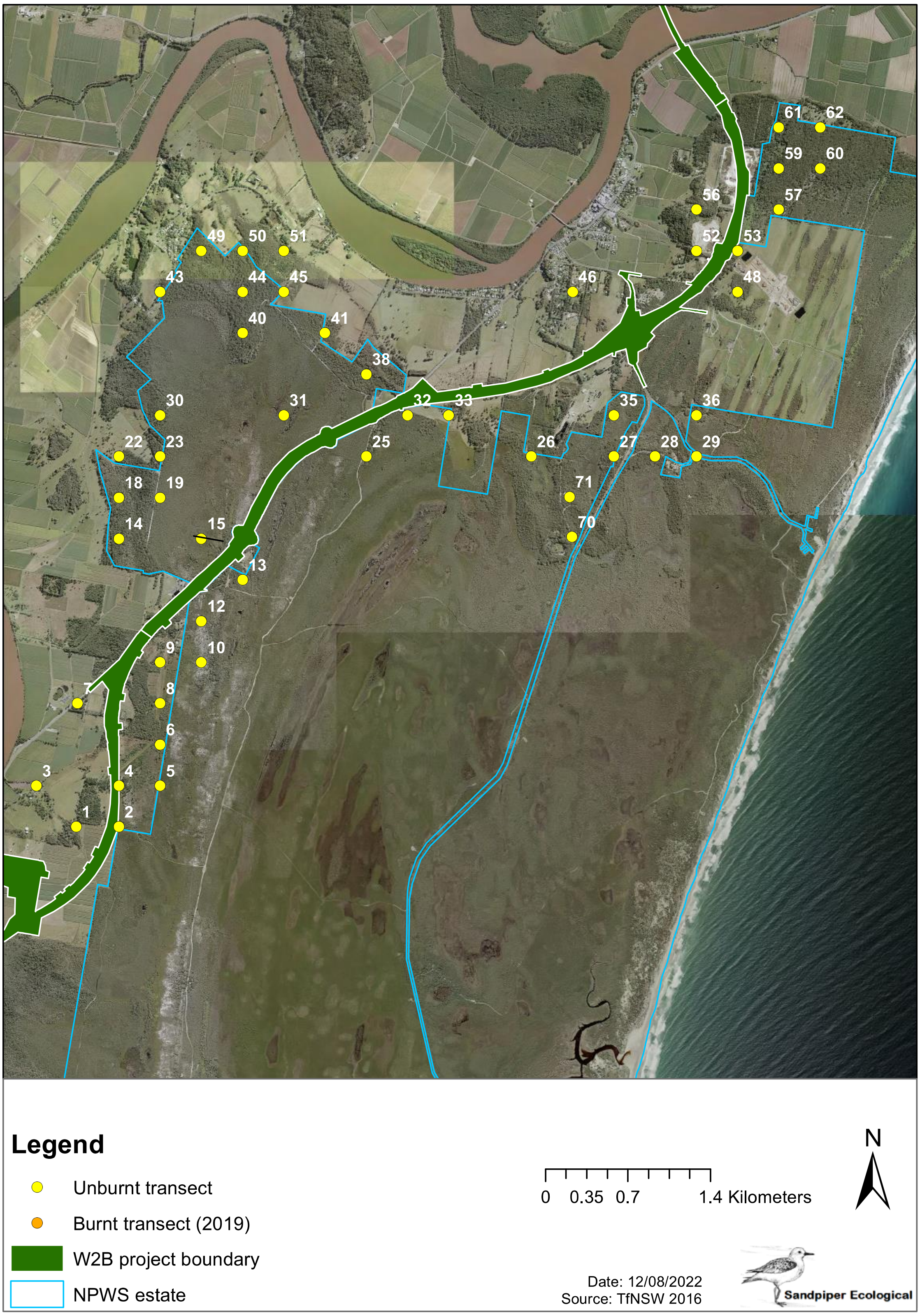

Figure 2: Broadwater (sections 8-9) sample sites.

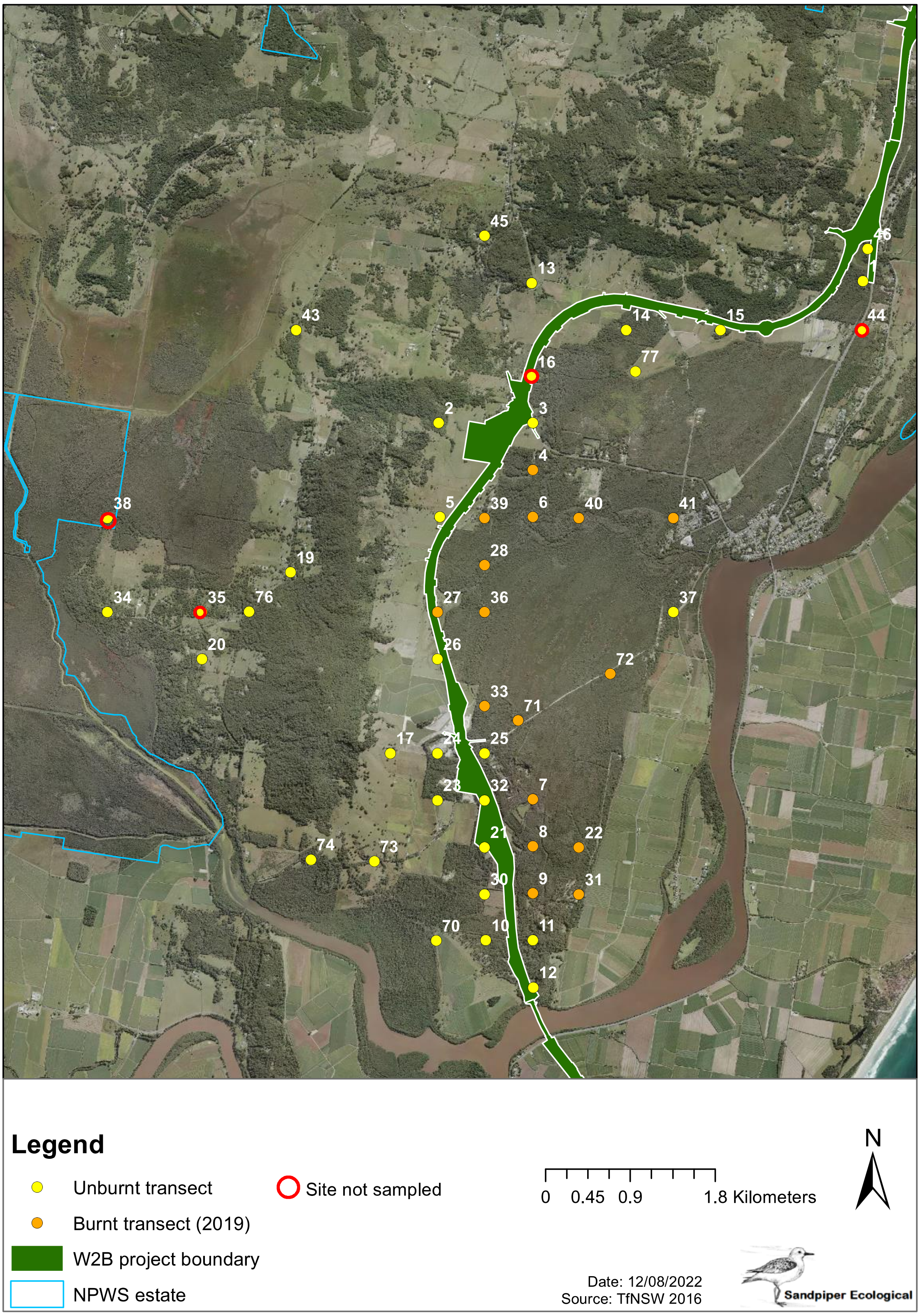

Figure 3: Bagotville (section 10) sample sites, including sites that were burnt during the spring 2019 wildfire.

3.2 Koala density and population size estimates

A detailed report on statistical analysis of koala population data is provided by Rankin (2022) and included in Appendix A. Following is a summary of analysis methods.

3.2.1 Bayesian modelling

A Bayesian estimation exercise was used to estimate densities at Broadwater and Bagotville for year 5 spring and autumn, and year 5 combined. The procedure included multi-model uncertainty for effects: night-time vs day-time effect, a radial- vs linear-transect effect; a seasonal effect; log-linear trend vs no trend vs each year having its own unique density, and five different amounts of over dispersion (excess count-variation). Each of these core specifications was repeated five times for five different Negative Binomial over dispersion priors (which broadly represented a spectrum of high-to-low over dispersion, the latter being equivalent to a Poisson). For this exercise, there were a total of 280 models. To acknowledge multi-model uncertainty, these models were model-averaged using posterior probabilities derived from the Watanabe-Akaike Information Criterion (Watanabe 2010, Gelman *et al.* 2014), as in previous reports (Sandpiper 2019a, 2019b, 2020, 2021).

In using the model-based approach, as described above, a Bayesian regression model is applied to the entire dataset to project the population back to Year zero based on the overall population trend. In so doing, this approach smooths-over the high natural-variation in counts and more accurately reflects the population density. The estimates include multiple sources of uncertainty resulting in conservative trend estimates. A disadvantage of this approach is that baseline and trend estimates need to be re-calculated as more data are acquired, which is why density estimates for the same years vary between reports. By contrast, a 'fixed baseline' approach employs a simple descriptive statistic, calculated as the mean (and standard deviation) of the raw counts during the baseline year. The baseline value is not updated as more data are acquired.

A disadvantage of the fixed-baseline approach is that due to the large natural-variation in koala counts (i.e., much year-on-year variation) and the sparsity of koala counts (i.e., few koalas in any year) the fixed-baseline reflects the random-variation during the baseline year, rather than the overall population density. Because the fixed baseline is sensitive to high variation in counts, it is more alarmist as a decision-making tool. In contrast, the model-based baseline focuses on the magnitude of the overall population trend, rather than the exact density in any one year. The trend is less sensitive to alarmist changes in koala counts although it may react quickly to a catastrophic drop in density, which is more likely with a fixed baseline approach. Due to high variability in counts between years and the focus on temporal population trends the model-based baseline approach was more consistent with the intent of the KMP/PVA.

For the Bagotville focal area, baseline density values derived from Bayesian modeling were then extrapolated across the total area of preferred koala habitat prior to clearing (i.e., 2,152 ha) and post-clearing/monitoring years (2,135 ha – as used in the PVA (Kavanagh 2016)), to derive a population size estimate for each period. To be consistent with the PVA population estimation methodology (Kavanagh 2016), a correction factor of 0.204 was then applied to Bagotville population estimates to account for the unsampled 0-1 year-old age cohort. The derived population estimates are referred to as 'revised population estimates'.

It should be noted that in applying the above approach, the Bagotville baseline population estimate presented in the PVA/KMP differs from the revised Bayesian modelling-derived baseline population estimate presented in the current report. Whereas population estimates are presented, determining population trends is focused on comparison of density estimates rather than population estimates. Focusing on density trends is more robust and reduces bias (Rhodes *et al*. 2015). Density estimates are also more reliable because the extrapolated area of preferred koala habitat differs between baseline and post-clearing (and differs between actual area cleared (i.e., 28 ha) and that predicted in the PVA (i.e., 17 ha)) and its quality and extent will likely change during the 15 year-long monitoring program.

For the Broadwater focal area, which is not informed by a PVA and will be assessed according to a statistically significant decline at year 15, population trends are assessed according to density estimates.

3.2.2 Supplementary analysis to estimate trend in density estimates

A supplemental analysis was conducted to further investigate evidence for or against the presence of a trend in density estimates. The intent of the supplementary analysis was to complement and contextualise the main results. The supplementary analysis used frequentist Negative-Binomial GLMs models and performed model-averaging by AICc weights (Akaike 1974, 1998, Schwarz 1978) to estimate the trends at Broadwater and Bagotville.

These models can be thought of as pseudo-Bayesian models whereby the i) priors-on-parameters have been weakened to zero-influence, and ii) priors-on-model-probabilities are adaptive (i.e., they become more conservative with less data, and more liberal with more data). In other words, the AICc “reacts” faster to new data as compared to the static Bayesian priors used in the main analyses. The trade-off is that: the AICc may be more sensitive to developing trends but may result in some over fitting and be alarmist, as compared to the Bayesian models with stronger priors.

3.3 Prospective power analysis

The KMP includes background information on use of a Power Analysis (PA) to determine minimum survey effort to reliably detect a decline in focal koala populations. It states survey effort that achieved 70% power (or confidence) to detect a 30% decline in the Bagotville population was acceptable (RMS 2016). Using baseline data for each focal population and a diurnal search detection probability of 1.0/observer, the KMP PA determined that to achieve the 70%/30% target 50 survey sites within each focal area would need to be double sampled (i.e., two surveys/session) every six months (J. Rhodes unpub. data).

A subsequent prospective PA, which included current density data, would then be completed at the end of each reporting period to determine the minimum survey effort required going forward. Whereas the PA used to inform the KMP was based on a frequentist/null hypothesis testing approach, the prospective PA used in the current and previous reporting periods was based on a Bayesian estimation analysis.

The prospective analysis uses a Monte Carlo simulation procedure. The goal of the power analyses is to estimate the rate of Type-II errors (falsely rejecting the hypothesis of a trend, $H_a : \beta_t \neq 0$) while detecting a -30% decline from baseline levels at Broadwater and Bagotville between years 2015 and 2031. The error rates were conditional on:

1. a negative trend of -30% from baseline levels until Year 15 of monitoring.
2. a cap on the rate of Type-I errors at $\alpha \leq 0.3$.
3. a monitoring effort of 400 transects per year each at Broadwater and Bagotville (i.e., 50 sites surveyed twice/season and two seasons/year at each area).
4. marginal effects for survey-design factors (daytime/night-time, spring/autumn, line-transect/radial-search transects) empirically derived from the Bayesian analysis.
5. baseline koala densities derived from the Bayesian estimation analysis.

The prospective analyses were conducted in the same manner as previous reports with no supplements. Because the prospective analysis assumed the (simulated) existence of 15 years of data, it was considered less sensitive to prior distributions and issues of small sample-sizes. However, because the analysis is conditional on some empirically estimated features, the results are still somewhat sensitive to the estimated baseline conditions and the models used to estimate those conditions.

3.4 DNA analysis

3.4.1 Faecal pellet (scat) collection

Faecal pellets (i.e., scats) were collected from koalas observed in the Bagotville area during year 5 surveys for DNA analysis. When a koala was observed, the base of its tree was searched for fresh scats. If fresh scats were found, they were collected in accordance with the "Collection of Scats Protocol" and the methods for collection and storage described by Piggott (2004) and Wedrowicz *et al.* (2013). As per year 3 surveys, the collection method was refined to involve placement of scats into a paper bag which was then stored in an esky and transferred to a freezer once field surveys were complete. Scat collection data included location, tree species, tree DBH, koala sex/health (if possible) and weather at time of collection.

In year 5, the collection of scats was affected by consistent heavy rain during the spring and autumn sample periods. Faecal pellets exposed to moisture and rain from inclement weather, heat and UV from sunlight have higher amplification failure and genotyping failures compared to scats collected from weather-protected positions (Hulse 2022). Scats exposed to rain were avoided, which meant that the total number of samples collected in year 5 was substantially less than in previous years. In addition, scats were collected from a reduced geographic area due to the combined effect of limited suitable weather conditions and the sampling program.

Effort was made to collect 75-100 scats for DNA analysis during the reporting period, however, the total number collected in year 5 was 45-55 from 12 separate samples.

3.4.2 DNA extraction and analysis

Genomic DNA was isolated using the NucleoSpin® DNA Stool kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Each DNA isolate was tested for quality and concentration using spectrophotometry (Nanodrop, ThermoFisher Scientific, VIC, Australia). The presence of koala genomic DNA (*Phascolarctos cinereus* beta-actin mRNA) successfully isolated from epithelial cells exfoliated onto the surface of the pellets was confirmed via real-time PCR (Hulse e*t al.*, 2018). One out of 12 samples did not have genomic DNA isolated. DNA degradation occurs over time and is expedited the longer biological samples are exposed to the environment. Pellets exposed to moisture and rain from inclement weather, heat and UV from sunlight have higher amplification failure and genotyping failures compared to scats collected from weather-protected positions. In addition, the presence of volatile organic compounds and phenolics derived from the koala's diet of Eucalyptus leaves may also impede isolation and amplification of DNA. Eucalypt molecules are excreted in koala faeces and are known to damage cell membranes, while phenolics can accelerate DNA degradation.

3.4.3 Genotypes and samples

Genotypes across 32 microsatellite loci for 11 scat koalas were generated from genomic DNA. There were no departures from Hardy Weinberg Equilibrium from the population, therefore a total of 32 loci were retained for analysis. Detection of repeated genotypes within the 2022 dataset to identify duplicate samples was performed using the software GENALEX version 6.5 (Peakall and Smouse, 2012) which revealed no identical multilocus genotypes present within the dataset.

3.4.4 Genetic diversity

Analysis of genetic diversity was performed using the software GENALEX version 6.5 (Peakall and Smouse, 2012) to calculate mean number of alleles and observed and expected heterozygosity. FSTAT (Goudet 2001) was used to calculate allelic richness, a measure of allelic diversity that considers differences in sample sizes by standardising to the smallest number of individuals typed for a locus in a sample, to enable comparison among populations. FSTAT was also used to estimate the inbreeding coefficient (F_{IS}) for which a positive value indicates that individuals in a population are more related than you would expect under a model of random mating, and a negative value indicating that individuals in a population are less related.

3.4.5 Pairwise genetic differentiation (F_{st})

Restrictions to gene flow among populations results in a genetic differentiation or divergence of the populations. F_{ST} is a measure of population genetic differentiation that quantifies the proportion of variance in allele frequencies among populations relative to the total variance. As a measure of genetic differentiation among populations, F_{ST} is calculated to evaluate how genetically different koala populations are to one another. A common reason for populations becoming more genetically different is reduced breeding movements of koalas among populations. The greater the genetic differentiation between populations, the less breeding movements there are between them and the more isolated they are from one another. F_{ST} can range from zero to one, where zero means populations show no genetic separation; a value of 0.25 or greater indicates strong differences among populations. Assessment of genetic differentiation between koala populations was calculated using FSTAT (Goudet 2001).

3.4.6 Genetic relatedness

Genetic relatedness was estimated to indicate the proportion of shared ancestry in pairs of individuals. Expected values are 0.5 for parent-offspring or full-sib pairs and 0.25 for half-sib pairs. However, genetic relatedness values will form a distribution around these expected values. Genetic relatedness of within-population individuals was calculated in GENALEX version 6.5 (Peakall and Smouse 2012) using the Queller and Goodnight estimator of relatedness.

3.4.7 Population structure

The clustering of koalas into genetic populations, termed population structuring, was determined using the Bayesian clustering program STRUCTURE version 2.3.4 (Pritchard *et al*., 2000). STRUCTURE implements a model-based clustering method for inferring population structure using genotype data of unlinked markers. This method demonstrates the presence of population structure, identifies distinct genetic populations, and assigns individuals to populations or clusters without any prior information about geographical location. The notion of a genetic cluster is that individuals within the cluster share on average more similar allele frequencies to each other than to those in other clusters.

Analysis of koala population genotype data involved 5 replicates of K = 1 to K = 10 (K = genetic cluster) using 150,000 iterations with 150,000 iterations discarded as burn-in. The number of K clusters was determined using both the maximum likelihood and the deltaK method of Evanno *et al*., (2005).

3.5 Road mortality surveys and fauna fence inspections

Koala road mortality surveys were undertaken on two occasions, once in winter (26/8/2021) and once in spring (10/11/2021). Whereas year 1 and 2 surveys involved walking along the side of the highway, year 3, 4 and 5 surveys were changed to car-based to address safety concerns. In year 5, car-based surveys covered all of sections 1-11 (155 km), Wardell Road (Gubay Lane to Thurgates Lane – 1.54 km), and the old Pacific Highway (Carlyle Street to Coolgardie interchange – 3.3 km).

Car-based surveys entailed a driver and passenger/observer travelling the length of the subject road in both directions. The survey vehicle featured a 'Vehicle Frequently Stopping' sign on the back and flashing light and travelled at 80-90 km/h in the left-hand lane. Surveys involved the passenger scanning the road surface and road shoulder for animal carcasses. The location of each carcass was recorded on an Ipad running Motion-X and details on the species/group was recorded on a notepad. Unidentified mammal carcasses were scored as either small (e.g., rodent, bat, glider, brush-tailed phascogale), medium (i.e., long-nosed potoroo, rufous bettong, koala, bandicoot, cat, spotted-tail quoll, possum), or large (i.e., wallaby, kangaroo, dog, fox). If roadkill was suspected of being a koala the site was revisited and the carcass inspected from a safe location. If safe to do so, a hair sample was collected from any unidentifiable carcass suspected of being a threatened mammal. Samples were sent to a recognised hair analyst for identification. Road mortality results were supplemented by other data sources including incidental observations from Sandpiper staff while traveling focal roads, TfNSW staff, and road mortality reports from Lismore-based Friends of the Koala (FOK).

4. Results

4.1 Population survey koala observations

4.1.1 Broadwater focal area

During spring 2021 surveys, three koalas were observed on transects during diurnal searches and two on transects during nocturnal searches (Table 4; Figure 5). These included three males and two of unknown sex. Two individuals, a probable female and a probable male, were observed within radial plot areas. A further six koalas were observed incidentally off-transect while moving between sites. The body condition of individuals that could be viewed was generally good.

During autumn 2022 surveys, one koala was observed on transect during diurnal searches, and one during nocturnal surveys (Table 4). One individual was recorded within a radial plot at night and a further three koalas were observed incidentally off-transect while moving between sites. Two males and two females were confirmed, with the sex of the remaining two individuals unknown. The body condition of individuals that could be viewed was good. Full details of Broadwater koala observations are provided in Table B1, Appendix B.

Table 2: Broadwater focal area koala observations – baseline to year 5 (2021/22). Sp = spring; A = autumn. Number of sample sites shown in parentheses.

Time & type	Baseline (54)	Year 1		Year 2		Year 3		Year 4		Year 5	
		Sp (52)	A (50)	Sp (50)	A (50)	Sp (50)	A (50)	Sp (50)	A (50)	Sp (50)	A (50)
Diurnal transect	7	1	4	1	1	2	2	3	1	3	1
Nocturnal transect	NA	2	4	1	2	3	5	2	1	2	1
Diurnal radial	1	0	1	0	0	0	1	0	0	2	0
Nocturnal radial	NA	0	1	0	0	0	2	0	0	0	1
Incidental	1	2	8	11	3	6	4	2	2	6	3

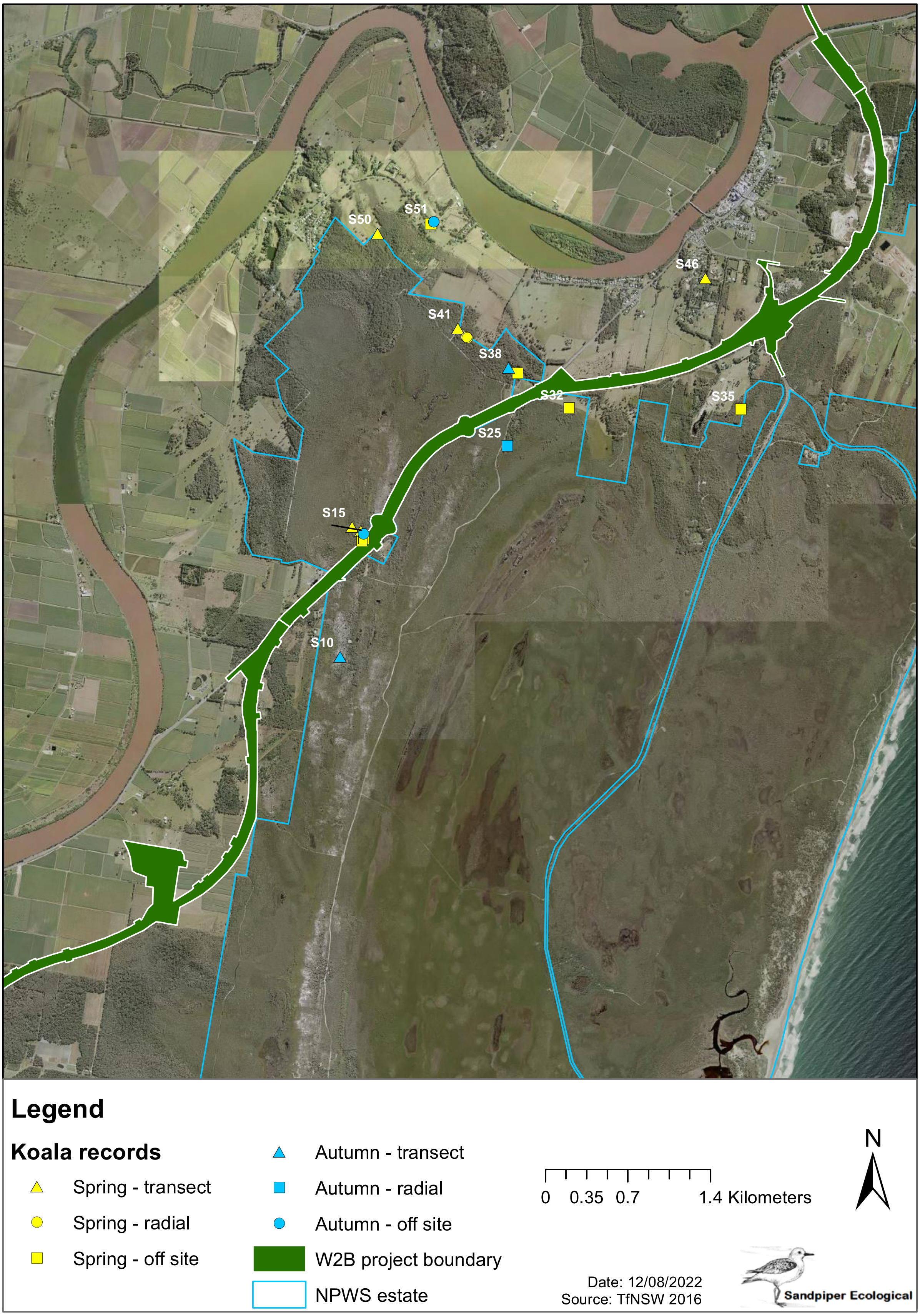

Figure 4: Broadwater survey sites and location of koalas observed during spring 2021 and autumn 2022 surveys.

4.1.2 Bagotville focal area

During spring 2021 surveys, three koalas were observed on transects during both diurnal and nocturnal searches, and four individuals were recorded off-transect (Table 5, Figure 6). No individuals were recorded within the radial plot areas. Of the koalas observed, four males and two females were confirmed, and the remainder could not be sexed. One female at site 73 had advanced back young. The body condition of individuals' that could be viewed was generally good except one individual at site 20 which had minor evidence of a "dirty tail". Transects affected by the spring 2019 wildfire were in varying stages of recovery. Over the spring and autumn surveys koalas were recorded on two previously burnt transects (#28 & 33).

During autumn 2022 surveys, six koalas were observed on transect during diurnal searches and four during night searches (Table 5). No individuals were observed within radial plot areas. An adult female recorded on transect 21 had back young. A further six koalas were observed incidentally off-transect while moving between sites. The body condition of individuals that could be viewed was generally good except one individual at site 34, which had evidence of a "dirty tail".

Full details of Bagotville koala observations are provided in Table B2, Appendix B.

Table 3: Bagotville focal area koala observations - baseline to year 5 (2021/2022).

Time & type	Baseline (46)	**Baseline (42)**	Year 1		Year 2		Year 3		Year 4		Year 5	
			Sp (43)	A (50)	Sp (50)	A (50)	Sp (50)	A (50)	Sp (50)	A (49)	Sp (48)	A (46)
Diurnal transect	3	NA	2	5	3	3	6	5	3	3	3	6
Nocturnal transect	NA	NA	3	5	5	3	5	4	5	4	3	4
Diurnal radial	NA	1	0	1	1	0	1	0	1	0	0	0
Nocturnal radial	NA	NA	0	1	2	0	0	0	0	0	0	0
Incidental	5	NA	5	8	4	3	6	3	4	4	4	6

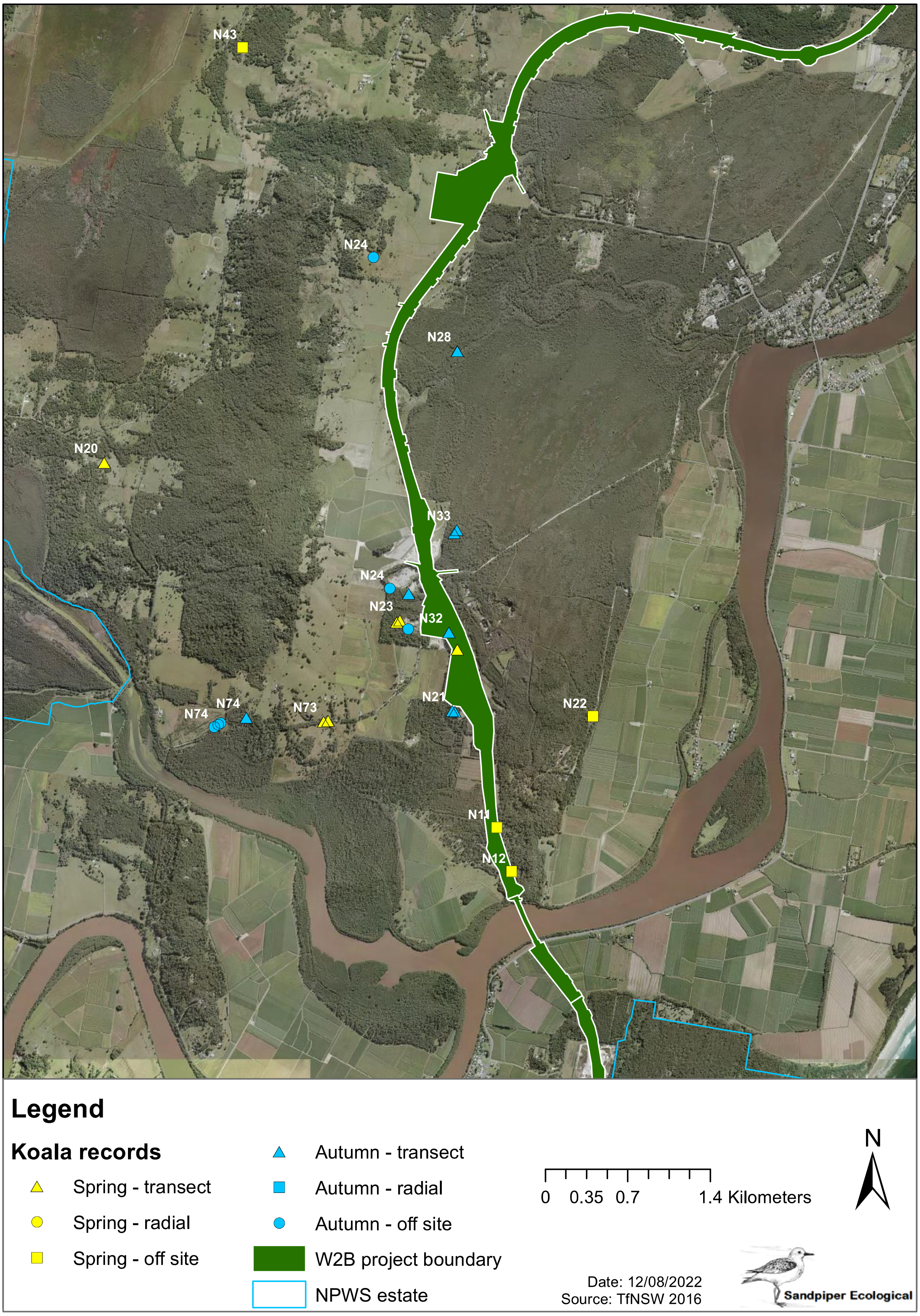

Figure 5: Bagotville survey sites and location of koalas observed during spring 2021 and autumn 2022 surveys.

4.2 Koala density, population size estimate and trend estimate

4.2.1 Broadwater

Based on the Bayesian estimation analysis, the density estimate for spring 2021 was 0.045 koalas ha^{-1} (95%CI: 0.031-0.064) and autumn 2022 was 0.045 koalas ha^{-1} (95%CI: 0.031-0.063). Overall, the Year 5 density estimate for Broadwater was 0.045 koalas ha^{-1} (95%CI: 0.031-0.063). This compares to a modeled baseline density estimate of 0.062 (95%CI: 0.041-0.089) koalas ha^{-1} (Figure 7).

The estimated trend in density estimates at Broadwater was a 3.3%/year decline (SE: 0.041; 95%CI -0.124-0.023) with a 0.752 posterior probability of a decline. These values are almost identical to the year 4 estimates, but with slightly less uncertainty (e.g., compare the Year 5 SE of 0.041 vs 0.043 in the Year 4 report). The hypothesis-testing posterior odds ratio (Bayes Factor) was 1.97, which is slight evidence of a declining trend. However, according to conventional categories, a value of 1.97 is considered 'barely worth mentioning' (Jeffreys 1961; Kass & Raftery 1995).

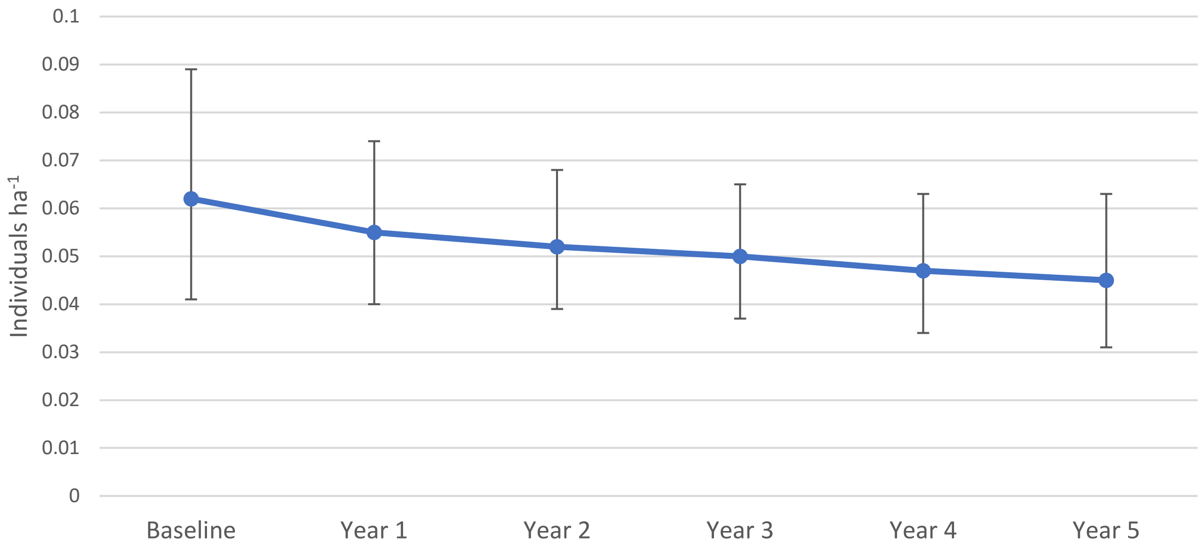

Figure 6: Comparison of Broadwater focal area (Bayesian) density estimates (± 95%CI) for the modeled baseline and monitoring years.

4.2.2 Bagotville

Based on the Bayesian estimation analysis, the density estimate at Bagotville for spring 2021 was 0.080 koalas ha^{-1} (95%CI: 0.058-0.107) and for autumn 2022 was 0.080 koalas ha^{-1} (95%CI: 0.058-0.106). The overall Year 5 density estimate was 0.080 koalas ha^{-1} (95%CI: 0.059-0.105). This compares to a modeled baseline density estimate of 0.082 (95%CI: 0.058-0.111) koalas ha^{-1} (Figure 8). The estimated trend in density estimates at Bagotville was nil/year change (SE: 0.023; 95%CI -0.060-0.053) with a 0.512 posterior probability of a decline. The hypothesis-testing posterior odds ratio (Bayes Factor) was 0.72, which is slight evidence against a decline. However, according to conventional categories, a value of 0.72 is considered 'barely worth mentioning' (Jeffreys 1961; Kass & Raftery 1995).

Extrapolated population size estimate for year 5 overall was 171 koalas (95%CI: 122-239) across 2,135 ha of preferred koala habitat (Figure 9). This compares to a modeled extrapolated baseline population estimate of 174 koalas (95%CI: 122-237) across 2,152 ha.

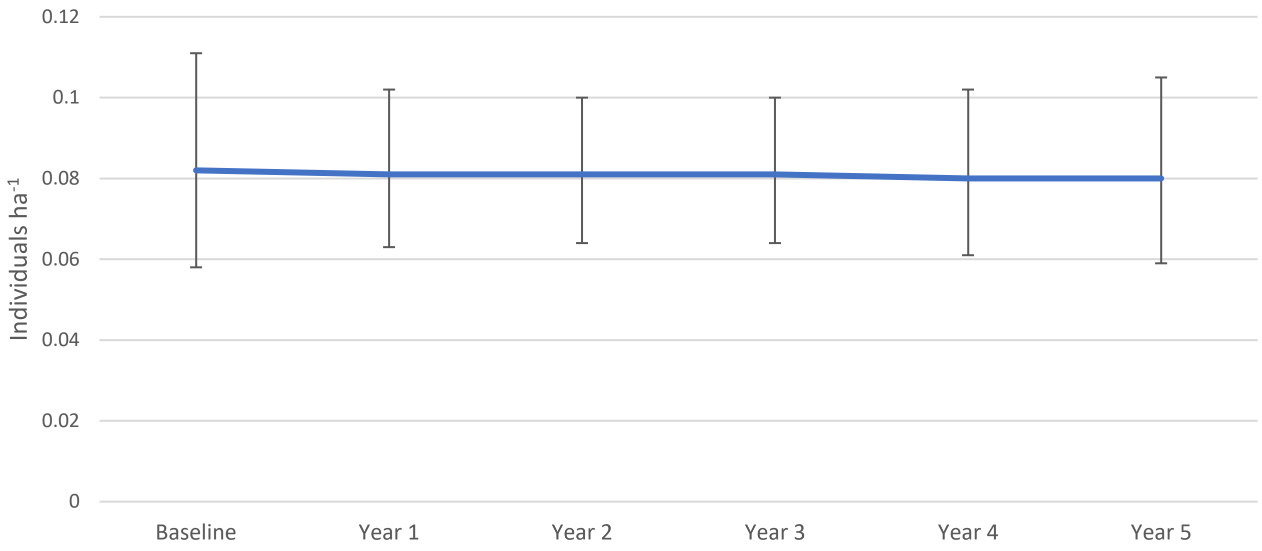

Figure 7: Comparison of Bagotville focal area (Bayesian) density estimates (± 95%CI) for the modeled baseline and monitoring years.

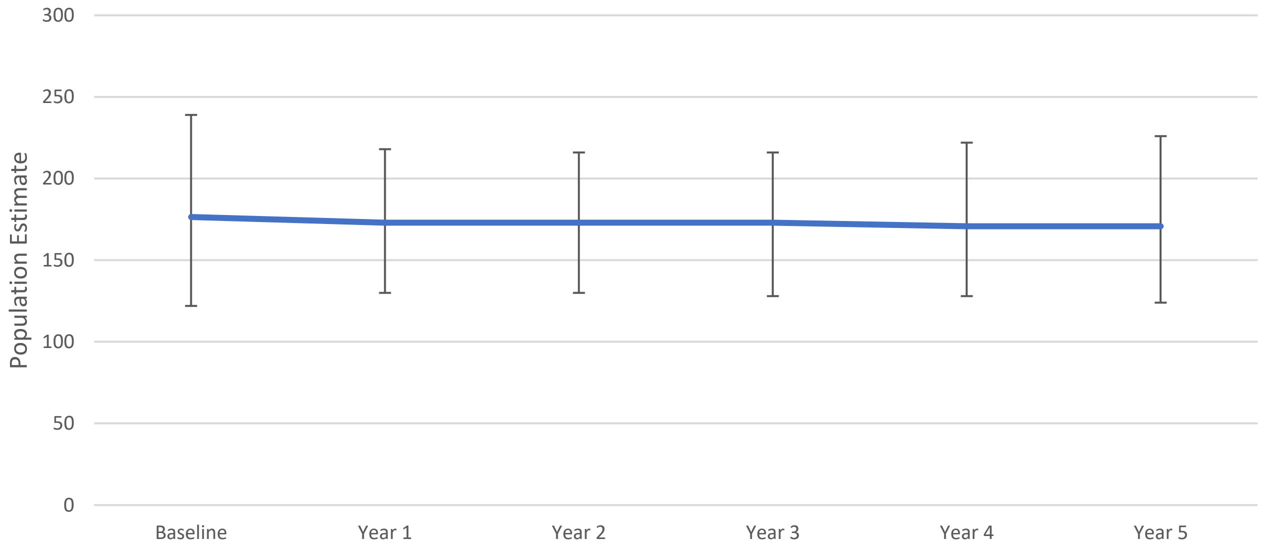

Figure 8: Comparison of Bagotville focal area population estimates (± 95%CI) for the modeled baseline and monitoring years. Population estimates are based on 2152 ha (baseline) and 2135 ha (monitoring years) of preferred koala habitat, as informed by the PVA (Kavanagh 2016).

4.3 Power analysis

For a maximum Type-I error rate of 0.3, the estimated power for Broadwater and Bagotville were 0.657 and 0.738, respectively (Figure 10). The Broadwater power decreased slightly from the Year 4 report (previously estimated to be 0.661), and improved slight for Bagotville (previously 0.726). For a maximum Type-I error rate of 0.35, the estimated power for Broadwater and Bagotville were 0.694 and 0.770 respectively. The Broadwater power decreased slightly from the Year 4 report (0.702), and improved slight for Bagotville (0.764).

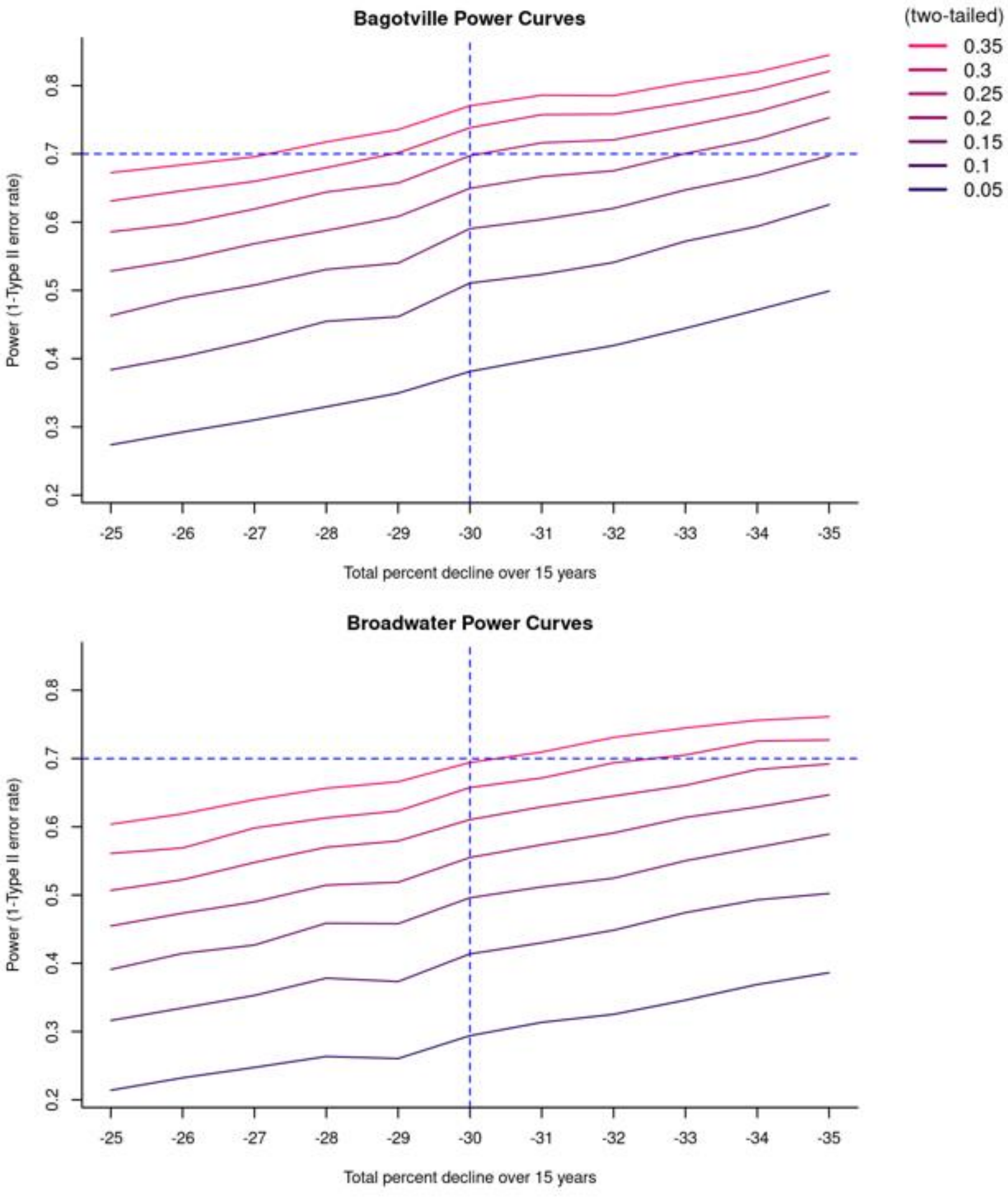

Figure 9: Statistical power to detect a 30% decline in baseline densities over a 15-year monitoring period for different maximum levels of Type-I errors (lines).

4.4 DNA extraction and analysis

4.4.1 Genetic diversity

Genetic diversity values, estimated through expected heterozygosity and allelic richness, were compared between the 2018, 2020 and 2022 surveys (Table 4) and revealed low to moderate diversity of local koalas has been maintained throughout the 2018, 2020 and 2022 surveys. The 2022 survey revealed a reduction in allelic diversity resulting in a decrease of mean number of alleles per locus, compared with 2018 and 2020 surveys. Whilst this result could indicate that koalas within the survey site are becoming isolated, variability in samples between years is likely to influence results. Despite a significant reduction of inbreeding observed in the 2020 survey, the 2022 survey revealed moderate to high inbreeding of koalas indicating relatively recent reductions in population size or gene flow within and between local koala populations, further suggesting the koalas within the study site are becoming isolated. Individual heterozygosity has not significantly deviated between the three samples. In 2022 individual animal heterozygosity varied from 78.0% down to 37.5%, with a median value of 56.3%. For comparison in 2020 individual animal heterozygosity ranged from 80.6% down to 41.9%, with a median value of 60.0%; and in 2018 individual heterozygosity ranged from 75.0% down to 35.5%, with a median value of 53.1%.

Table 4: Genetic diversity statistics for the three Bagotville population samples based on 30 loci. Allelic richness, which is the number of alleles per locus corrected for sample size to enable comparison among populations, was estimated for n=11. N: Number of individuals sampled; A_{mean}: Mean number of alleles per locus; A_r: Allelic richness; H_o: Observed heterozygosity; H_e: Expected heterozygosity; F_{IS}: Inbreeding coefficient - the proportion of variance in a population that is contained within an individual; F_{IS} >0 indicates high levels of homozygosity and can suggest inbreeding.

Population	N	A_{mean}	A_r	F_{IS}	H_o	H_e
2018 Survey	19	6.63	4.19	0.204	0.539	0.672
2020 Survey	22	6.16	3.94	0.114	0.594	0.655
2022 Survey	11	5.59	4.26	0.205	0.555	0.687

4.4.2 Pairwise genetic differentiation

Table 5 presents genetic differentiation between the 2022, 2020 and 2018 northern NSW koala population and regional koala populations. There is weak differentiation between the three Bagotville samples indicating gene flow has occurred over time within the study area. The degree of genetic differentiation between the 2022 northern NSW population and regional populations increases with distance. The Bagotville population has low genetic differentiation to Byron Bay, Lismore and Tweed koalas but increasing differentiation to Sunshine Coast and Oakey populations, and the island population on St Bees.

Table 5: Pairwise FST values between 2022, 2020, and 2018 northern NSW koala surveys and regional koala populations.

	2020 Survey	2018 Survey	Grandchester	Sunshine Coast	Gold Coast	Byron Bay	Lismore	Tweed	Clark Connors	Mt Byron	Oakey	St Bees	Yarrabilba
2022 Survey	0.027	0.011	0.152	0.146	0.119	0.146	0.130	0.115	0.165	0.162	0.191	0.240	0.117
2020 Survey		0.002	0.178	0.165	0.132	0.172	0.138	0.142	0.201	0.187	0.214	0.258	0.149
2018 Survey			0.161	0.153	0.122	0.157	0.131	0.127	0.186	0.176	0.192	0.242	0.134

	2020 Survey	2018 Survey	Grandchester	Sunshine Coast	Gold Coast	Byron Bay	Lismore	Tweed	Clark Connors	Mt Byron	Oakey	St Bees	Yarrabilba
Grandchester				0.058	0.066	0.102	0.119	0.071	0.102	0.095	0.119	0.158	0.065
Sunshine Coast					0.048	0.075	0.092	0.054	0.074	0.052	0.080	0.125	0.062
Gold Coast						0.033	0.063	0.020	0.091	0.082	0.089	0.141	0.052
Byron Bay							0.059	0.025	0.122	0.120	0.155	0.193	0.076
Lismore								0.035	0.141	0.133	0.162	0.200	0.094
Tweed									0.089	0.097	0.105	0.166	0.055
Clark Connors										0.090	0.098	0.075	0.091
Mt Byron											0.069	0.153	0.083
Oakey												0.169	0.103
St Bees													0.155

<0.05 = **weak** genetic differentiation

0.05-0.15 = **moderate** genetic differentiation

0.15-0.25 = **strong** genetic differentiation

>0.25 = **very strong** genetic differentiation

Pairwise comparison of genetic relatedness between the east and west sides of the Pacific Highway using all samples collected from 2018-2022 showed weak differentiation (F_{ST} <0.05) indicating overall evidence of geneflow. Evidence of moderate genetic differentiation (F_{ST} between 0.05 and 0.15) between east sites in 2018 and west sites in 2020, and west sites in 2020 and east sites in 2022 was recorded (Table 6). These differences may be due to the location of west side 2020 samples which included a cluster of samples further west than in 2018 and 2022.

Table 6: Pairwise F_{ST} values between 2022, 2020 and 2018 for scats samples collected in the Bagotville area of northern NSW.

	West of Pacific Highway		West of Pacific Highway 2018	East of Pacific Highway 2020	West of Pacific Highway 2020	East of Pacific Highway 2022	West of Pacific Highway 2022
East of Pacific Highway	0.0254	East of Pacific Highway 2018	0.0366	0.0038	0.0871	0.0000	0.0224
		West of Pacific Highway 2018		0.0151	0.0115	0.0365	0.0000
		East of Pacific Highway 2020			0.0383	0.0064	0.0186
		West of Pacific Highway 2020				0.0860	0.0346
		East of Pacific Highway 2022					0.0000

4.4.3 Genetic relatedness

Genetic relatedness was estimated and compared for the 2018, 2020 and 2022 northern NSW koala populations separately. Figure 11 presents the average relatedness for each survey and revealed a wider distribution in relatedness values for koalas identified in the 2022 survey, compared to the 2018 and 2020 surveys. Noticeably, the 2020 data shows a mean relatedness that was higher than the confidence interval, suggesting that koalas are significantly more related than expected. In Figure 11, red lines indicate the upper

(U) and lower (L) 95% confidence interval expected for that population under the null hypothesis of no difference among populations and r = relatedness.

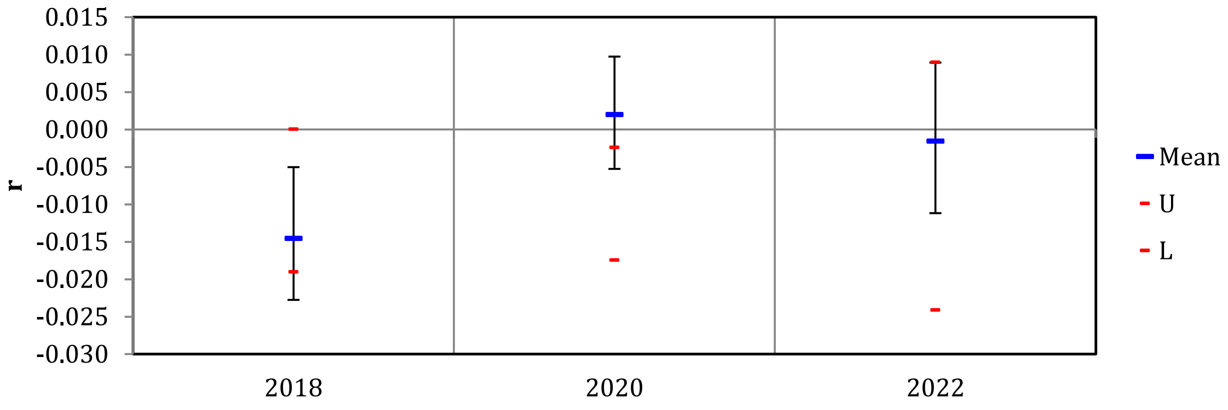

Figure 10: Mean genetic relatedness (r) for 2018, 2020 and 2022 koala site surveys.

4.4.3 Population structure

STRUCTURE analysis identified two genetic clusters of koalas in both the 2022 and 2018 surveys, with both surveys comparable to each other (K = 2, Figures 12 and 14, respectively) compared to genetic clusters from the 2020 samples (K = 3, Figure 13). The notion of a genetic cluster is that individuals within the cluster share on average more similar allele frequencies to each other than to those in other clusters. Each bar (in Figures 12-14) represents an individual scat sample and colours indicate the proportion of the population cluster to which an individual was assigned. The additional genetic cluster identified from the 2020 sample suggested evidence of gene flow occurring within the population; however, the 2022 sample identified reduced genetic clustering, indicative of reduced gene flow and genetic isolation of the population.

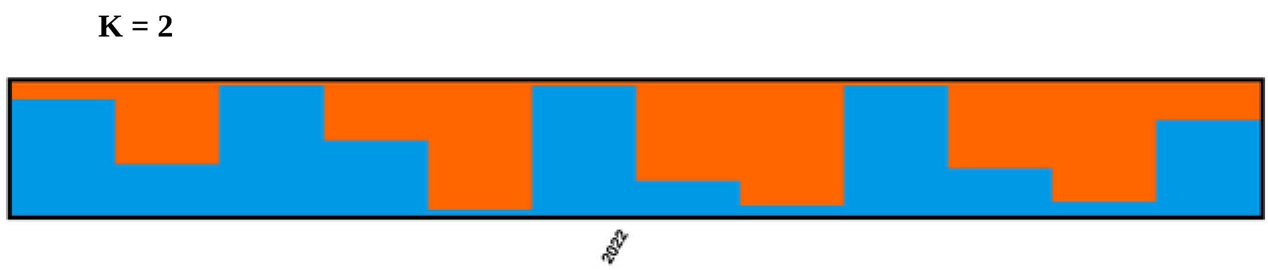

Figure 11: Population substructure of 2022 northern NSW koala populations using STRUCTURE based on 32 loci.

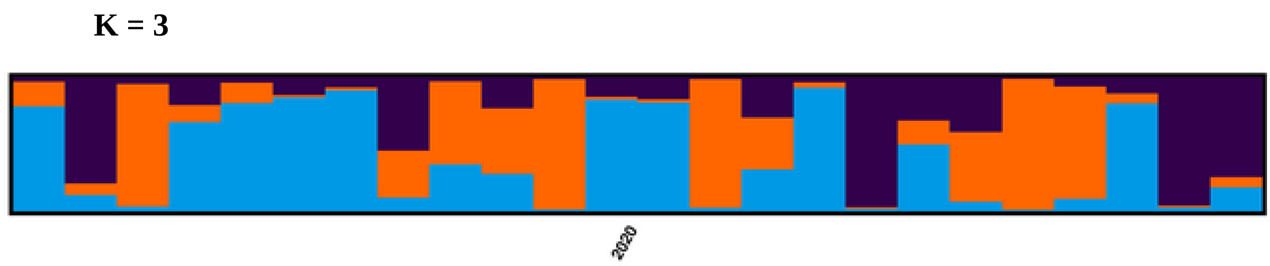

Figure 12: Population substructure of 2020 northern NSW koala populations using STRUCTURE based on 32 loci.

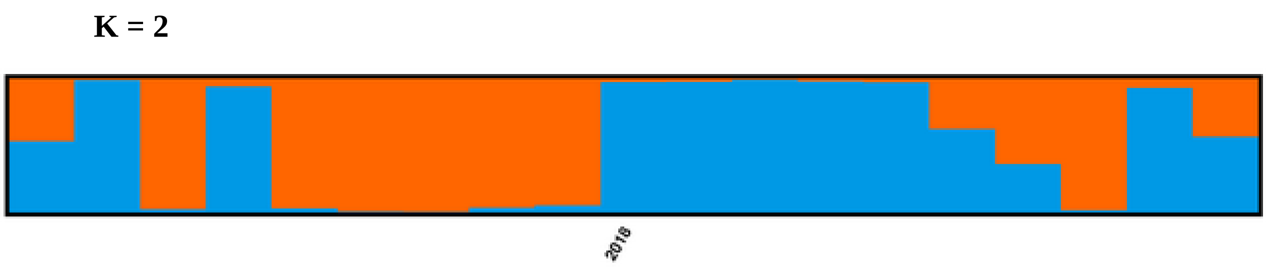

Figure 13: Population substructure of 2018 northern NSW koala populations using STRUCTURE based on 32 loci.

Figure 15 A, B and C depict each scat sample location represented by a pie chart. The pie chart details that individual's proportional assignment to each of the clusters from the STRUCTURE analysis (i.e., Figure 12).

4.4.4 Effective population size

Determination of sex was tested using Y-linked markers designed to amplify a 569-bp region of sex determining region of the Y chromosome (SRY gene). Effective population size (N_e) was estimated using the molecular co-ancestry method of Nomura (2008), as implemented in NeEstimator V2.1 (Do et al., 2014).

Effective population size estimates of males and females from 2018, 2020 and 2022 koala surveys are presented in Table 7. Based on the sample sizes for both male and female koalas for all survey years, the confidence intervals for females in 2018 and 2020 are not notably wide indicating the N_e value is informative enough to predict the effective population size. However, N_e predicted within the male cohort for each survey year is infinity, indicating there is no evidence for variation in the genetic characteristic caused by a finite number of parents and can be due to sampling error. Assessment of heterozygote excess (D) returned negative values for all cohorts, except for the male cohort in 2018, indicating a deficit of heterozygote samples in populations and therefore a difference in allele frequencies between population males and females.

Table 7: Effective population size of males and females for NSW koalas. n = Number of samples; Ne: Effective population size (P = 0.05); 95% CI: 95% confidence interval; and D: Heterozygote excess estimate.

Year	Population	n	N_e (P=0.05)	95%CI		D
2018	Female	14	14.8	11.6	19.7	-0.310
	Male	5	∞	14.5	∞	0.041
2020	Female	19	12.5	10.8	14.5	-0.165
	Male	5	∞	30.9	∞	-0.043
2022	Female	6	30.4	10.4	∞	-0.195
	Male	5	∞	∞	∞	-0.097

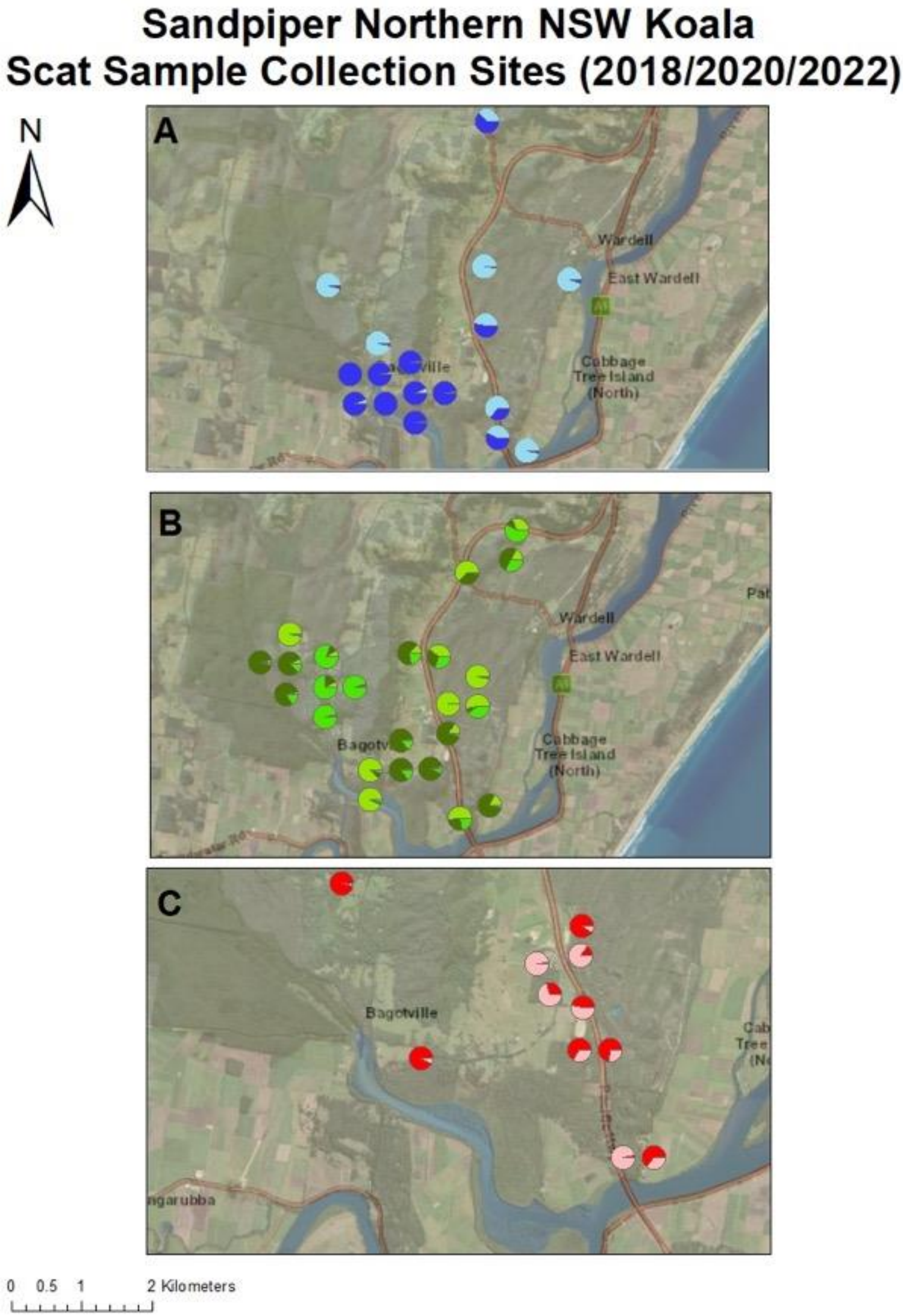

Figure 14: Inferred cluster assignments of (A) 2018 (K = 2); (B) 2020 (K = 3) and (C) 2022 (K = 2) northern NSW koalas.

4.5 Road mortality surveys and fauna fence condition

4.5.1 Road mortality

No koalas were recorded during road mortality surveys in August and November 2021 (Tables D1 & D2, Appendix D). Road mortality surveys detected 56 individuals in August and 63 individuals in November 2021, with 54 and 60 individuals recorded in sections 1-11 during the respective periods. Three individuals were recorded on Wardell Road during each period and one individual was recorded on the old Pacific Highway in August. Unless carcasses are fresh accurate identification of fauna is difficult from vehicle-based surveys. Koala falls into the "medium mammal" category, and seven and 13 "medium mammals" were recorded in August and November respectively. A further five "unidentified mammals" were recorded in August.

The likelihood that the "medium" or "unidentified mammal" categories include a koala is possible yet unlikely. Pelage colour is a key diagnostic feature used during road mortality surveys and mammals with grey pelage, which could be koala, would be inspected more closely. Most of the medium and unidentified mammals had dark pelage and were likely, bandicoots, short-eared brushtail possum, or remnants of swamp wallaby.

Twenty species were recorded during road mortality surveys, including 11 species of bird, six species of mammal, two species of reptile and one amphibian (Appendix D). A further 14 fauna groups were identified. The density of road-killed mammals within sections 1-11 ranged from 0.23 – 0.25/km (Table 6). Wardell Road had the highest density of 1.29 individuals/km. No mammals were recorded on the old Pacific Highway.

Table 8: Road mortalities recorded during two surveys conducted in spring/summer 2021. * = excludes bats

Location (survey distance)	August 2021			November 2021		
	Total roadkill	Number of mammals*	Mammal Roadkill/km	Total roadkill	Number of mammals*	Mammal Roadkill/km
Wardell Road (1.54 km)	2	0	0	3	2	1.29
Old Pacific Highway (3.3 km)	0	0	0	0	0	0
Sections 1-11 (155 km)	54	35	0.23	60	38	0.25
Total (159.84 km)	56	35		63	40	

One koala road mortality was recorded by TfNSW Roads Maintenance Division on 8/9/2021 on the northbound carriageway south of Devils Pulpit, at chainage 104500 -approximate location E 520941, N 6760800 (GDA94). The fence was inspected in the vicinity of the strike and no breaches were detected. Friends of the Koala had not recorded any vehicle strike within the sampled sections of road between July 2021 and June 2022.

4.5.2 Fauna fence

No detectable breaches were observed in fauna fence on Wardell Road, old Pacific Highway or along sections 1-11 of the Pacific Highway.

5. Discussion

5.1 Koala population surveys

5.1.1 Koala counts, density estimates and trend estimates

Broadwater

The aggregate count of koalas (i.e., spring + autumn) in the Broadwater focal area during year 5 was the same as year 4, like year 2, and less than during the baseline, years 1 and 3. The number recorded during the baseline is expected to be higher due to greater survey effort at that time. The number recorded in spring year 5 was equivalent to spring years 3 and 4. Numbers declined substantially during the autumn sample, which was consistent with year 4. Bayesian modelling of density, which largely controls for differences in survey effort between survey periods, suggests a downward trend in koala density from baseline to year 5 {i.e., from 0.062 koalas ha^{-1} (95%CI: 0.041-0.089) to 0.045 koalas ha^{-1} (95%CI: 0.031-0.063)}. Analysis shows increasing evidence of a downward trend, however, the strength of the trend remains weak.

Overall, at Broadwater, the estimated densities in year 5 were lower than previous years and the downward trend was more pronounced. The estimate of the year 5 density at Broadwater was 0.045 koalas/ha (SE: 0.008; CI: 0.031-0.063), which was lower than the preceding years. The estimated trend (-3.3%/a) was equivalent year 4 and double the magnitude of the year 3 analysis (-1.6%/year). However, given the uncertainty in the estimates, the Bayes Factors do not provide strong evidence of a decline.

Rankin (2022) also presented supplemental analysis using frequentist model-averaging. The AICc-based model-averaged estimate showed a 13.5%/year decline with a Fisher p-value against a decline of 0.184. The estimated percentage decline is half that recorded in year 4 and the p-value is much higher than year 4 (0.094) and is moving in the direction of accepting the “no-trend” null hypothesis. Rankin (2022) points out “For both odds-ratios and Fisher p-values, the evidence of a trend is weak and undermined by high variance and data-sparsity.”

Despite a high degree of variability in results there is consistent evidence of a decline at Broadwater, with a Bayesian trend estimate that is nearly identical to that produced in Year 4 (Sandpiper Ecological 2021). The sequence of trend estimates from the Year 3 analysis to Year 5 suggests a steadily narrowing statistical certainty about a population decline.

The AICc-based methods continue to estimate a very large decline at Broadwater, despite the relatively high survey counts during Year 5. The AICc-based Year 5 densities (0.036 koalas/ha) are less than half the values at Baseline (0.087). However, the sequence of AICc-based trend estimates have continued to moderate over time: from an alarming -43%/year in the Year 2 report, to -13%/year estimated in the present report. The latter estimate is still very large for a mammalian population, but we anticipate that as more data are collected, the AICc-based estimates and the Bayesian estimate will slowly converge (assuming no strong deterioration in the underlying population).

Bagotville

Compared with Broadwater, counts for the Bagotville focal area have been relatively consistent across the survey period. The aggregate count (i.e., spring + autumn) in year 5 (16 individuals) was equivalent to years 1 (15 individuals), 2 (14 individuals) and 4 (15 individuals) and less than year 3 (20 individuals). Density estimates have been stable between the baseline {0.082/ha^{-1} (95%CI: 0.058-0.111)} and year 5 {0.080/ha^{-1} (95%CI: 0.059-0.105)} and the per-year densities have lower overall standard errors, suggesting improving certainty in the estimates. There is no evidence of a trend, which was estimated to be 0.001%/year and had a high frequentist p- value of 0.512. There is increasing confidence that the koala population at Bagotville is stable (Rankin 2022).

5.1.2 Power analysis

The current update to the prospective power analysis found that Bagotville has exceeded the 0.70 target, while Broadwater remains below the target threshold with an estimated power of 0.657, slightly lower than the year 4 value. The power analysis relies heavily on the empirical estimates from the other analyses which, given the high uncertainty in the density estimates and covariate-effects, are likely contributing to a persistent inability to gain higher statistical power.

Improvements in statistical power has not been as large as the improvements in other statistical measures, such as declining p-values or reductions in standard errors of density estimates. Statistical power is sensitive to the overall uncertainty in the entire system because it incorporates empirical estimates of variance in the Markov Chain Monte-Carlo (MCMC) routine (Rankin 2022).

To improve statistical power and improve statistical certainty at Broadwater it may be necessary to add more transects. An alternative method to boost power, without adding more sites, could be to remove sites whose habitat is not suitable for koalas. In other words, reduce the dilution on estimates from zero-koala sites. Further discussion on this option is included in Section 5.4.

5.1.3 Catastrophic events and other exogenous factors

As with any long-term population monitoring program, the focal koala populations may be affected by a range of catastrophic events and exogenous factors outside of the control of the upgrade project. The wildfire that burnt through approximately 470 ha of the Ngunya Jargoon IPA was one such catastrophic event. It followed a wildfire in the eastern part of the Ngunya Jargoon IPA that burnt 350 ha in September 2017. The PVA modelling for Bagotville estimated catastrophic fire events at a frequency of once every 35 years with each event encompassing only 10% (i.e., 215 ha) of the 2152 ha study area (Kavanagh 2016). However, within the first three years of the monitoring program wildfire has occurred twice and encompassed 16-22% of the study area on each occasion. This suggests that the frequency and extent of wildfire modelled in the PVA was underestimated.

The other ‘catastrophe’ input in the PVA was drought (Kavanagh 2016). Drought was modelled to occur at a frequency of every 4-5 years. Records from the closest long-term weather station (i.e., Bureau of Meteorology Weather Station No. 58171, Meerschaum Vale) show that for the first three years of the monitoring program (i.e., July 2017 to June 2020) annual rainfall totals were 16.4% - 21.8% below average. Moreover, the calendar year of 2019 was 44.2% below average and the latter half of 2019 was by Bureau of Meteorology definitions a “serious to severe drought”. It was also the lowest annual rainfall total on record (since records began in 1977). Further monitoring years will be required to determine the veracity of PVA drought predictions.

Successive La-Nina years from 2020-2022 resulted in above average rainfall with cumulative rainfall totals exceeding the long-term average by 19%, 16% and 40% in 2020, 2021 and 2022 respectively. The 2022 value of 2779.2 mm does not include data for November or December. In March 2022 large parts of the study area were inundated by a major flood event with some transects remaining inundated for several weeks. Flooding was not included as a ‘catastrophe’ input in the PVA (Kavanagh 2016). Whilst floods are likely to have a less severe impact than drought and fire some negative effects are likely particularly when feed trees remain inundated for long periods of time. Two dead koalas were recorded during the Feb/March flood event, both on Old Bagotville Road. The origin of these individuals is unknown (M. Mathes pers comm).

Other exogenous factors may include local land development, clearing activities, euthanasia of diseased individuals, and the emergence of other diseases and/or pathogens. One such pathogen – myrtle rust – was observed in and around site 14 during autumn 2020 surveys. Myrtle rust is a fungal pathogen that infects plants in the Myrtaceae family, which includes plants of the genus *Eucalyptus* (DPI NSW 2015). The potential impact on koalas would primarily be the loss of food resources within infected areas. Infection has not been observed at other sites. To reduce the risk of spreading myrtle rust the site 14 transect was shifted from the infested area prior to the spring year 4 survey.

5.2 Genetic analysis

Genetic analysis has shown that the allelic mean (i.e., mean number of alleles per locus) and Allelic richness (i.e., number of alleles per locus) were higher in each of the population monitoring samples than during the baseline (Neaves *et al.* 2015). In addition, the baseline survey did not identify any genetic structuring within the population whereas 2-3 genetic clusters have been identified from population monitoring samples (Neaves *et al.* 2015; Hulse 2022).

Genetic analysis of koala scats collected in years 1, 3 and 5 of the monitoring program (Hulse 2018, 2020, 2022) indicates some slight concerning trends, including:

1. Weak genetic differentiation within the sample population – indicating minimal gene flow.
2. Moderate to strong genetic differentiation to other regional populations – indicating minimal gene flow with other populations.
3. Low-moderate genetic diversity within the population – indicating that inbreeding and isolation is occurring.

The absence of a systematic approach to scat collection has resulted in variability in the location and number of samples collected between years and persistent wet weather in 2021/22 exacerbated this effect. The slight trends identified from genetic analysis can be attributed to the sampling protocol. Samples collected in year 5 (2021/22) were from a smaller geographic area and included fewer samples from east of the highway. In contrast, 2020 samples occurred throughout the study area and included almost equal sample numbers east and west of the highway and a cluster of samples further west than in 2018 and 2022 (Hulse 2022). The 2020 samples showed less inbreeding and isolation than the 2022 sample and greater genetic sub-structuring of the population with three genetic clusters recorded. This is likely due to a cluster of samples from the western part of the Bagotville study area. Genetic cluster analysis from the three samples (i.e., 2018, 2020, 2022) showed no genetic differentiation between the east and west sides of the highway. This was supported by pairwise analysis of genetic relatedness, which revealed only weak differentiation between the east and west sides of the highway and suggests there is no genetic differentiation at this stage.

It is likely to take several generations for genetic differences to become apparent between koalas east and west of the highway. In a landmark study Frere *et al.* (2023) found that a koala population sub-divided by a highway could experience between 12% and 69% loss in genetic diversity after 10 generations. They also estimated that a minimum of eight koalas would need to disperse from each side of the highway per generation to maintain genetic connectivity. According to NSW Scientific Committee (2022) generation length of koalas is estimated at 6-8 years and longevity for wild animals at 15 years for females and 12 years for males. To date, monitoring of the Bagotville koala population is unlikely to have covered a sufficient timescale to confirm the occurrence of population isolation and its effect on genetic diversity. The absence of confirmed crossings by koalas under the highway in the Bagotville area is concerning and over time could contribute to genetic isolation.

5.3 Road mortality and fauna fence

5.3.1 Road mortality

Road mortality rates on the upgraded section of the Pacific Highway declined from 2.36 individuals/km during year 2 to 0.26 individuals/km in year 3, 0.17 individuals/km in year 4 and 0.29/km in year 5. The substantial decline in road mortality rates from year 2 is partly due to the change in survey method from foot-based to vehicle-based surveys and likely habituation to the highway by fauna. Detectability trials of car-based surveys found them to be highly effective at detecting medium-sized fauna (see Taylor & Goldingay 2004), however, our experience is that fewer small birds, and reptiles are recorded during vehicle-based surveys. Other factors likely to influence mortality rates, include time since opening and environmental conditions.

The presence of one koala vehicle strike in section 1-11 in 2021/22 is consistent with year 4 when one individual was recorded near the Devils Pulpit rest area. Koala vehicle strikes recorded in years 1, 2 and 3 (see Sandpiper Ecological 2019a, 2019b, 2020) occurred on the old Pacific Highway or local roads near the upgrade alignment. Results suggest that the upgrade has reduced koala vehicle strike on the Pacific Highway. The single year 5 mortality in S1-11 equates to a strike rate of 0.006 individuals/km/year, which is less than a quarter the rate of 0.026 ind/km cited by RMS (2016) for the old Pacific Highway in S10. Both koala mortalities recorded in years 4 and 5 occurred in an area with standard exclusion fence that does not contain metal sheeting or a floppy top. The 2021 record occurred 200m south of a property access and it is possible that a gate was left open, which allowed the koala to access the highway.

5.3.2 Fauna fence

The fauna fence was generally in good repair, although observation has shown that koalas can move through small gaps at gates and the point of entry may not always be obvious. No breach of the fauna fence was detected near chainage 104500 where a road-killed individual was recorded.

5.3.3 Performance indicators

Koala population trends in Sections 8/9 and 10

1. Koala population sizes at or above the minimum expected targets including rate of population change/decline at/above the minimum expected target of 195-276 at five years; 147-272 at 10 years and 103-261at 15 years.
    a. *The year 5 Bagotville koala population estimate of 171 individuals is slightly below the revised Bayesian estimate of 174 individuals.*

Road mortality

1. No injury to an individual koala as a result of vehicle strike across all upgraded sections.
    a. *One koala was struck and killed during the 2021/22 sample period.*
2. Section 10: no koala road mortality within the fenced areas of the upgrade, on existing Pacific Highway or Wardell Road.
    a. *No koala road mortalities observed or reported.*

Fauna exclusion fence.

1. No breaches in fauna exclusion.
    a. *Exclusion fence in the vicinity of the roadkill at chainage 104500 was inspected and no gaps in the fauna fence were recorded.*
    b. *The presence of a koala within the alignment suggests that the fence may have been breached, however, there is a property access gate 200m to the north.*

c. *Vehicle strike occurred in an area with standard exclusion fence that does not include a metal sheet or a floppy top.*

5.4 Program review

5.4.1 Changes to the monitoring program

The ability of the Koala Monitoring Program to detect changes in the koala population is influenced by the low abundance and patchy distribution of koalas (and koala habitat) within both the Broadwater and Bagotville study areas. These issues result in high statistical variance, which has repeatedly been identified as a factor contributing to low statistical power and variable density estimates (Rankin 2022). The influence of low abundance and patchy distribution is evident in the analysis of Preece and Rhodes (2016) who conducted an *apriori* power analysis on baseline data and found that to detect a 10% decline with 80% certainty over a three-year sample period more than 20,000 sample sites would be required in each study area.

Despite some limitations, the monitoring program is achieving the required objective of 70% power to detect a 30% decline in koala abundance in the Bagotville focal area and has identified a high degree of stability in that area's koala population. Monitoring of the Broadwater focal area is not achieving 70% power.

Any change to the monitoring method should be carefully evaluated as data collected by a new method must be comparable to the baseline sample. Changing methods during a long-term monitoring program is generally not recommended, however, limitations of the existing method and advances in technology mean that consideration of alternatives is warranted. Such an approach is also consistent with the Koala Management Plan (RMS 2016).

Some alternate methods that could be considered to replace walk transects include:

1. Remotely Piloted Aircraft Systems (RPAS) or drones, equipped with thermal sensors.
2. Acoustic surveys using song meters.
3. A targeted transect sampling design focused on areas with koala habitat.

RPAS is the most viable alternative as it is being widely used for koala surveys and provides an opportunity to collect equivalent data more efficiently than the present design. Song meters have proven effective in surveying koalas (see Law *et al.* 2019 & 2022), and with further research may become a viable means of monitoring density when existing population data are available as is the case at Bagotville and Broadwater. Song meters may collect comparable data to human surveys at a more cost-effective rate, however, they may not increase statistical power to the point where survey duration could be reduced. The focus of song-meters on bellowing males would make direct comparison difficult as baseline data would need to be adjusted to include males only.

A more targeted approach to transect sampling to reduce the dilution on estimates from zero-koala sites would introduce sampling bias and compromise survey results. To avoid such bias, the removal process would ideally pick sites while blind to their counts of koalas. For example, the removal process could be based on an objective "koala habitat index" that is independent of the actual number of koalas present, such as a composite of vegetation/habitat indicators. A simulation-study may be useful to investigate the effects on power from targeted removal of sites vs. adding more sites. A major limitation on using vegetation/habitat indicators is the absence of accurate vegetation mapping, particularly mapping that includes details on the occurrence of primary feed tree species. Any attempt to utilise existing data to reduce survey effort would likely encounter the same power problems identified by Preece and Rhodes (2016).

RPAS

RPAS equipped with a thermal camera are increasingly becoming the preferred method to survey wildlife as they tend to be more cost effective and accurate than human-based surveys (Gonzalez *et al.* 2016). Direct comparison of ground-based (human) and aerial (RPAS) surveys for koalas have concluded that RPAS surveys are less biased (Corcoran *et al.* 2021), more accurate (Witt *et al.* 2020) and more cost-effective (Howell *et al.* 2021). Issues associated with observer error may be overcome as automated detection systems become more refined (Winsen *et al.* 2022).

RPAS surveys typically occur in large plots where the drone is flown in parallel (overlapping) transects to provide near complete coverage of the plot and obtain an accurate population estimate (Spaan *et al.* 2019; McKellar *et al.* 2020; Witt *et al.* 2020). RPAS can also be targeted to smaller areas such as flying-fox camps or wetlands (McCarthy *et al.* 2021; Dundas *et al.* 2021) and they can be programmed to sample smaller belt transects.

Key questions to consider in determining whether RPAS represent a viable alternative to the existing ground-based survey method include:

1. Are density estimates derived from RPAS survey comparable to those derived from ground-based transect surveys?
2. What is the detection probability of RPAS verses ground-based surveys?
3. If RPAS data are comparable to ground-based data will survey power increase and can survey effort be reduced?

There are several means of using RPAS to determine koala density, including:

1. Sampling the entire study area (i.e., the entire area containing existing transects)
2. Sampling random quadrats (i.e., 10-50ha) within the Bagotville and Broadwater study areas
3. Sampling the same 1ha transects used in previous surveys.

Whilst the detection probability of RPAS is likely to be higher than ground surveys (see Witt *et al.* 2020) sampling the same transects is still likely to result in high sample variance as several transects are unlikely to ever support koalas. Indeed, recent RPAS surveys have shown that some large blocks of forest, in the Broadwater study area, containing multiple transects did not support koalas (Sandpiper Ecological in prep).

Whilst RPAS are well suited to landscape scale surveys there are several limitations associated with sampling the entire project area, including: landowner permission, accuracy of koala identification, potential for double-counting due to koala movement within a quadrat and between adjacent quadrats, and assumption that all koalas are being detected. Whilst some of these issues could be overcome by developing a set of decision rules and using an automated detection system obtaining landowner approval across both study areas would be difficult. Limiting surveys to the larger blocks of forest or public land, where access is easy, may introduce a sampling bias as these areas tend to support more koalas. Sampling randomly selected quadrats within each study area would overcome some of the issues associated with large-scale sampling. In essence, a robust assessment and modelling program is required to confirm whether RPAS could replace ground-based human surveys. This program would also determine the level of statistical power associated with RPAS surveys and determine future survey effort.

To determine a way forward, input was sought from Professor Jonathan Rhodes who provided the following advice on two key questions:

1. Is changing methods part-way through a monitoring program feasible?

Yes, but this would have to be dealt with in the statistical modelling by explicitly modelling the two different observation processes. The survey design and analysis would have to be set up from the start to ensure this is possible. One option to help with calibration is to use both approaches initially before switching over to only drones.

2. Whether the type of data collected by drones would be comparable to ground-based surveys and the baseline.

Ideally the information collected by drones should be as similar as possible to the information collected by ground-based surveys. For example, if you are collecting count data now, you'd want to collect count data from the drones as well.

The following actions are recommended to progress the assessment of an alternative survey method:

1. Undertake statistical modelling to compare drone surveys with ground-based surveys to determine if the two methods (can) provide comparable data. Such an exercise would also determine if drones can provide greater statistical power and therefore reduce survey duration. Initially, the existing drone survey data from the Ngunya Jargoon IPA and Broadwater National Park should be used. If these data are not sufficient then additional comparative surveys would be required.
2. Concurrent with the modelling, approval for drone surveys from all affected landowners (i.e., properties with transects and properties within 30m of transects) should be obtained. This is required to determine how many of the existing sample sites could be included in a drone survey.

5.4.2 Population estimates and trend analysis against PVA predictions for the Bagotville study area

Section 8.1 and Table 8-4 of the KMP require the year five program review to "Determine whether the population is tracking according the predictions of the PVA (i.e. whether Koala mortality has been reduced by an initial four animals/year thus slowing population decline such that the population is greater than the lower bound of the 90% confidence interval of the PVA Scenario 6 (195 at year 5, 147 at year 10 and 104 at year 15), which equates to an approximate 1.2% decline over five years, 13.7% decline over 10 years and 27.3% decline over 15 years."

Prior to evaluating year five population estimates it is important to acknowledge that the Bayesian analysis method used to analyse population data recalculates the baseline estimate as more data are acquired (Sandpiper 2020). Benefits and limitations of this approach are discussed in the year three monitoring report. One consequence of using the Bayesian analysis method is that the baseline population estimate derived in year five (and each preceding year) differs to that used in the Population Viability Analysis (PVA, Kavanagh 2016) and stated in the KMP.

The year five population estimate of 171 individuals within the Bagotville focal area is less than the population estimate range of 195-276 specified in the KMP. However, it is only slightly lower than the revised baseline estimate of 174 individuals and well within the 90% confidence interval which ranged from 122-239. The year 5 density estimate of 0.08 koalas ha^{-1} is only slightly less than the "refined" baseline density estimate of 0.09 koalas ha^{-1} and higher than the original ("unrefined") estimate of 0.07 koalas ha^{-1} obtained from similar survey effort (Phillips *et al.,* 2015). Stability in the koala population has occurred despite a severe drought, two wildfires and a severe flood. Both fire and drought were considered to have a 1.5% and 4.8% reduction in breeding success respectively and fire was considered likely to reduce annual survival by 4% (Kavanagh 2016).

Determining if the current population estimates are consistent with PVA predictions is difficult as most projects showed reasonable stability in population size over the first five years. Nonetheless, the result is broadly consistent with the modelled population projection for "road_1.98 dispersal_with revegetation_reduced mortality by 8" of the PVA. High sample variance within and between surveys means it is difficult to make valid comparisons between the initial baseline population estimate and the current estimate. The 40% difference in koala density (i.e., 0.07 to 0.12 koala ha^{-1}) recorded between the two surveys reported by Phillips *et al.* (2015) is a good example of the variation between samples.

Confirming changes in koala mortality is difficult in the absence of quantitative data collected in a systematic manner. Unfortunately, vehicle strike data from local koala care groups was unavailable at the time of completing this report. Consistent and targeted control of feral canids in section 10 since 2017 is also likely to have reduced predation pressure, although the quantitative benefit to the local koala population is unknown.

The absence of koala vehicle strike on roads with exclusion fence (i.e., Wardell Road, old Pacific Highway and new Pacific Highway) over the period 2018-2022 indicates that annual mortality has declined by at least 1-2 individuals per year since exclusion fence was installed. This is likely to benefit the long-term population trend.

5.4.3 Consideration of any population information resulting from genetic analysis undertaken for the project.

Genetic analysis of faecal pellets has been compromised by the sample method (i.e., opportunistic collection), and weather conditions. The 2022 analysis concluded that there is limited genetic exchange between the Bagotville population and other nearby populations and there was some evidence of inbreeding within the population. The degree of isolation between Bagotville and other regional populations has remained stable throughout the monitoring program and is consistent with published data (Dennison *et al.* 2017). Genetic analysis of scats collected in 2022 was skewed by a small sample size and limited geographic extent of sampling. This has contributed to the slight evidence of genetic isolation east and west of the highway, differences in the effective koala population between sample years and evidence of inbreeding. Genetic analysis of faecal pellets represents a power method of determining if the highway has isolated koalas, however, it is too early to draw conclusions and a more robust systematic sampling program is required to ensure confidence in analysis results.

5.4.4 A review of road-kill data obtained from rehabilitation groups

At the time of preparing this report data on koala mortality from vehicle strikes in the study area were unavailable. These data will be included in subsequent monitoring reports.

5.4.5 Requirement for corrective actions in accordance with the KMP

The requirement for corrective actions relating to koala population and vehicle strike monitoring are summarised in Tables 7 (Section 10) and 8 (Sections 1-9). Actions relating to fauna crossing structures, fauna exclusion fence and predator attack are addressed in a separate report (Sandpiper Ecological in prep) and the action relating to koala use of food tree plantations has not been triggered. No corrective actions are recommended based on findings of the year five monitoring period, however, remedial action will be required should another koala vehicle strike occur in the Devils Pulpit area (Table 8).

Table 9: Assessment of corrective actions for section 10.

Performance measure	Performance threshold	Threshold achieved	Corrective action required
Koala population trend	Koala population sizes at or above the minimum expected targets including rate of population change/decline at/above the minimum expected target of 195-276 at five years in Section 10	Yes – koala population within section 10 is within the 90% confidence interval and remains stable declining from 176 individuals during the baseline to 171 individuals in year 5.	No
Road mortality	No koala road mortality within the fenced areas of the Upgrade, on existing Pacific Highway or Wardell Road.	Yes – no koala vehicle strike detected during the first five years of monitoring.	No

Table 10: Assessment of corrective actions for section 8/9.

Performance measure	Performance threshold	Threshold achieved	Corrective action required
Koala population trend	No significant decline in koala population (within sections 8/9) at year 15	N/A – statistical analysis of koala population to occur at year 15.	No
Road mortality	No injury to an individual Koala as a result of vehicle strike.	No – Two koalas have been struck and killed by vehicles in the Devils Pulpit area in 2020 and 2021.	Yes – exclusion fence in the vicinity of vehicle strike was inspected within 3 days. Retro-fitting of exclusion fence has not been required at this stage. Should additional koala vehicle strike occur in the Devils Pulpit area retrofitting of exclusion fence to exclude koalas should be considered.

6. Recommendations

The following actions are recommended for year six of the koala monitoring program:

1. Continue the koala population monitoring program in year 6 (2022/23).
2. Continue collecting koala faecal pellets for genetic analysis.
3. Undertake concurrent koala drone surveys with ground-based koala surveys during the spring year 6 sample period and compare results of the two methods.
4. Undertake statistical modelling to compare drone surveys with ground-based surveys to determine if the two methods (can) provide comparable data. Such an exercise would also determine if drones can provide greater statistical power and therefore reduce survey duration.
5. Depending on the findings of underpass monitoring and statistical modelling of drone verses ground-based surveys consider implementing a more robust scat sampling and genetic analysis program.

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Appendix A: Bayesian estimation analysis and power analysis report Year 5

Year 5 Monitoring Report: Updated Analysis of the W2B Koala Monitoring Programs in Bagotville and Broadwater, NSW, Australia.

5 June 2023

By Robert W Rankin, Ph.D.

under contract by Rankin Holdings, 1035179 Ontario Inc.

Summary

This report provides an updated analysis for Year 5 of the W2B Pacific Highway Upgrade koala population monitoring program being conducted in sections 8/9 (Broadwater) and 10 (Bagotville) in accordance with the Koala Management Plan (RMS 2016). The analyses have been updated using the latest data following completion of the spring 2021 and autumn 2022 field seasons.

This report presents four analyses: i) estimation of koala densities; ii) estimation of possible emerging trends; iii) hypothesis-testing of a night-time *vs.* day-time effect; and iv) an updated prospective power analysis.

<u>Summary of results</u>:

- In general, the estimates of trend, densities and power were nearly-identical to those of the Year 4 report. The overall statistical uncertainty remains quite high, making it difficult to make inferences about trends.
- The estimated population densities at Broadwater and Bagotville for Year 5 2021/22 were, respectively, 0.045 koala/ha (SE: 0.008; 95%CI: 0.031-0.063) and 0.080 (SE: 0.012; 95%CI: 0.059-0.105). At Broadwater, the Year 5 density estimate was slightly lower than the Year 4 estimate of 0.047 (SE: 0.007; CI: 0.034-0.063), while at Bagotville, there was little-to-no change, either between years as well as between revised estimates between the Year 5 vs. Year 4 reports.
- The trend at Broadwater was estimated to be -3.3%/year ($\bar{\beta}_t$= -0.033 per year; SE: 0.041; 95%CI -0.124-0.023), which is nearly identical to the estimate from the previous report. The estimated trend at Bagotville was 0.0%/year increase ($\bar{\beta}_t$= -0.001 per year; SE: 0.023; 95%CI -0.060-0.053). There was no strong evidence of a significant trend at either location, according to Bayesian hypothesis testing. The alternative AICc-based method continues to estimate a large decline at Broadwater, but these estimates have moderated from their extremes in previous reports.

- There was slight evidence *against* the presence of a "night-time effect", i.e., there was no meaningful difference between night-time and day-time surveys. The statistical evidence to support this conclusion continues to get stronger and stronger with each additional year of data.
- The estimated statistical power for Broadwater and Bagotville were 0.657 and 0.738 respectively. The power at Broadwater was down slightly compared to the Year 4 report.

1 Introduction

1.1 Background

This report presents the fifth statistical analysis of koala densities and trends, commissioned in support of Sandpiper Ecological Survey's ongoing koala population monitoring in sections 8/9 (Broadwater) and 10 (Bagotville) of the W2B Pacific Highway Upgrade which are being conducted in accordance with the Koala Management Plan (RMS 2016). These analyses evaluate the program's goal to detect a potentially large decline in koala densities. Specifically, the survey effort and statistical modelling should be able to detect a 30% decline over 15 years with a power of at least 70% and a Type-I error rate (α) of 0.30.

This report updates the statistical analyses of previous reports, including a Bayesian trend analysis and simulation-based power-analysis. The methodological details have been described in previous reports (Sandpiper Ecological 2020), and will be summarised here.

1.2 Objectives

There are four objectives addressed in this report:

Objective #1. Update the koala population density estimates at Broadwater and Bagotville for Year 5, including segregated estimates for Spring (2021), Autumn (2022), and a pooled estimate for Year 5 (both seasons).

Objective #2. Update the trend analyses and evaluate the evidence of an emerging trend at either Broadwater or Bagotville. The method of evaluation consists of Bayesian trend estimation, as developed in the original report from Year 1 (Sandpiper Ecological 2019a), as well as an alternative frequentist method that was developed in the Year 2 and Year 3 reports (Sandpiper Ecological 2019b; Sandpiper Ecological 2020).

Objective #3. Evaluate whether there is an important difference between densities during night-time *vs.* day-time surveys.

Objective #4. Update the prospective power analyses; determine whether the program can detect a 30% decline over 15 years with a power of 0.70 ($\alpha \leq 0.3$ and $power > 0.3$).

2 Comments on Year 4 Changes

The previous Year 4 report highlighted two important methodological changes. One was a modelling improvement that accommodated inter-annual variation. The second was an admission of a small error in the analysis of the Year 3 report. These improvements/changes are highlighted in the present report only to affirm their continued implementation in the Year 5 analyses.

2.1 Multi-model Inference: Accounting for Inter-Annual Differences

Throughout these reports, we have employed a model-averaging approach to pool estimates from multiples models (trend vs. no trend, night-time effect vs. no-effect, inter-annual variation, and more) based on their predictive accuracy. Beginning with the Year 4 report, and continuing with the represent analysis, the way we modelled the trend and inter-annual variation was parameterised according to three different functional-forms: i) no trend, ii) trend, iii) inter-annual variation around a dominant trend.

The above update seemed warranted given that most ecological processes include both inter-annual variation as well as a long-term trend. The above update was and is currently only possible for the Bayesian models.

3 Methods

The following sections will review major methodological features of the statistical analyses. More extensive details about the methodologies can be found in the Year One and Year Two reports (see Sandpiper Ecological 2019a, b & 2020).

3.1 Statistical Model for Counts and Density

We are interested in modelling koala density $\eta_{l,t,j}$, using observations of the counts of koalas $y_{l,t,j}$ at location *l (Broadwater vs. Bagotville)*, in year *t*, at transect *j*. Each transect *j* also has a record for its area A_j, and indicator variables X_j denoting: i) whether the survey occurred at night-time or daytime, ii) whether it was a radial survey or line-transect, and iii) whether the survey happened during the autumn or spring.

We combine these variables into a log-linear GLM statistical model according to the following reasoning. We start with the formula for density (number of koalas per area):

$$\eta_{l,t,j} = \frac{N_{l,t,j}}{A_{l,t,j}} \iff N_{l,t,j} = \eta_{l,t,j} A_{l,t,j}$$

Where η is the density of koalas at location *l* at time *t* and transect *j; N* is the (true) number of koalas; and *A* is the area at transect *j*. We substitute *N* for its statistical expectation *E[y]* from a count distribution like the Negative Binomial, and take the natural logarithm of both sides to yield:

$$\mathbb{E}[y]_{l,t,j} = \eta_{l,t,j} \cdot A_{l,t,j}$$
$$\log(\mathbb{E}[y]_{l,t,j}) = \log(\eta_{l,t,j}) + \log(A_{l,t,j})$$

Finally, we substitute the density term η for its linear-model decomposition ($\boldsymbol{\beta}^\intercal \mathbf{x}_{l,t,j}$), thus arriving at our familiar equation of a line with an area offset.

$$\log(\mathbb{E}[y]_{l,t,j}) = \boldsymbol{\beta}^\intercal \mathbf{x}_{l,t,j} + \log(A_{l,t,j})$$

This means we can use a Negative Binomial distribution to model counts *y* and perform linear regression to estimate parameters β, as well as estimate other interesting quantities, such as the koala densities for each year, location and season. Estimating the densities per year satisfies Objective #1, while a trend parameter in $\boldsymbol{\beta}$ helps satisfy Objective #2.

3.2 Parameters, Priors and MCMC

The regression parameters β and the covariates in the model-matrix *X* include different features like: year, daytime *vs.* night-time effect, radial- *vs.* line-transects, and a seasonal effect. According to the Bayesian estimation paradigm, each of these parameters requires a prior distribution.

The priors used in this analysis were the same as used in the Year 1, 2 and 3 reports. The motivation and description of the priors can be found in the Year 1 report; the values are reported here without extensive expository detail.

Priors.

- The prior distribution on the trend parameters were set to $\pi(\beta_{t,l}) = \mathcal{N}(0, 0.05^2)$.
- The prior on the (log) baseline density at Bagotville was given a Gaussian distribution $\pi(\beta_0) = \mathcal{N}(\log(0.091), 0.41^2)$.
- The prior on the marginal difference between the Broadwater log-density *vs.* Bagotville was $\pi(\beta_l) = \mathcal{N}(0, 0.54^2)$.
- The prior on the marginal affect of the radial- *vs.* line-transects was $\pi(\beta_r) = \mathcal{N}(0, 0.54^2)$.
- The marginal effects of night-time *vs.* day-time was the same as the above prior on radial- vs line-transects, and likewise for and autumn *vs.* spring effects.
- Finally, for the Negative Binomial overdispersion parameter θ, a Gamma prior was used with a prior mean of 5. The strength of this prior was determined according to a model-

selection exercise (see next section) where the shape and rate parameters of the Gamma distribution were: {(5,1), (10,2), (20,4), (40,8),(500,100)}. Put simply, we let the count distribute vary between an overdispersed Negative-Binomial distribution as well as tight Poisson distribution, as determined by the strength of the Gamma prior on θ, and where the strength of the Gamma prior was decided according to model selection.

MCMC. Given the data and the priors, the regression coefficients β could be estimated according to Monte Carlo Markov Chain (MCMC) algorithm, in particular, using the statistical package JAGS (Plummer 2007, 2014) in R (R Core Team 2016). Each model used 80000 MCMC samples plus a 5000 sample burn-in period. Posteriors were inspected for adequate mixing and convergence.

3.3 Multi-Model Inference

In the regression analyses, the challenge is to estimate a high number of plausible explanatory covariates (time, location, time-of-day, season, etc.), but only a small amount of survey data. In such situations, it is common in ecological studies to employ “multi-model inference” (Johnson and Omland 2004). This technique was used in previous reports, and the technique is summarised here.

Briefly, the core idea is that one never knows which subset of covariates are “best” *a priori*, and so a prediction-based criteria, such as the corrected Akaike Information Criteria (AIC/AICc; Akaike 1974, 1998, Hurvich and Tsai 1989) or the Watanabe-Akaike Information Criteria (WAIC; Watanabe 2010), are useful to weight models and combine their estimates according to each models’ predictive performance. Specifically for the Bayesian models, the model-averaging uses model-weights based on the WAIC criterion (Watanabe 2010, Link and Sauer 2015):

$$p(m|\mathbf{y}) \approx \frac{e^{-0.5\mathrm{WAIC}^{(m)}}}{\sum_m^M e^{-0.5\mathrm{WAIC}^{(m)}}}$$

where *m* indexes a particular model with its own unique specification of covariates β_m.

Model-averaging is important because some models are bad at prediction because they are *overfitting* the data (they have too many covariates with too little data) and some models are *underfitting* the data (they omit an important covariate). Using a predictive criteria like the WAIC or AICc helps find the best combination of parameters which yield the highest predictive accuracy, while minimising the influence of spurious covariates.

There were 280 possible models, which included various combinations of the following:

1. a night-time *vs.* day-time effect, or not;
2. a radial vs. line-transect effect, or not;
3. a season-effect, or not;
4. a log-linear trend *vs.* no trend *vs.* each year has its own density; and

5. 5 different amounts of overdispersion (excess count-variation).

As mentioned in Section 3.1, there was a slight modification the analyses regarding the specification of (4) “each year has its own density”: in prior years, this meant that each years' density was independently estimated, but there was no explicit trend. The growing availability of data and number of years allows us to incorporate both a trend-component and an inter-annual variability component, and estimate them jointly i.e., the years' densities vary around a main trend.

Previously, with fewer years and fewer data-points, this type of model would be over-determined and unestimable. It is preferable to use this model going-forward because it better reflects reality.

3.4 Hypothesis Testing

Objectives #2 and #3 pertained to evaluating hypotheses, such as: whether there was a trend, and whether there was a night-time *vs.* day-time effect.

As was developed in previous reports, these hypothesis-type objectives were addressed through a Bayesian quantitative technique called posterior odds-ratios (also known as Bayes Factors; Jeffreys 1961, Kass and Raftery 1995).

The odds ratios are calculated by taking the ratio of two quantities, which, respectively, represent the strength of support for a hypothesis H_1 *vs.* its complimentary alternative hypotheses H_0 (i.e., a “null-hypothesis” of no effect). In this report, we used the sum of WAIC model probabilities for those models that supported the H_1 (the numerator of the odds-ratio), *vs.* those models that constituted the null hypothesis (the denominator of the odds-ratio). For example, the odds-ratio in favour of a trend would be:

$$BF_{\text{trend>no trend}} \approx \frac{\sum_{k\in\mathcal{M}_{\text{trend}}} \text{WAIC}_k}{\sum_{k\in\mathcal{M}_{\text{no trend}}} \text{WAIC}_k}$$

where $\mathcal{M}_{\text{trend}}$ represents the set of models that included a trend, and $\mathcal{M}_{\text{no trend}}$ represents models without a trend (which thereby act as a composite null hypothesis). The *BF* ratio must be substantially greater than 1 to provide evidence in favour of a trend. A *BF* of ~1 suggests that there is no meaningful difference between the H_1 and its compliment. A *BF* << 1, suggests strong refutation of the existence of a trend.

Similarly, another analysis used Bayesian odds-ratios to evaluate the evidence in favour of *no* night-time effect *vs.* evidence of a difference between night-time and day-time surveys.

As was done in previous reports, the strength of the odds-ratios were evaluated against established quantitative cut-offs (Jeffreys 1961, Kass and Raftery 1995). For instance, a ratio above 10:1 is considered “strong” evidence in favour of a trend; a ratio above 3.2:1 is “substantial” evidence; and a ratio between 3.1:1 to 1:1 is considered “barely worth a mention”.

3.5 Prospective Power Analysis

The power analysis used the same Monte Carlo simulation method as in the Year 1 report. The goal of the power analyses was to estimate the rate of Type-II errors (falsely rejecting the hypothesis of a trend, $H_a : \beta_t \neq 0$) while simultaneously detecting a 30% decline from baseline levels at Broadwater and Bagotville, between years 2015 to 2031. The error rates were conditional on:

1. a negative trend of -30% from baseline levels until Year 15 of monitoring;
2. a cap on the rate of Type-I errors at $\alpha \leq 0.3$;
3. monitoring effort of 400 transects per year per location (Broadwater and Bagotville separately);
4. marginal effects for other independent covariates (such as day-time/night-time, spring/autumn, and line-transect/radial-search transects) empirically derived from the Bayesian estimation analysis (from Objective #1); and
5. baseline koala densities in 2015 derived from the Bayesian estimation analysis.

One key-point to note is item (3), whereby the number of transects/samples has been kept constant over 15 years of prospective data. If there are actual changes in effort, as the study removes or expands sites, then the prospective power-analysis should be updated to incorporate such changes, inasmuch as they can be anticipated.

Another key-point pertains to item (4): notice how the Monte-Carlo procedure incorporates several sources of empirically-derived uncertainty. First, there is the uncertainty in the baseline densities at Bagotville and Broadwater, as quantified by the posterior distributions of Year 0 densities from the Bayesian estimation exercise (Objective #1). Secondly, there is the uncertainty in the magnitude of marginal effects (such as day-time/night-time, spring/autumn, and line-transect/radial-search). This uncertainty was incorporated by using the posterior distributions from the Bayesian estimation exercise. Finally, there is the *multi-model* uncertainty due to multiple plausible models for estimating statistical power. The latter point reflects the fact that a future analyst will want to improve their statistical accuracy by including or excluding certain covariates, and will likely perform model-selection by AIC (Akaike 1974, 1998).[1]

These three sources of uncertainty make the calculation of Type-II errors non-trivial. They are best estimated through Monte Carlo simulations. This Monte Carlo power analysis proceeded as follows:

1. set the annual percent decline to $-\delta$, and set parameters $\beta_t = \log(1-\delta),\ \beta_{t,bw} = 0$;
2. set the desired Type-I error rate to α;
3. for *i* in 1 to 4000 Monte Carlo iterations, do:

   I. get a sample of parameter values from the Bayesian posteriors (e.g., baseline densities, overdispersion, marginal effects of day-time/night-time, spring/autumn, and line-transect/radial-search) $\boldsymbol{\beta}_{\neg t}^{(i)} \sim \pi(\boldsymbol{\beta}_{\neg t}|\mathbf{y}),\ \theta^{(i)} \sim \pi(\theta|\mathbf{y})$, and combine these samples with the specified trend in (1) above: $\boldsymbol{\beta}^{(i)} = (\boldsymbol{\beta}_{\neg t}^{(i)}, \beta_t, \beta_{t,bw})^{\intercal}$;

   II. simulate count data ***y*** using the linear model in Eqn. 1 and parameters $\boldsymbol{\beta}^{(i)}$

   $$y_{l,t,j}^{(i)} \sim \text{NB}\left(e^{(\mathbf{x}_{l,t,j}\boldsymbol{\beta}^{(i)} + \log(p_d \cdot A_{l,t,j}))}, \theta^{(i)}\right);$$

   III. use the simulated data $\mathbf{y}^{(i)}$ to get maximum-likelihood estimates of the trend and standard error $(\hat{\beta}_t^{(i)}, \hat{\text{se}}(\beta_t)^{(i)})$ for both Broadwater and Bagotville, including:

      i. option 1: use the Poisson *full-model* (model m_8 in Eqn. 2), or

      ii. option 2: use the best AIC Poisson model from models m_1 to m_8

   (this analysis proceeded with option 2, but I also ran option 1 for comparison purposes)

   IV. for each location *l* (Broadwater and Bagotville) compare the two-tailed Fisher p-value to α and calculate the score statistic *I*

   $$I_l^{(i)} = 2\left(1 - \text{PDF}_{\mathcal{N}}\left(\frac{|\hat{\beta}_t^{(i)}|}{\hat{\text{se}}(\beta_t)^{(i)}}\right)\right) \leq \alpha$$

   Over all 4000 iterations, the estimated Type-II error rate (per *l* location Broadwater and Bagotville) was $\hat{b}_{l,\alpha,\beta_t} \approx \frac{1}{4000}\sum_{i=1}^{4000} I_l^{(i)}$ and the power is $1 - \hat{b}_{l,\alpha,\beta_t}$

1 Note: in the future, there will be a lot of data, which will make the difference between the AICc vs AIC unimportant. The AICc is corrected for small-sample sizes, and converges to the AIC with increasing data.

3.6 Supplemental Analyses

The Year 2 report (Sandpiper Ecological 2019b) introduced several supplementary analyses that were continued in the Year 3, Year 4 and Year 5 studies. These supplementary analyses were meant to investigate alternative methods of estimating trends and evaluating evidence for or against the presence of a trend.

These supplementary analyses varied according to the hypothetical strength of prior information, and were meant to help to contextualise the results from the main Bayesian analysis. In particular, the AICc-based model-averaging method is more sensitive to changes, and has yeilded sometimes extreme estimates of trends (e.g., ~40% decline in Year 2) compared to the more conservative Bayesian model-averaging technique.

3.6.1 Estimation According to AICc Model-Averaging

This analysis used frequentist Negative-Binomial GLMs and performed model-averaging by AICc weights (Akaike 1974, 1998, Schwarz 1978) to estimate the trends at Broadwater and Bagotville. As described in the Year 2 report, these models can be thought of as pseudo-Bayesian models whereby i) the priors-on-parameters have been weakened to zero-influence, and ii) priors-on-model-probabilities are adaptive (i.e., they become more conservative with less data, and more liberal with more data). In other words, the AICc "reacts" faster to new data compared to static Bayesian priors used in the main analyses.

The trade-off is that while the AICc may be more sensitive to developing trends, it may result in some overfitting and be alarmist, as compared to the Bayesian models with stronger priors. See the Year 2 report (Sandpiper Ecological 2019b) for more discussion on the difference between the Bayesian-WAIC models and the frequentist-AICc models.

3.6.2 Hypothesis Testing According to AICc-Evidence Ratios

In the same way that one can garner evidence for or against a hypothesis according to Bayesian posterior odds-ratios (see above), the sum-of-AICc weights can also be used to produce odds-ratios (Lukacs *et al.* 2007). The AICc-based odds-ratios are analogous to the WAIC-based Bayes Factors but are simply called "evidence" ratios, according to the "Evidentialist" approach (Taper and Ponciano 2016). The interpretation is largely the same as for the Bayesian approach: high ratios > 1 are evidence in favour of a trend *vs.* no trend (except that the AIC controls Type-I errors more consistently across sample sizes, Taper and Ponciano 2016). The sum-of-AICc ratios was used to assess the evidence in favour of a trend *vs.* no-trend, to supplement the Bayesian odd-ratios.

4 Results

4.1 Descriptive Statistics

The following are descriptive summaries about the observed counts and empirical (unmodelled) densities of koalas at Broadwater and Bagotville.

At Broadwater, both the counts and empirical densities were higher in Year 5 than Year 4 (counts of 10 vs. 7 koalas, and densities of 0.056 vs. 0.018 koalas/ha). However, year 5 counts and empirical densities are lower than those observed in Year 3.

In Bagotville, the koala counts in Year 5 were the same as in Year 4 (16 koalas), but the empirical density was lower (0.044 vs 0.051 koalas/ha). This was due to the higher prevalence of observed koalas occurring in small-area radial transects in Year 4, which inflated the Year 4 density. The Year 5 Bagotville empirical density was the lowest in our entire time-series.

Table 1: Unmodelled empirical counts, aggregated by year and location

Location	Baseline	Year 1	Year 2	Year 3	Year 4	Year 5
Broadwater	8	13	5	15	7	10
Bagotville	4	17	18	22	16	16

Table 2: Unmodelled empirical densities (koalas/ha), pooled per year and location

Location	Baseline	Year 1	Year 2	Year 3	Year 4	Year 5
Broadwater	0.117	0.053	0.013	0.069	0.018	0.056
Bagotville	0.093	0.069	0.077	0.063	0.051	0.044

4.2 Results for Objective #1: Density Estimation

The following tables show the updated statistical estimates for all years, segregated by location and season. Table 3 shows pooled estimates; Table 4 shows seasonal estimates. The values were calculated by model-averaging Bayesian estimates, such that Bayesian per-model estimates were weighted by WAIC model probabilities.

The statistical results show that Broadwater's densities continue to decrease from earlier years (although the 95%CI are strongly overlapping between years). There was a high of 0.062 koalas/ha during the Baseline year, to a low of 0.045 koalas/ha in Year 5.

Bagotville continues to show near-consistent densities across the years, from 0.083 koalas/ha during the Baseline to 0.080 koalas/ha in Year 5.

The updated Year 5 modelling-results provide minor revisions to the density estimates for past years: across all years and locations, the Year 5 retrospective/revised estimates are approximately 0-4% higher than those we reported in Year 4 (for example, we previously estimated the Bagotville and Broadwater Baseline densities to be 0.081 and 0.060 koalas/ha, respectively, which have been revised to 0.082 and 0.062 koalas/ha; likewise, we estimated the Year 4 densities to be 0.080 and 0.046 koalas/ha, respectively, which have been revised to 0.08 and 0.047 koalas/ha). These slight revisions are much more subdued compared to past revisions in the Year 4 and Year 3 reports.

Table 3: Bayesian estimates densities (koalas/ha), pooled, per year and location

Location	Baseline	Year 1	Year 2	Year 3	Year 4	Year 5
Broadwater	0.062 (SE: 0.012; CI: 0.041-0.089)	0.055 (SE: 0.009; CI: 0.040-0.074)	0.052 (SE: 0.007; CI: 0.039-0.068)	0.050 (SE: 0.007; CI: 0.037-0.065)	0.047 (SE: 0.007; CI: 0.034-0.063)	0.045 (SE: 0.008; CI: 0.031-0.063)
Bagotville	0.082 (SE: 0.014; CI: 0.058-0.111)	0.081 (SE: 0.010; CI: 0.063-0.102)	0.081 (SE: 0.009; CI: 0.064-0.100)	0.081 (SE: 0.009; CI: 0.064-0.100)	0.080 (SE: 0.010; CI: 0.061-0.102)	0.080 (SE: 0.012; CI: 0.059-0.105)

Table 4: Bayesian estimates of densities (koalas/ha), per year and season

Location	Baseline	Year 1 Spring	Year 1 Autumn	Year 2 Spring	Year 2 Autumn	Year 3 Spring	Year 3 Autumn	Year 4 Spring	Year 4 Autumn	Year 5 Spring	Year 5 Autumn
Broadwater	0.062 (SE: 0.012; CI: 0.041-0.089)	0.055 (SE: 0.009; CI: 0.040-0.075)	0.055 (SE: 0.009; CI: 0.039-0.074)	0.052 (SE: 0.008; CI: 0.038-0.069)	0.052 (SE: 0.008; CI: 0.038-0.069)	0.050 (SE: 0.008; CI: 0.037-0.067)	0.050 (SE: 0.008; CI: 0.037-0.067)	0.047 (SE: 0.008; CI: 0.034-0.064)	0.047 (SE: 0.008; CI: 0.033-0.064)	0.045 (SE: 0.008; CI: 0.031-0.064)	0.045 (SE: 0.008; CI: 0.031-0.063)
Bagotville	0.082 (SE: 0.014; CI: 0.058-0.111)	0.081 (SE: 0.011; CI: 0.062-0.104)	0.081 (SE: 0.011; CI: 0.062-0.103)	0.081 (SE: 0.010; CI: 0.063-0.103)	0.081 (SE: 0.010; CI: 0.062-0.101)	0.081 (SE: 0.010; CI: 0.062-0.102)	0.081 (SE: 0.010; CI: 0.062-0.101)	0.080 (SE: 0.011; CI: 0.061-0.104)	0.080 (SE: 0.011; CI: 0.060-0.103)	0.080 (SE: 0.012; CI: 0.058-0.107)	0.080 (SE: 0.012; CI: 0.058-0.106)

4.2.1 Supplementary Analysis: AICc-based model-averaged model estimates

Table 5 shows the supplementary density estimates using AICc model-weights to produce model-averaged estimates. As explained in past reports, the AICc-based model-averaging approach is more sensitive to variations in the data, as compared to the more conservative Bayesian approach. The updated estimates of koala-densities demonstrate this sensitivity.

The AICc-method revealed a decrease in koala densities in Year 5 compared to Year 4, at both locations. For instance, at Broadwater, the Year 5 estimate was 0.036 vs. 0.040 koalas/ha in Year 4; while at Bagotville the Year 5 estimate was 0.077 vs. 0.079 koalas/ha in Year 4.

However, in the prior year report, the AICc-based estimates of the Year 4 densities were 0.045 koalas/ha at Broadwater and 0.082 at Bagotville. These highlight how the AICc-based estimates are getting less extreme and more similar to the Bayesian estimates, as data accumulates.

Table 5: AICc-based estimates of densities, by year and location.

Location	Baseline	Year 1	Year 2	Year 3	Year 4	Year 5
Broadwater	0.087 (SE: 0.054; CI: 0.050-0.256)	0.063 (SE: 0.015; CI: 0.035-0.095)	0.053 (SE: 0.018; CI: 0.010-0.070)	0.048 (SE: 0.018; CI: 0.035-0.104)	0.040 (SE: 0.013; CI: 0.016-0.065)	0.036 (SE: 0.014; CI: 0.022-0.077)
Bagotville	0.087 (SE: 0.039; CI: 0.033-0.182)	0.084 (SE: 0.018; CI: 0.051-0.125)	0.082 (SE: 0.017; CI: 0.052-0.119)	0.081 (SE: 0.020; CI: 0.061-0.139)	0.079 (SE: 0.017; CI: 0.045-0.112)	0.077 (SE: 0.019; CI: 0.046-0.119)

4.3 Objective #2: Emerging Trends

4.3.1 Trend Estimate

The estimated log-linear trend at Broadwater was -0.033/year (SE: 0.041; 95%CI -0.124-0.023), i.e. a 3.3% decline per year, with a 0.752 posterior probability of decline. These values are identical to the estimates from the Year 4 report, but with slightly less uncertainty (e.g. compare the Year 5 SE of 0.041 vs 0.043 in the Year 4 report).

The estimated log-linear trend at Bagotville was -0.001/year (SE: 0.023; 95%CI -0.060-0.053), with a 0.512 posterior probability of a decline. This is nearly the same as the prior year’s estimate of 0.000/year, with slightly less uncertainty (the SE from the Year 4 report was 0.025).

Table 6 shows the change in trend estimates from the Year 3 report to the present report.

Table 6: Sequence of Bayesian Trend Estimates by Report-Year

Location	Year 3	Year 4	Year 5

Broadwater Trend	-0.016 (SE: 0.037; 95%CI -0.105-0.048)	-0.033 (SE: 0.043; 95%CI -0.127-0.028)	-0.033 (SE: 0.041; 95%CI -0.124-0.023)
Broadwater Bayes Factor	1.17	2.07	1.97
Bagotville Trend	0.001 (SE: 0.029; 95%CI -0.068-0.073)	-0.000 (SE: 0.025; 95%CI -0.061-0.060)	-0.001 (SE: 0.023; 95%CI -0.060-0.053)
Bagotville Bayes Factor	0.85	0.78	0.72

4.3.2 Trend Hypothesis-Testing

The trend at Broadwater had a posterior odds ratio (Bayes Factor) of 1.966 in favour of a trend *vs.* no-trend. This provides slight evidence in favour of a trend. The Bayes Factor value is considered 'barely worth mentioning' according to conventional cut-offs (Jeffreys 1961, Kass and Raftery 1995). This Bayes Factor is close to the estimate from the Year 4 report (2.071).

The trend at Bagotville had a posterior odds ratio (Bayes Factor) of 0.724, which is slightly lower than last year's estimate of 0.782. It is slight evidence *against* there being a trend. This ratio falls into the conventional descriptive bin 'barely worth mentioning'.

Table 6 shows the sequence of Bayes Factors from Year 3 to 5. The sequence of Bayes Factors at Broadwater shows varying levels of statistical confidence in favour of a trend, with values between ~1 and 2. In contrast, at Bagotville, there seems to be a monotonic decrease in the values from 0.85 to 0.72, which suggest gradually increasing confidence about the non-existence of a trend a Bagotville. However, the Bayes Factor differences at Bagotville are small and should not be over-interpreted.

4.3.3 Supplementary Trend Analysis by AICc Model-Averaging

According to the frequentist AICc-based model-averaged estimates, the estimated log-linear trend at Broadwater was -0.135 /year (SE: 0.101), i.e. a 13.5%/year decline. Despite the extreme trend, it is less extreme than the value reported in Year 4, which was estimated to be -0.218/year (SE 0.131), i.e. 21.8% decline.

The Year 5 variance was so high that the hypothesis-testing statistics do not provide overwhelming evidence in favour of a trend: the AICc-based odds-ratio in favour of a trend at Broadwater was 1.374 which is 'barely worth mentioning'. This odds-ratio is lower than the reported value in Year 4 which was 2.563. The Fisher p-value against a trend was 0.184, and higher than the Year 4 p-value of 0.094. For both odds-ratios and Fisher p-values, the evidence of a trend is weak and undermined by high variance and data-sparsity.

Table 7: Sequence of AICc-Based Trend Estimates by Report-Year

Location	Year 3	Year 4	Year 5
Broadwater Trend	-0.259/year (SE: 0.177)	-0.218/year (SE 0.131)	-0.135 /year (SE: 0.101)
Broadwater AICc Odds Ratio	0.97	2.56	1.37
Bagotville Trend	0.042/year (SE: 0.123)	-0.006/year (SE: 0.085)	-0.015/year (SE: 0.065)
Bagotville AICc Odds Ratio	0.39	0.16	0.16

Table 7 shows the time-series of AICc trend estimates from Year 3 to Year 5. These statistics demonstrate: i) a continual reduction in trend-magnitude, declining from extremes of ~-43% in the Year 2 report to -13% is the present report; and ii) a high variance that undermines the confidence in such estimates. Further on in the Discussion section, we speculate more about the pattern of statistics and what may be happening "under the hood" at Broadwater.

The estimated AICc-based log-linear trend at Bagotville was -0.015/year (SE: 0.065), i.e., a 1.5% decline per year. The magnitude of the trend is greater than the Year 4 estimate of -0.006/year (SE: 0.085). At Bagotville, the AICc-based odds-ratio in favour of a trend was 0.163, which is considered 'barely worth mentioning', and smaller than the Year 4 reported value (0.155). The Fisher p-value against the no-trend null-hypothesis was 0.813 (i.e., we are far away from the 0.05 threshold that would allow us to reject the no-trend null-hypothesis). This was similar to the p-value reported in Year 4 (p=0.945). However, in order to use these high p-values to *accept* the no-trend hypothesis, we would need to have high power.

4.4 Objective #3: Day-Time VS. Night-Time Effect

A posterior odds-ratio (Bayes Factor) was employed to assess whether night-time surveys had meaningfully different densities than day-time surveys. In this case, the favoured hypothesis of *no*-difference constituted the numerator of the odds-ratio, and the alternative hypothesis of *yes*-difference constituted the denominator. Therefore, odds-ratios values above 1 support the conjecture that there is *no* difference between night-time and day-time surveys, and values <1 support the conjecture that there *is* a night-time effect.

The posterior odds-ratio was 2.598, i.e., there was slight evidence of no difference between night-time and day-time surveys. This estimate was approximately 21% higher than the Year 4 estimate of 2.141, which was itself ~60% higher than the Year 3 estimate. The Year 5 ratio is within the ratio-category which is conventionally described as "barely worth mentioning" (Kass and Raftery 1995), although it is trending in the direction of the 3.16 threshold for a “noteworthy” effect (cut-off of 3.16).

4.5 Objective #4: Prospective Power Analysis

The results of the prospective power analysis are showing in figure 1. For a maximum Type-I error rate of 0.3, the estimated power for Broadwater and Bagotville were 0.657 and 0.738, respectively. The Broadwater power decreased slightly from the Year 4 report (previously estimated to be 0.661), and improved slight for Bagotville (previously 0.726).

For a maximum Type-I error rate of 0.35, the estimated power for Broadwater and Bagotville were 0.694 and 0.770 respectively. The Broadwater power decreased slightly from the Year 4 report (0.702), and improved slight for Bagotville (0.764).

The Bayes’ p-values[2] for Broadwater and Bagotville were 0.920 and 0.947, which improved upon the Year 4 report estimates of 0.919 and 0.945, respectively. To interpret these values, it means that if there was a 30% decline, a Bayesian analyst would be able to conclude that there was a trend with 92.0% certainty at Broadwater, and likewise 94.7 certainty at Bagotville. In other words, there is more certainty about the Bagotville system than there is at the Broadwater system.

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2 Recall that the definition of “detecting a trend” according to the Bayes’ p-value approach is very different from frequentist approaches. In particular, Bayesians use a “balance of probabilities” approach: if the probability of a trend is >51%, we say there is a trend. In contrast, the frequentists set a Type-I error rate (conventionally 0.05), and only reject ‘no trend’ if that error-rate is below the threshold (which is like saying they want to be 95% sure of no-trend before accepting the conclusion). In this study, our frequentist error-rate threshold was 0.3.

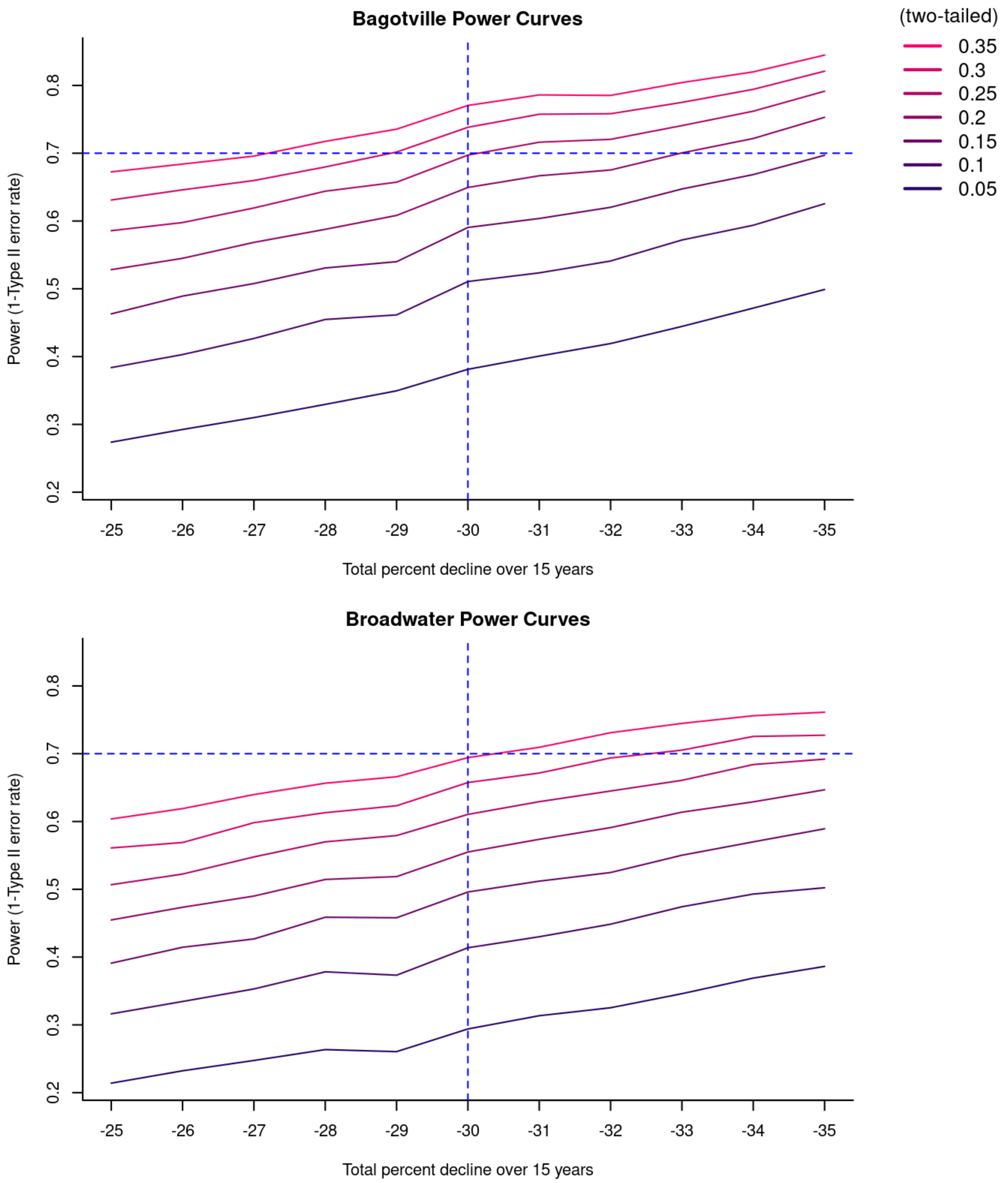

Figure 1: Statistical power to detect a -30% drop in baseline densities vs. Year 15 of the monitoring program, for different maximum levels of Type-I errors (lines)

5 Discussion and Conclusions

This report presents a quantitative assessment of the Year 5 (2021/22) W2B Pacific Highway Upgrade koala population monitoring program at Bagotville (section 10) and Broadwater (section 8/9).

Overall, the Bayesian statistical results are very similar to those of the Year 4 report, such as near identical trend estimates, density estimates, and power estimates, with little-to-no improvement in statistical certainty measures.

For instance, Broadwater showed a continued statistical decline of ~3.3%/year, even while the empirical (un-modelled) densities at Broadwater jumped by over 3.1x compared to Year 4 densities (i.e., 0.018 koalas/ha in Year 4 vs. 0.056 in Year 5). Bagotville seems to be stable with a near-zero statistical trend.

5.1 Trends and Densities

5.1.1 Bagotville

Across the various reports, and continuing with this Year 5 report, the statistical story of Bagotville has been one of little-to-no significant trend in koala densities. Most of the statistical evidence in this report, including p-value tests, Bayes Factors and AICc-based evidence ratios, continue to find no evidence of a trend at Bagotville.

The Bayesian estimates of koalas densities at Bagotville continue to fluctuate slightly around 0.080 (SE: 0.012; CI: 0.059-0.105). The Year 5 density estimate is nearly identical to the estimate from the Year 4 report, and is very close to the revised Baseline estimate of 0.082 (SE: 0.014; CI: 0.058-0.111).

The only concerning statistics for Bagotville has been the empirical (un-modelled) densities and the AICc-based point estimates. These suggest that there was a modest decline at Bagotville in Year 5 compared to Year 4, as well as compared to the Baseline, albeit only slightly.

5.1.2 Broadwater

There is consistent evidence of a decline at Broadwater, with a Bayesian trend estimate that is nearly identical to that produced in the previous Year 4 report: a decline of 3.3%/year (SE: 4.1%). The sequence of trend estimates from the Year 3 analysis to Year 5 suggest a steadily narrowing statistical certainty about a population decline (see Table 6).

The consistency of the Year 4 and Year 5 estimates is interesting, especially in the face of underlying swings in the empirical (un-modelled) density at Broadwater. For instance, the Year 5 empirical density is more than 3x larger than that of Year 4 (0.018 koalas/ha in Year 4 vs. 0.056 in Year 5), although the Year 5 empirical density is still down from a high of 0.117 koalas/ha in the Baseline year.

This consistency in trend estimates, in the face of underlying swings, is likely a reflection of the way in which the log-linear trend-model only captures long-term trends, and not year-by-year variation. In other words, we are fitting a straight-line through a noisy process. As more data accumulates, the line varies less and less, and the long-term trend gets more certain.

The annual swings in counts are more influential on the AICc-based methods, as opposed to the more conservative Bayesian methods. The AICc-based methods continue to estimate a very large decline at Broadwater, despite the relatively high survey counts during Year 5. The AICc-based Year 5 densities (0.036 kioalas/ha) are less than half the values at Baseline (0.087). However, the sequence of AICc-based trend estimates have continued to moderate over time: from an alarming -43%/year in the Year 2 report, to -13%/year estimated in the present report. The latter estimate is still very large for a mammalian population, but we anticipate that as more data is collected, the AICc-based estimates and the Bayesian estimate will slowly converge (assuming no strong deterioration in the underlying population).

In the prior Year 4 report, we anticipated that a further decline at Broadwater could elicit a Fisher p-value (test against a trend) that would be close to the conventional cut-off of 0.05 (i.e., a 95% Type I error rate), at which point we would no longer be able to reject the null-hypothesis of no trend. Instead, the Fisher p-value in Year 5 is higher at 0.184, up from the Year 4 p-value of 0.094.

5.2 Night-Time vs. Day-Time Surveys

The analysis of differences between night-time vs. day-time surveys continues to lead to the same conclusion as in prior reports, albeit with a slow and continuous strengthening in the conviction of the conclusion: there is slight evidence *against* the presence of a night-time effect.

In other words, there does not seem to be a difference between night-time and day-time surveys. The strength of this conviction, as measured by Bayes Factors, is up 21% compared to the Year 4 report, which was approximately 60% higher than the Year 3 report.

5.3 Prospective Power Analysis

At Broadwater, the sequence of power-estimates from Years 3 to 5 were 0.661, 0.667, and 0.657, respectively. These are remarkably consistent, but also below the 0.7 target.

The power-measure is representative of the overall statistical uncertainty and natural variation in the entire system (including inter-annual population variability and variance in MCMC estimates). It is a useful indicator of statistical weakness, as manifests in other estimates and statistical tests. It also reflects our (in)ability to make strong inferences at Broadwater.

For example, the discrepancy between the large estimated declines at Broadwater (according to the AICc-based method) versus a weak p-value that cannot rule-out a “no-trend” conclusion is another manifestation of being underpowered.

It may be necessary to add more transects at Broadwater in order to meaningfully improve its power and statistical certainty. An alternative method to boost power, without adding more sites, could be to remove sites whose habitat is definitely not suitable for koalas. In other words, reduce the dilution on estimates from zero-koala sites.

However, the risk site-removal is that we may be inadvertently "picking our own data to prove our hypothesis". In order to avoid such bias, the removal process would ideally pick sites while blind to their counts of koalas. For example, the removal process could be based on an objective "koala habitat index" that is independent of the actual number of koalas present, such as a composite of vegetation/habitat indicators.

A simulation-study may be useful to investigate the effects on power from targeted removal of sites vs. adding more sites.

5.4 A Note on Change Points vs. Linear Trends

All of the statistical analyses thus far have assumed either a log-linear trend (per location), or no trend, or some trend with a slight amount of annual variation. This means that the analyses can only measure and test long-term population changes.

The present analyses do not accommodate more complex population functional-forms, such as reversals of trend, or ‘V’-bottoms. For example, imagine an initial decline, followed by some intervention which causes the population to increase. Such “change point” functional-forms were not possible at the beginning of the study with only a few years of data. It may be possible to do a change point analysis in the near-future as more data are collected, and the koala populations manifest different short-term trends that deviate from the initial and/or long-term trends.

We highlight this, not only to mention the possibility of new change point analyses, but also to highlight the baked-in assumptions of log-linear trend modelling over long time horizons. Consider a future scenario, in which the koala population continues to grow at Broadwater: in order for the log-linear statistical analyses of Broadwater to register an increase, or merely to return to the baseline, there would have to be several years of higher-than-present densities, such that the (hypothetical) increase would have to overpower the earlier declines, and push the log-linear trend back above 0.

We also highlight this potential population pattern, because it could explain what we are witnessing in the sequence of trend estimates (Tables 6 and 7), whereby initial extreme trend estimates have moderated in recent years. However, without further analysis, this is only speculation.

Modelling more-complex population dynamics is difficult when we only have sparse-data. It is likely that a change point would not be useful at this time. But, with additional years of data, there may be a time when such change point analyses could be considered.

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Appendix B: Population survey koala detections year 5

Table B1: Details of koala observations during year 5 population monitoring in the Broadwater focal area. Uk = unknown, M = male, F = female, pr = probable

Site	Date	Day/ Night	Individuals	Koala Easting	Koala Northing	Tran, Rad, Off	Tree sp.	DBH	Sex	Notes/Condition
Spring 2021										
S15	6/10/21	D	1	538960	6787804	Tran	Swamp mahogany	21	M	Healthy
S15	8/11/21	N	1	539037	6787771	Tran	Swamp mahogany	22	Uk	Healthy
S35	8/11/21	N	1	542259	6788803	Off	Swampy mahogany	55	Uk	Healthy
S38	8/11/21	N	1	540367	6789107	Off	Swamp mahogany	45	Uk	Healthy
S41	8/10/21	D	2	539857	6789491	Tran	Swamp mahogany	61	Mpr & Fpr	Healthy, clean rump
S41	8/10/21	D	1	539940	6789411	Radial	Blueberry ash	10	Fpr	Healthy
S41	8/10/21	D	1	539934	6789416	Radial	Swamp mahogany	28	Mpr	Healthy
S46	7/10/21	D	1	541958	6789917	Tran	Flooded gum	49	Uk	Healthy, clean rump, couldn't see eyes
S50	4/11/21	N	2	539179	6790298	Tran	Swamp mahogany	36	Mpr & Fpr	Healthy, clean rump and eyes
S32	8/10/21	D	1	540804	6788812	Off	Blackbutt	58	Uk	Clean rump and eyes
S51	8/11/21	N	1	539629	6790379	Off	Forest red gum	68	Uk	Unclear
S15	9/11/21	N	1	539061	6787702	Off	Swamp mahogany	52	F	Healthy
S15	9/11/21	N	1	539046	6787685	Off	Swamp mahogany	63	Uk	Healthy
Autumn 2022										
S10	18/5/22	D	1	538861	6786699	Tran	Coastal Cyprus pine	15	F	Healthy
S15	8/7/22	N	1	539059	6787743	Off	Broad-leaved paperbark	23	Uk	Healthy
S25	29/6/22	N	1	540279	6788493	Radial	Swamp mahogany	38	F	Healthy
S38	7/7/22	N	1	540289	6789154	On	Scribbly gum	45	Uk	Healthy
S51	27/6/22	D	1	539655	6790396	Off	Forest red gum	45	M	Healthy
S51	5/7/22	N	1	539655	6790396	Off	Forest red gum	45	M	Healthy

Table B2: Details of koala observations during year 4 population monitoring in the Bagotville focal area. F = female, M = male, by = back young, pr = probable, Uk = unknown.

Site	Date	Day/ Night	Koalas	Koala Easting	Koala Northing	Tran, Rad, Incidental	Tree sp.	DBH	Sex	Condition/notes
Spring 2021										
N20	9/11/21	N	1	539259	6796071	Tran	*Eucalyptus* spp.	11	Fpr	Dirtybum,left eye pink
N22	9/11/21	N	1	543406	6793913	Off	*Eucalyptus* spp.	80	Uk	Healthy
N23	14/10/21	D	1	541739	6794711	Tran	NR	24	Uk	Healthy
N23	1/11/21	N	1	541766	6794730	Tran	Grey ironbark	9	M	Healthy, clean bum
N32	1/11/21	D	1	542254	6794480	Tran	White mahogany	22	Uk	Bum clean
N43	21/10/21	D	1	540433	6799600	Off	Uk	Uk	M	Heard call
N73	11/10/21	D	1	541156	6793874	Tran	Camphor laurel	23	M	
N73	21/10/21	N	2	541120	6793869	Tran	Swamp box	21	F&uk	Female and joey healthy
N11	9/11/21	N	Nil	542591	6792968	Off	Tallowwood	49	Uk	
N12	9/11/21	N	Nil	542716	6792596	Off	*Eucalyptus* spp.	10	M	Dirty bum, not wet, clear eyes.
Autumn 2022										
N21	20/5/22	D	1	542234	6793954	Tran	Tallowwood	55	Uk	Healthy
N21	4/7/22	N	1	542212	6793960	Tran	Tallowwood	55	F&by	
N23	18/7/22	D	1	541837	6794653	Off	Tallowwood	NR	NR	NR
N24	20/5/22	D	1	541843	6794956	Tran	Ironbark	37	Uk	Uk
N24	4/7/22	N	2	541684	6795001	Off	Tallowwood	57	Uk	Clear eyes
				541545	6797813	Off	Blackbutt	Uk	Uk	Clear eyes
N28	20/7/22	D	1	542256	6797018	Tran	Swamp mahogany	43	Uk	Healthy?
N28	20/7/22	N	1	542256	6797018	Tran	Swamp mahogany	43	UK	Healthy?
N32	30/6/22	N	1	542184	6794628	Tran	Blackbutt	62	M	Bum clean, eyes appeared clear
N33	18/7/22	D	2	542229	6795471	Tran	Tallowwood	NR	NR	
				542250	6795496	Tran	Tallowwood	NR	NR	
N34	20/5/22	D	1	538280	6796477	Tran	Tallowwood	48	Uk	Dirty bum
N74	30/6/22	N	4	540463	6793901	Tran	Red gum	48	Fpr	All healthy
				540246	6793856	Off	Red gum	52	Uk	All healthy
				540226	6793842	Off	NR	39	Uk	All healthy
				540191	6793822	Off	*Eucalyptus* spp.	60	M	All healthy

Appendix C: Koala genetics report

Final Report

Meta-Population Koala Genetics of Northern New South Wales

Prepared for Sandpiper Ecological Surveys Pty Ltd

November 2022

By Dr Lyndal Hulse BAppSc MScAg PhD

1 EXECUTIVE SUMMARY

This report presents the findings of a study into koala population genetics for 11 individual koalas as assessed via non-invasive sampling, with koala faecal scat samples collected by personnel from Sandpiper Ecological Surveys from sites located in northern New South Wales during surveys conducted in 2021/2022. Scat samples are indicative of koala activity, and representative of koala populations located within the survey site. Koala genomic DNA isolated from faecal scat samples were analysed for genetic diversity and relatedness, assessment of gene flow, population structure, and compared with regional koala population diversity values based on 32 microsatellite genetic markers.

Genetic analysis of the sampled population reveals a decrease in diversity of the population, compared with the 2020 genetic analysis survey of the same site, with the current population showing low to moderate genetic diversity with a loss of alleles over time and possibly indicative of population isolation and genetic drift. Inbreeding value of the population has increased since the previous site survey in 2020, and the current inbreeding value calculated from the 2022 survey further suggests the koala population is becoming genetically isolated with limited geneflow. However, the loss of genetic variation observed in the current 2021/2022 survey may be due to the small sample size analysed, in addition to a reduced geographic survey area, with the limited subset of individuals genetically analysed producing erroneously elevated relatedness and inbreeding values. Analysis with distant regional koala populations revealed moderate to strong genetic differentiation, to be expected given the geographical distances between the populations.

There is genetic sub-structuring into two distinct genetic clusters within the 2022 population, indicating a decrease of gene flow occurring within the population, compared with 2020 genetic analysis whereby three distinct sub-populations were present.

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2 INTRODUCTION

2.1 Background

Koala faecal scat samples (N = 12) were provided by Sandpiper Ecological Surveys, collected from transects of a survey site located in northern New South Wales. Collection of scats were undertaken between November 2021 and July 2022.

For the purposes of this report, the selected samples collected are considered to genetically represent the koala sub-population located within the study site, although there is the possibility that there is some bias in genetic diversity or divergence within the sample.

2.2 Purpose

The purpose of this study is to evaluate current koala presence/absence across the survey site and assess population structure and genetic diversity of a sub-sample of northern NSW koalas. This study aims to provide data that can be used to inform effective measures and strategies to conserve or recover koala populations in northern NSW.

2.3 Study Area

The study area is located adjacent to the Pacific Highway, between Wardell and Broadwater, northern NSW. Figure 1 depicts survey site and locations of koala scat retrieval between the months of November 2019 - May 2020, January – May, 2018 and November 2021 – July 2022.

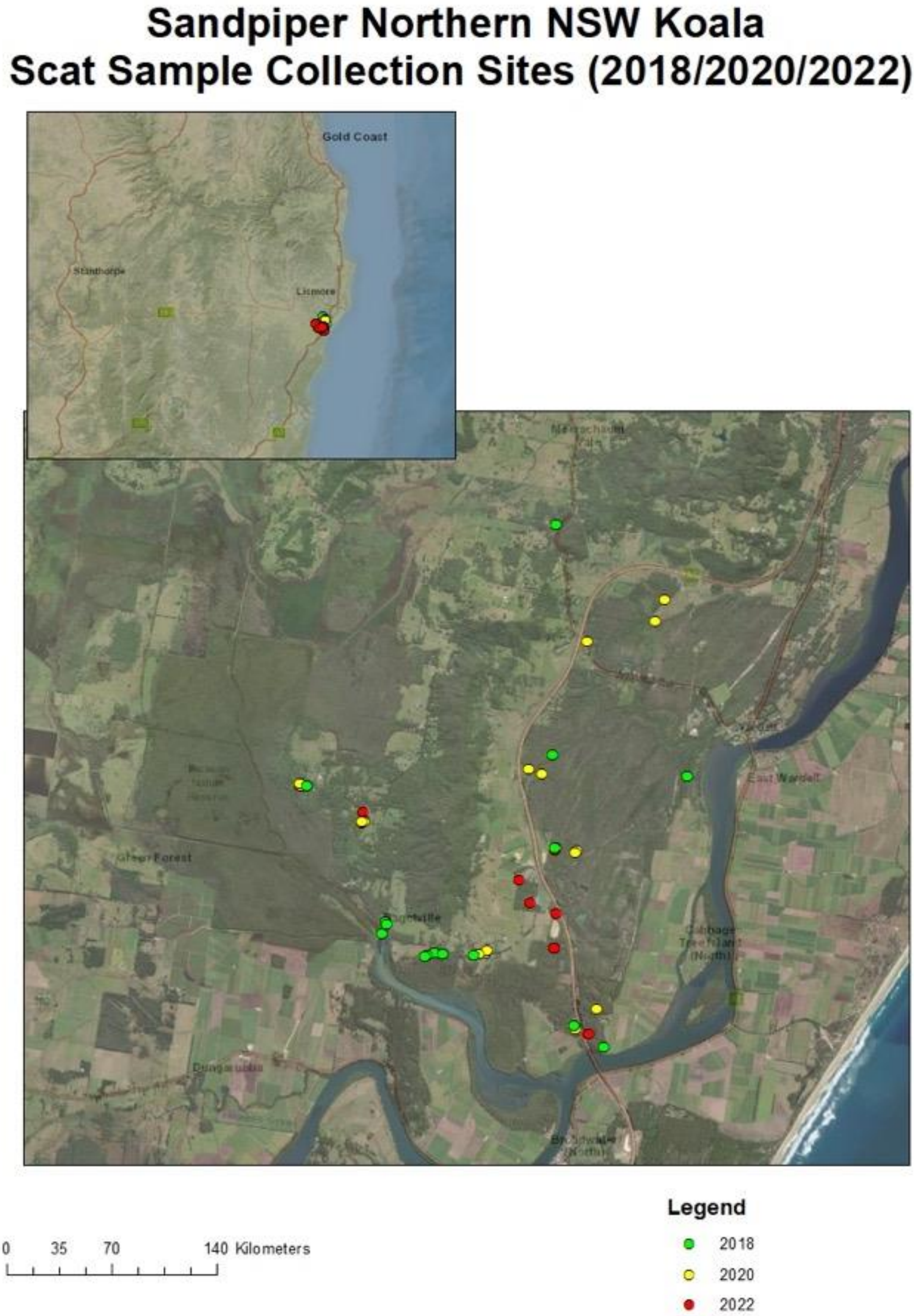

Figure 15 Northern NSW koala survey site with koala scat collection sites for 2018, 2020 and 2022.

3 SCAT ANALYSIS METHODOLOGY

3.1 Scat Analysis Protocol

Koala faecal scats received via mail from Sandpiper Ecological were processed upon arrival. Koala DNA from presumptive mucosal epithelial cells was recovered by scraping the surface of the faecal sample with a scalpel blade.

3.2 DNA Isolation

Genomic DNA was isolated using the NucleoSpin® DNA Stool kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Each DNA isolate was tested for quality and concentration using spectrophotometry (Nanodrop, ThermoFisher Scientific, VIC, Australia). The presence of koala genomic DNA (*Phascolarctos cinereus* beta-actin mRNA) successfully isolated from epithelial cells exfoliated onto the surface of the faecal scats was confirmed via real-time PCR (Hulse et al., 2018). One out of 12 faecal scats did not have genomic DNA isolated. DNA degradation occurs over time and is expedited the longer biological samples are exposed to the environment. Faecal scats exposed to moisture and rain from inclement weather, heat and UV from sunlight have higher amplification failure and genotyping failures compared to scats collected from weather-protected positions. In addition, the presence of volatile organic compounds and phenolics derived from the koala's diet of Eucalyptus leaves may also impede isolation and amplification of DNA. Eucalypt molecules are excreted in koala faeces and are known to damage cell membranes, while phenolics can accelerate DNA degradation.

4 GENETIC ANALYSIS

4.1 Genotypes and Samples

Genotypes across 32 microsatellite loci for 11 scat koalas were generated from genomic DNA. There were no departures from Hardy Weinberg Equilibrium from the population, therefore a total of 32 loci were retained for analysis. Detection of repeated genotypes within the 2022 dataset to identify duplicate samples was performed using the software GENALEX version 6.5 (Peakall and Smouse, 2012) which revealed no identical multilocus genotypes present within the dataset.

4.2 Genetic Diversity

Genetic diversity is the variability of genes in a species; high genetic variability is associated with the potential fitness of a population and ultimately its long-term persistence. In population genetics, the concept of heterozygosity is commonly extended to refer to the population as a whole, i.e., the fraction of individuals in a population that are heterozygous for a particular locus. It can also refer to the fraction of loci within an individual that are heterozygous. High heterozygosity (closer to 1.0) indicates high genetic variability, whereas, low heterozygosity (closer to 0.0) means little genetic variability.

Gene diversity is affected by two elements; 1) the number of alleles and 2) the abundance (or evenness) of the alleles. Increases in either of these leads to an increase in the expected heterozygosity. If a population consists of an excess of homozygotes for different alleles this leads to a low observed heterozygosity but does not affect the expected heterozygosity calculated from Hardy-Weinberg Equilibrium.

Analysis of genetic diversity was performed using the software GENALEX version 6.5 (Peakall and Smouse, 2012) to calculate mean number of alleles and observed and expected heterozygosity. FSTAT (Goudet, 2001) was used to calculate allelic richness, a measure of allelic diversity that takes into account differences in sample sizes by standardising to the smallest number of individuals typed for a locus in a sample, so as to enable comparison among populations. FSTAT was also used to estimate the inbreeding coefficient (F_{IS}) for which a positive value indicates that individuals in a population are more related than you would expect under a model of random mating, and a negative value indicating that individuals in a population are less related.

Genetic diversity values, estimated through expected heterozygosity and allelic richness, were compared between the 2018, 2020 and 2022 surveys (Table 1) and revealed low to moderate diversity of local koalas has been maintained throughout the 2018, 2020 and 2022 surveys. The 2022 survey has revealed a reduction in allelic diversity resulting in a decrease of mean number of alleles per locus, compared with 2018 and 2020 surveys, suggesting koalas located within the survey site becoming isolated. Despite a significant reduction of inbreeding observed in the 2020 survey, the 2022 survey reveals moderate to high inbreeding of koalas indicating relatively recent reductions in population size or gene flow within and between local koala populations, further suggesting the koalas within the study site are becoming isolated. Individual heterozygosity has not significantly deviated from the 2020 and 2018 analysis, where in 2022 individual animal heterozygosity varies from 78.0% down to 37.5%, with a median value of 56.3%, compared to 2020 data where individual animal heterozygosity ranged from 80.6% down to 41.9%, with a median value of 60.0%; and 2018 data where individual animal heterozygosity ranged from 75.0% down to 35.5%, with a median value of 53.1%. Figures 2 -

4 presents the frequency distribution of heterozygosity of individual scat samples collected in 2022, 2020 and 2018, respectively.

Table 11 Genetic diversity statistics representing 2018, 2020 and 2022 northern NSW koala populations.

(Based on 32 loci. Allelic richness, which is the number of alleles per locus corrected for sample size to enable comparison among populations, was estimated for n = 5)

Population	N	A_{mean}	A_r	F_{IS}	H_o	H_e
2018 Survey	19	6.63	4.19	0.204	0.539	0.672
2020 Survey	22	6.16	3.94	0.114	0.594	0.655
2022 Survey	11	5.59	4.26	0.205	0.555	0.687

N: Number of individuals sampled **A_{mean}**: Mean number of alleles per locus

A_r: Allelic richness **H_o**: Observed heterozygosity **H_e**: Expected heterozygosity

F_{IS}: Inbreeding coefficient - the proportion of variance in a population that is contained within an individual; F_{IS} >0 indicates high levels of homozygosity and can suggest inbreeding.

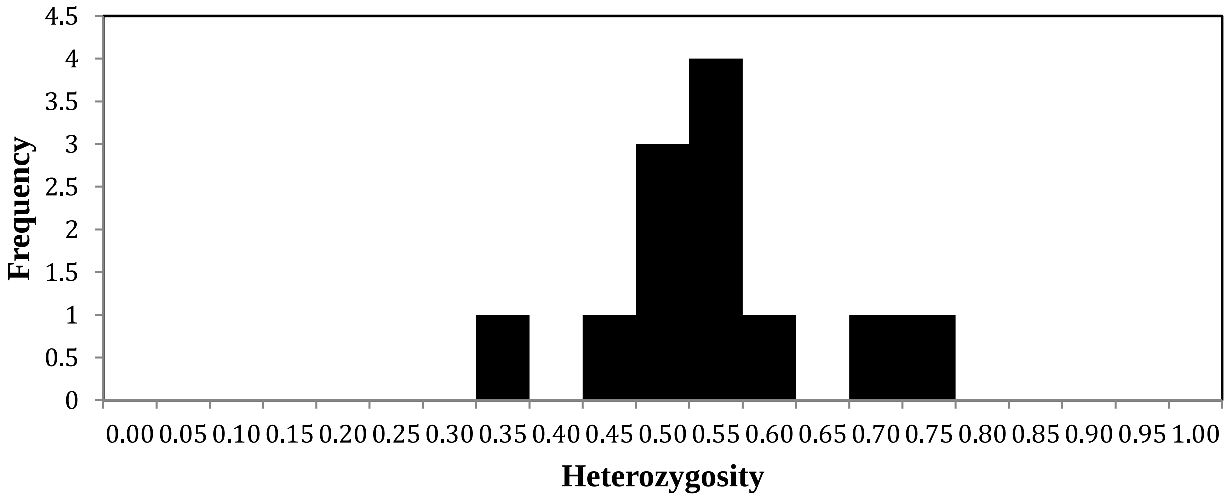

Figure 16 Frequency distribution of heterozygosity of individual scat samples collected in 2022.

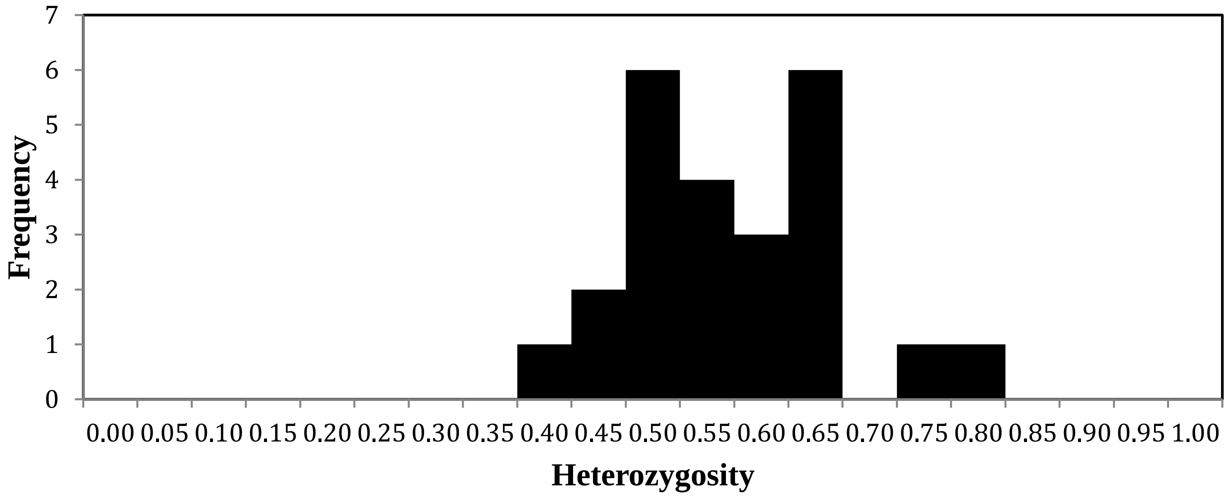

Figure 17 Frequency distribution of heterozygosity of individual scat samples collected in 2020.

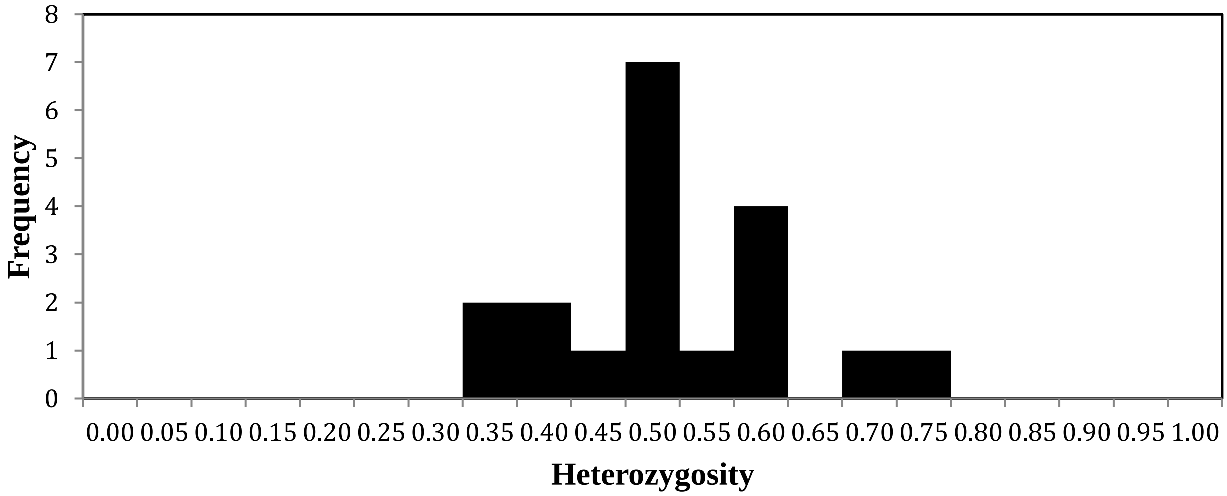

Figure 18 Frequency distribution of heterozygosity of individual scat samples collected in 2018.

Comparison of genetic diversity with previously typed regional koala populations provided further information as to the genetic health of the northern NSW koala population. Table 2 presents diversity values for previously typed regional koala populations located at Byron Bay, NSW (N -28° 38’ 30.88”, E 153° 36’ 37.86), Lismore, NSW (N -28° 48’ 33.95”, E 153° 17’ 16.41), Tweed Heads, NSW (N -28° 10’ 43.20”, E 153° 32’ 13.20), Grandchester, QLD (N -27° 43’ 01.51”, E 152° 28’ 01.65), Sunshine Coast, QLD (N -26° 39’ 00.00”, E 153° 04’ 00.00”) Gold Coast, QLD (N -28° 01’ 66.67”, E 153° 39’ 99.96”), Clarke Connors Range, QLD (N -21° 48’ 54.31”, E 150° 22’ 32.40”), Mt Byron, QLD (N -27° 14’ 79.55”, E 152° 64’ 63.75”), Oakey, QLD (N -27° 4’ 44.177”, E 151° 72’ 28.69”), St Bees, QLD (N -20° 55' 0.012”, E 149° 25' 59.988”) and Yarrabilba, QLD (N -27° 82’ 73.64”, E 153° 12’ 92.11”). These genetic data indicate that genetic diversity in the tested 2022 northern NSW koalas had similar expected heterozygosity and mean number of alleles per locus with Lismore and Tweed Heads koala populations, indicating the 2022 survey has identified the koala population may be collectively considered as a closed population.

Table 12 Comparison of genetic diversity statistics within New South Wales and Queensland koala populations (Allelic richness was estimated for n = 3).

Population	N	A_{mean}	A_r	F_{IS}	H_o	H_e
2018 NSW Survey	19	6.68	3.30	0.207	0.538	0.678
2020 NSW Survey	24	6.12	3.13	0.129	0.588	0.652
Byron Bay	4	4.08	3.49	-0.032	0.647	0.626
Lismore	24	7.28	3.33	0.177	0.570	0.684
Tweed Heads	8	5.76	3.68	0.086	0.639	0.709
Grandchester	26	6.96	3.36	0.103	0.610	0.696
Sunshine Coast	171	10.92	3.70	0.143	0.643	0.755
Gold Coast	173	10.80	3.71	0.171	0.631	0.764
Clarke Connors	54	9.44	3.67	0.229	0.580	0.732
Mt Byron	39	4.08	3.47	0.114	0.628	0.706
Oakey	16	6.20	3.28	0.042	0.624	0.663
St Bees	40	6.52	2.94	0.135	0.539	0.612
Yarrabilba	26	8.72	3.72	0.174	0.621	0.751
2022 NSW Survey	*11*	*5.88*	*3.47*	*0.193*	*0.576*	*0.699*

N: Number of individuals sampled **A_{mean}**: Mean number of alleles per locus

A_r: Allelic richness **H_o**: Observed heterozygosity **H_e**: Expected heterozygosity

F_{IS}: Inbreeding coefficient - the proportion of variance in a population that is contained within an individual; F_{IS} >0 indicates high levels of homozygosity and can suggest inbreeding.

4.3 Pairwise Genetic Differentiation (F_{ST})

Restrictions to gene flow among populations results in a genetic differentiation or divergence of the populations. F_{ST} is a measure of population genetic differentiation that quantifies the proportion of variance in allele frequencies among populations relative to the total variance. As a measure of genetic differentiation among populations, F_{ST} is calculated to evaluate how genetically different koala populations are to one another. A common reason for populations becoming more genetically different is reduced breeding movements of koalas among populations. The greater the genetic differentiation between populations, the less breeding movements there are between them and the more isolated they are from one another. F_{ST} can range from zero to one, where zero means populations show no genetic separation; a value of 0.25 or greater indicates strong differences among populations.

Assessment of genetic differentiation between koala populations was calculated using FSTAT (Goudet, 2001). Table 3 presents genetic differentiation between survey sites positioned east and west of the A1 Pacific

Highway. Inclusive of the site surveys of 2018, 2020 and 2022, there is weak differentiation between east and west koala sub-populations indicating overall evidence of gene flow occurring in koala populations within the range of the study site and that the Pacific Highway has not posed a barrier for koala gene flow. However, there is moderate genetic differentiation between both the east and west sites in 2018 and 2020; and 2020 and 2022 (highlighted yellow in Table 3) indicating there has been a change in genetic diversity of time and suggestive of the Pacific Highway decreasing gene flow between east and west koala sub-populations.

Table 4 presents genetic differentiation between the 2022, 2020 and 2018 northern NSW koala population and regional koala populations. There is weak differentiation between the 2022, 2020 and 2018 northern NSW populations indicating there is gene flow occurring over time in koala populations within the range of the study site. There is moderate to strong genetic differentiation between the 2022 northern NSW population and regional populations, as expected given the geographical distances between populations and isolation of the island population on St Bees.

Table 13 Pairwise F_{ST} values between 2022, 2020 and 2018 northern NSW koala surveys

	West of Pacific Highway
East of Pacific Highway	0.0254

	West of Pacific Highway 2018	East of Pacific Highway 2020	West of Pacific Highway 2020	East of Pacific Highway 2022	West of Pacific Highway 2022
East of Pacific Highway 2018	0.0366	0.0038	0.0871	0.0000	0.0224
West of Pacific Highway 2018		0.0151	0.0115	0.0365	0.0000
East of Pacific Highway 2020			0.0383	0.0064	0.0186
West of Pacific Highway 2020				0.0860	0.0346
East of Pacific Highway 2022					0.0000

<0.05 = **weak** genetic differentiation
0.05-0.15 = **moderate** genetic differentiation
0.15-0.25 = **strong** genetic differentiation
>0.25 = **very strong** genetic differentiation

Table 14. Pairwise F_{ST} values between 2022, 2020 and 2018 northern NSW koala surveys and regional koala populations.

	2020 Survey	2018 Survey	Grandchester	Sunshine Coast	Gold Coast	Byron Bay	Lismore	Tweed	Clark Connors	Mt Byron	Oakey	St Bees	Yarrabilba
2022 Survey	0.027	0.011	0.152	0.146	0.119	0.146	0.130	0.115	0.165	0.162	0.191	0.240	0.117
2020 Survey		0.002	0.178	0.165	0.132	0.172	0.138	0.142	0.201	0.187	0.214	0.258	0.149
2018 Survey			0.161	0.153	0.122	0.157	0.131	0.127	0.186	0.176	0.192	0.242	0.134
Grandchester				0.058	0.066	0.102	0.119	0.071	0.102	0.095	0.119	0.158	0.065
Sunshine Coast					0.048	0.075	0.092	0.054	0.074	0.052	0.080	0.125	0.062
Gold Coast						0.033	0.063	0.020	0.091	0.082	0.089	0.141	0.052
Byron Bay							0.059	0.025	0.122	0.120	0.155	0.193	0.076
Lismore								0.035	0.141	0.133	0.162	0.200	0.094
Tweed									0.089	0.097	0.105	0.166	0.055
Clark Connors										0.090	0.098	0.075	0.091
Mt Byron											0.069	0.153	0.083
Oakey												0.169	0.103
St Bees													0.155

<0.05 = **weak** genetic differentiation
0.05-0.15 = **moderate** genetic differentiation
0.15-0.25 **strong** genetic differentiation
>0.25 = **very strong** genetic differentiation

4.4 Genetic Relatedness

Genetic relatedness was estimated to indicate the proportion of shared ancestry in pairs of individuals. Expected values are 0.5 for parent-offspring or full-sib pairs and 0.25 for half-sib pairs. However, genetic relatedness values will form a distribution around these expected values. Genetic relatedness of within-population individuals was calculated in GENALEX version 6.5 (Peakall and Smouse, 2012) using the Queller and Goodnight estimator of relatedness.

Genetic relatedness was estimated and compared for the 2018, 2020 and 2022 northern NSW koala populations separately. Figure 5 presents the average relatedness for each survey and revealed a wider distribution in relatedness values for koalas identified in the 2022 survey, compared to the 2018 and 2020 surveys. Noticeably, the 2020 koala survey shows a mean relatedness that is higher than the confidence interval, suggesting that koalas are significantly more related than expected. A full list of individual pairwise genetic relatedness values for combined 2022, 2020 and 2018 individuals are shown in Appendix 1.

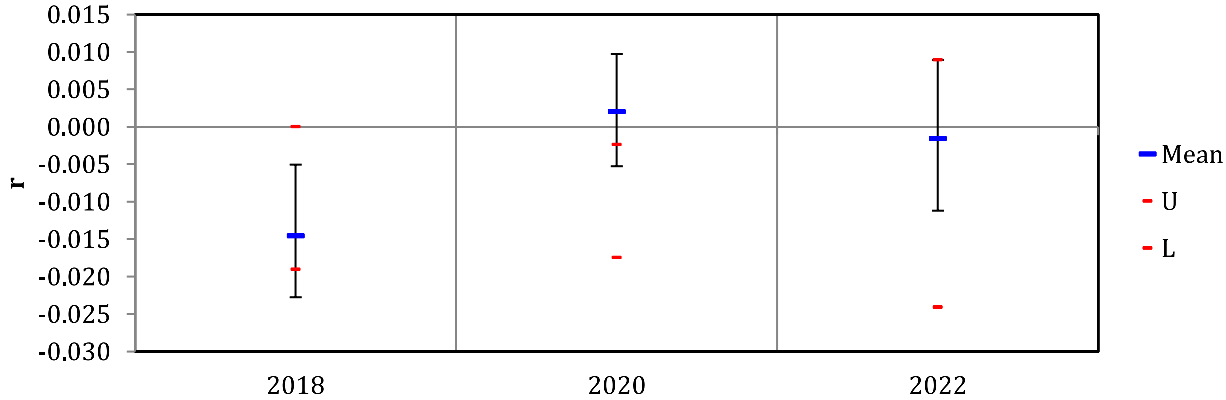

Figure 19 Mean genetic relatedness (r) for 2018, 2020 and 2022 koala site surveys.

The red lines indicate the upper (U) and lower (L) 95% confidence interval expected for that population under the null hypothesis of no difference among populations; r = relatedness.

4.5 Population Structure

The clustering of koalas into genetic populations, termed population structuring, was determined using the Bayesian clustering program STRUCTURE version 2.3.4 (Pritchard et al. 2000). STRUCTURE implements a model-based clustering method for inferring population structure using genotype data of unlinked markers. This method demonstrates the presence of population structure, identifies distinct genetic populations and assigns individuals to populations or clusters without any prior information about geographical location. The notion of a genetic cluster is that individuals within the cluster share on average more similar allele frequencies to each other than to those in other clusters.

Analysis of koala population genotype data involved 5 replicates of K = 1 to K = 10 (K = genetic cluster) using 150,000 iterations with 150,000 iterations discarded as burn-in. The number of K clusters was determined using both the maximum likelihood and the deltaK method of Evanno et al. (2005).

STRUCTURE analysis identified two genetic clusters of koalas in both the 2022 and 2018 surveys, with both surveys comparable to each other (K = 2, Figures 6 and 8, respectively) compared to genetic clusters at the 2020 survey site (K = 3, Figure 7). Identification of an additional genetic cluster at the 2020 survey site indicates evidence of gene flow occurring within the population; however, the 2022 survey has identified the reduction of a genetic cluster at this site, providing further evidence of reduced gene flow and genetic isolation of the koala population. Figure 9A, B and C depicts each scat sample location and represented by a pie chart, details that individual's proportional assignment to each of the clusters from the STRUCTURE analysis.

K = 2

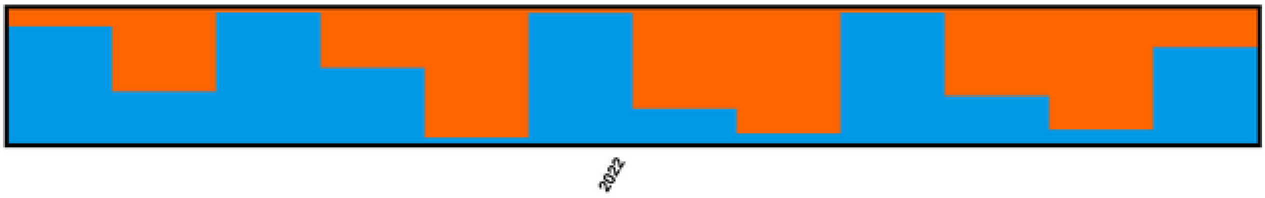

Figure 20 Population substructure of 2022 northern NSW koala populations using STRUCTURE based on 32 loci.

K = 3

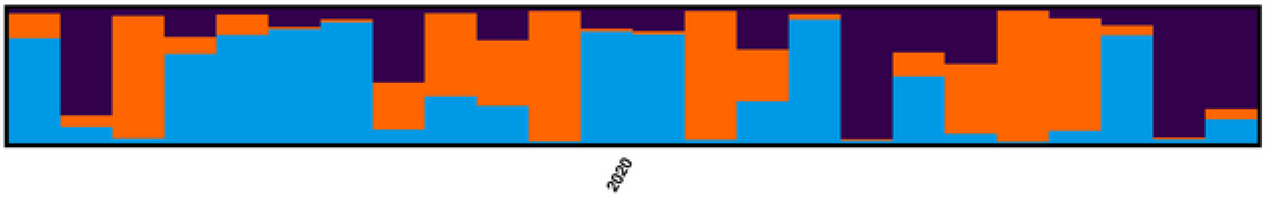

Figure 21 Population substructure of 2020 northern NSW koala populations using STRUCTURE based on 32 loci.

K = 2

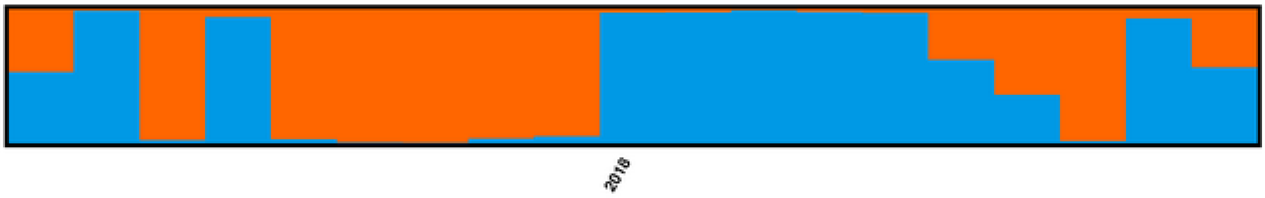

Figure 22 Population substructure of 2018 northern NSW koala populations using STRUCTURE based on 32 loci.

Each bar represents an individual koala and colours indicate the proportion of the population cluster to which an individual was assigned.

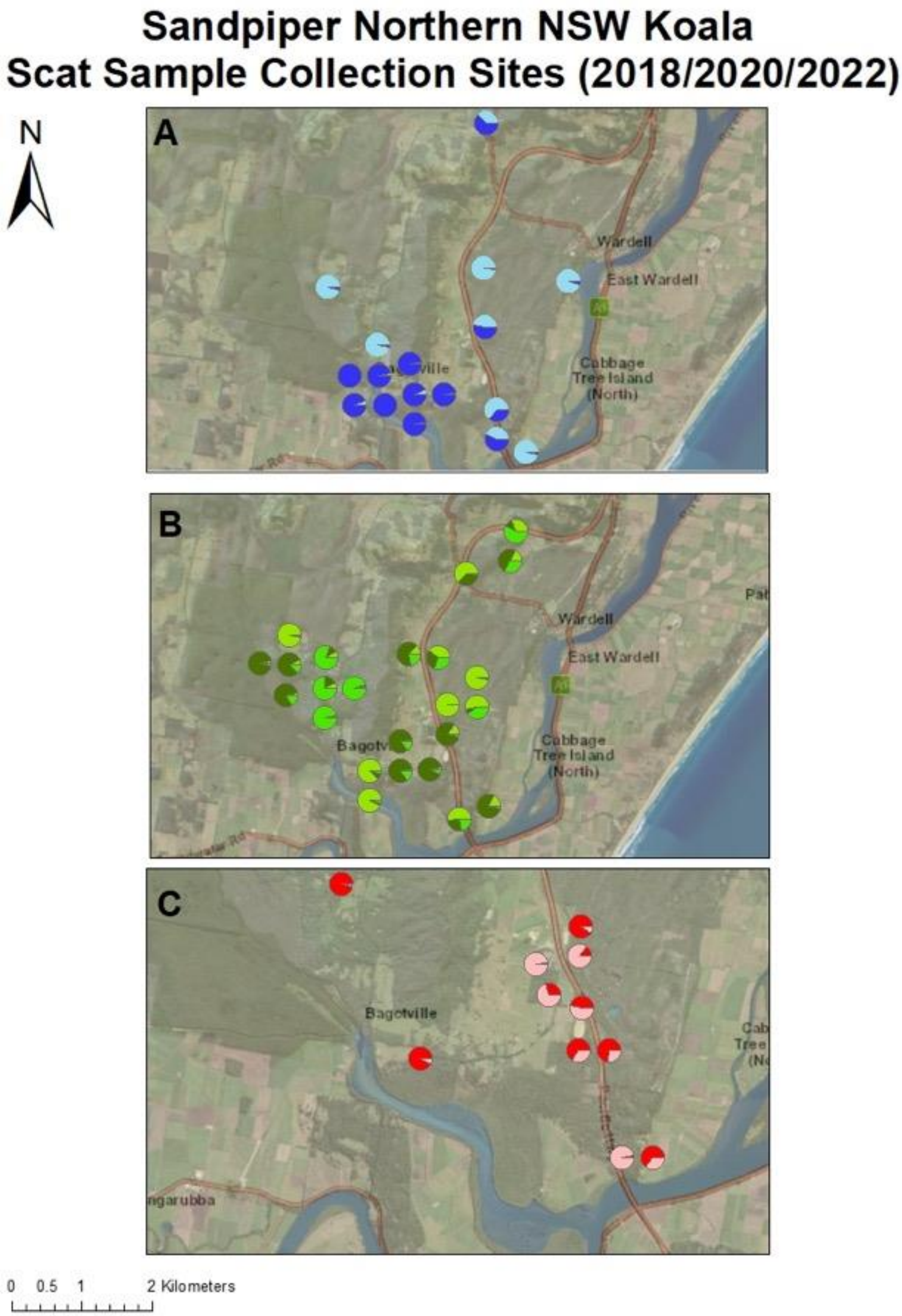

Figure 23 Inferred cluster assignments of (A) 2018 (K = 2); (B) 2020 (K = 3) and (C) 2022 (K = 2) northern NSW koalas.

Each koala is represented by a pie chart, which details that individual's proportional assignment to each of the clusters from the STRUCTURE analysis (Figures 6 - 8), where clusters are shown by proportion of colour.

4.6 Effective Population Size

The loss of genetic variation through the process of random genetic drift occurs much more rapidly when population sizes are small. When assessing effective population size (N_e) as a measure of genetic drift, it is essential to take into account that an ideal population constitutes equal numbers of males and females, all of which are able to reproduce and produce offspring, mating is random and the number of breeding individuals is constant from one generation to the next. Large variances between breeding male and female numbers in a population directly effects N_e. N_e estimates, which reflect N_e from the last one to several generations, generally mirror the severity of known bottlenecks (Funk et al, 2016). Determination of sex was tested using Y-linked markers designed to amplify a 569-bp region of sex determining region of the Y chromosome (SRY gene). Appendix 2 presents visualisation of sex determination from genomic DNA isolated from koala faecal scats collected from the survey site in 2022. N_e was estimated using the molecular co-ancestry method of Nomura (2008), as implemented in NeEstimator V2.1 (Do et al., 2014).

Tables 5 presents effective population size estimates of males and females from 2018, 2020 and 2022 koala surveys. Based on the sample sizes for both male and female koalas for all survey years, the confidence intervals for females in 2018 and 2020 are not notably wide indicating the N_e value is informative enough to predict the effective population size. However, N_e predicted within the male cohort for each survey year is infinity, indicating there is no evidence for variation in the genetic characteristic caused by a finite number of parents and can be due to sampling error. Assessment of heterozygote excess (D) returned negative values for all cohorts, with the exception of the male cohort in 2018, indicating a deficit of heterozygote samples in populations and therefore a difference in allele frequencies between population males and females.

Table 15. Effective population size of males and females for NSW koalas.

Year	Population	n	N_e (P=0.05)	95%CI		D
2018	Female	14	14.8	11.6	19.7	-0.310
	Male	5	∞	14.5	∞	0.041
2020	Female	19	12.5	10.8	14.5	-0.165
	Male	5	∞	30.9	∞	-0.043

2022	Female	6	30.4	10.4	∞	-0.195
	Male	5	∞	∞	∞	-0.097

n = Number of samples; Ne: Effective population size (P = 0.05); 95% CI: 95% confidence interval; and D: Heterozygote excess estimate.

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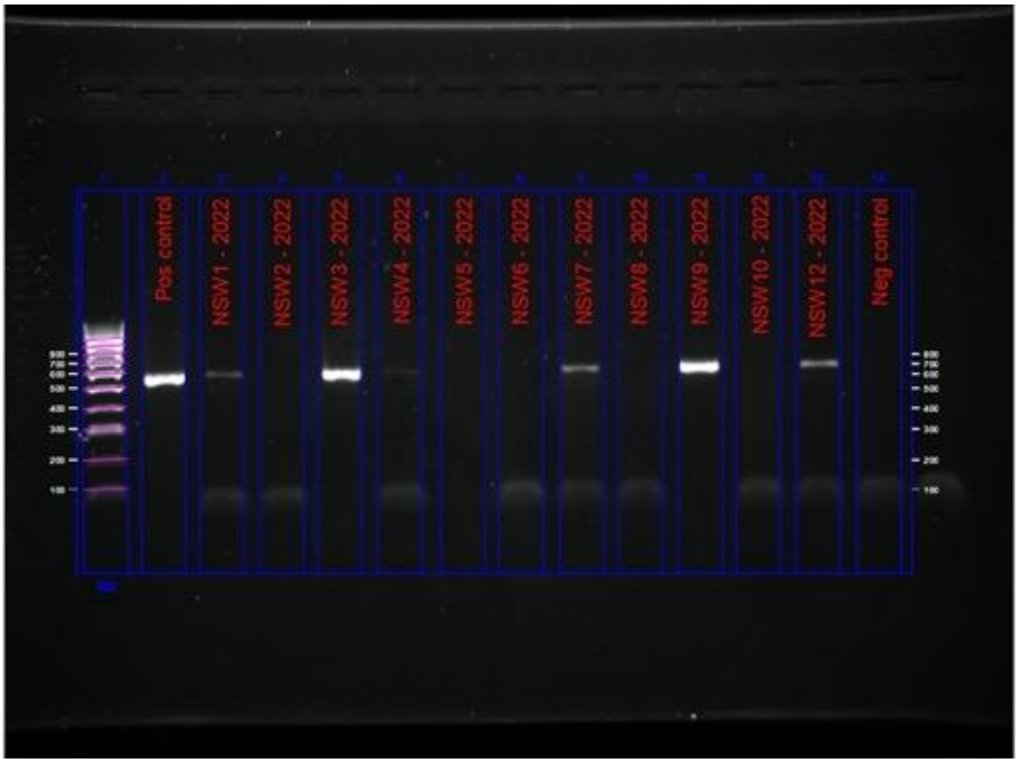
Appendix 1. Labelled pairwise relatedness matrix for 2018, 2020 and 2022 northern NSW Koala Population.

Pairs of individuals with high levels of genetic relatedness (i.e. values approximating those expected for full-sib pairs and parent-offspring, 0.5) are highlighted in yellow and those with genetic relatedness values approximating half-sib pairs (0.25) are highlighted in blue.

		2018																			2020																								2022										
		BB_KO1	BT_K2	BT_K3	BT_K4	BT_K5	KH3	KH4	SF_K05	SF_K06	SF_K07	SF_K08	SF_K09	SF_K10	SF_K11	SF_K12	SF_K13	SF_K14	SF_K15	SF_K16	BT_K10	BT_K11	BT_K12	BT_K13	BT_K14	BT_K1	BT_K2	BT_K3	BT_K4	BT_K5	BT_K6	BT_K7	BT_K8	BT_K9	SF_K10	SF_K1	SF_K2	SF_K3	SF_K4	SF_K5	SF_K6	SF_K7	SF_K8	SF_K9	NSW10	NSW12	NSW1	NSW2	NSW3	NSW4	NSW5	NSW6	NSW7	NSW8	NSW9
	BB_KO1	0.000	0.179	0.103	0.082	0.000	0.000	0.000	0.000	0.000	0.000	0.064	0.000	0.120	0.027	0.151	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077	0.068	0.000	0.000	0.061	0.103	0.168	0.006	0.000	0.000	0.000	0.264	0.000	0.073	0.083	0.675	0.000	0.000	0.000	0.096	0.000	0.000	0.000	0.000	0.054	0.000	0.093	0.142	0.000	0.000	0.638	0.000
	BT_K2		0.000	0.050	0.318	0.000	0.000	0.000	0.000	0.000	0.163	0.483	0.387	0.545	0.426	0.000	0.149	0.000	0.425	0.086	0.097	0.126	0.009	0.372	0.194	0.344	0.244	0.000	0.258	0.193	0.000	0.296	0.368	0.000	0.295	0.420	0.282	0.012	0.146	0.000	0.175	0.273	0.207	0.098	0.116	0.000	0.117	0.177	0.000	0.133	0.367	0.000	0.214	0.232	0.000
	BT_K3			0.000	0.000	0.100	0.040	0.000	0.000	0.069	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.281	0.000	0.000	0.000	0.101	0.000	0.175	0.000	0.000	0.000	0.065	0.000	0.000	0.000	0.064	0.061	0.000	0.000	0.000	0.232	0.000	0.118	0.000	0.148	0.000	0.099	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.081	0.000	0.000	0.000
	BT_K4				0.000	0.000	0.000	0.000	0.000	0.000	0.377	0.251	0.330	0.134	0.206	0.125	0.098	0.000	0.356	0.024	0.258	0.176	0.047	0.186	0.194	0.158	0.190	0.000	0.000	0.167	0.000	0.090	0.075	0.000	0.083	0.138	0.072	0.138	0.000	0.000	0.000	0.031	0.106	0.079	0.000	0.000	0.036	0.316	0.000	0.209	0.189	0.000	0.260	0.055	0.122
	BT_K5					0.000	0.059	0.051	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.057	0.000	0.000	0.000	0.000	0.058	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000
	KH3						0.000	0.053	0.039	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.043	0.036	0.000	0.000
	KH4							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.070	0.000	0.000	0.000
	SF_K05								0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.061	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.837	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.118	0.000	0.000	0.000	0.704	0.000	0.000	0.000	0.000	0.000	0.000
	SF_K06									0.000	0.000	0.000	0.000	0.000	0.000	0.057	0.000	0.062	0.000	0.000	0.067	0.000	0.135	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2018	SF_K07										0.000	0.452	0.285	0.063	0.128	0.229	0.000	0.000	0.385	0.100	0.123	0.101	0.171	0.279	0.351	0.132	0.187	0.060	0.000	0.085	0.000	0.092	0.202	0.000	0.009	0.182	0.093	0.121	0.000	0.000	0.101	0.044	0.037	0.286	0.000	0.000	0.235	0.090	0.000	0.169	0.107	0.000	0.149	0.000	0.088
	SF_K08											0.000	0.397	0.275	0.126	0.105	0.034	0.000	0.314	0.063	0.030	0.023	0.000	0.119	0.129	0.138	0.166	0.000	0.066	0.354	0.000	0.056	0.346	0.000	0.186	0.265	0.199	0.011	0.000	0.000	0.078	0.168	0.140	0.117	0.174	0.000	0.253	0.124	0.000	0.122	0.117	0.000	0.000	0.165	0.000
	SF_K09												0.000	0.162	0.453	0.108	0.064	0.000	0.155	0.108	0.131	0.139	0.029	0.119	0.167	0.437	0.541	0.066	0.000	0.129	0.000	0.412	0.157	0.000	0.104	0.276	0.191	0.157	0.000	0.000	0.030	0.038	0.140	0.301	0.006	0.000	0.164	0.228	0.000	0.288	0.226	0.000	0.135	0.000	0.057
	SF_K10													0.000	0.215	0.078	0.000	0.000	0.307	0.058	0.305	0.000	0.000	0.095	0.129	0.146	0.124	0.078	0.182	0.147	0.000	0.120	0.288	0.000	0.196	0.431	0.158	0.000	0.202	0.000	0.086	0.177	0.263	0.107	0.055	0.000	0.220	0.083	0.000	0.000	0.185	0.000	0.145	0.125	0.058
	SF_K11														0.000	0.143	0.132	0.000	0.010	0.238	0.192	0.224	0.000	0.345	0.106	0.765	0.477	0.353	0.107	0.000	0.000	0.716	0.286	0.000	0.000	0.211	0.212	0.048	0.000	0.000	0.000	0.200	0.187	0.137	0.021	0.000	0.000	0.262	0.000	0.225	0.231	0.000	0.309	0.031	0.052
	SF_K12															0.000	0.000	0.000	0.000	0.000	0.136	0.000	0.035	0.024	0.042	0.142	0.066	0.264	0.000	0.040	0.000	0.102	0.103	0.000	0.000	0.050	0.000	0.099	0.049	0.000	0.000	0.050	0.094	0.058	0.000	0.000	0.030	0.185	0.000	0.101	0.122	0.000	0.000	0.156	0.206
	SF_K13																0.000	0.000	0.000	0.204	0.000	0.000	0.000	0.105	0.011	0.039	0.000	0.000	0.000	0.055	0.000	0.020	0.017	0.000	0.000	0.058	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.124	0.084	0.000	0.109	0.000	0.000
	SF_K14																	0.000	0.054	0.000	0.000	0.003	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.219	0.000	0.000	0.000
	SF_K15																		0.000	0.000	0.144	0.185	0.045	0.195	0.222	0.000	0.133	0.000	0.000	0.236	0.000	0.000	0.234	0.000	0.218	0.279	0.228	0.000	0.000	0.000	0.118	0.169	0.078	0.207	0.184	0.000	0.227	0.000	0.000	0.082	0.060	0.000	0.162	0.000	0.000
	SF_K16																			0.000	0.000	0.058	0.000	0.097	0.041	0.184	0.065	0.095	0.035	0.098	0.000	0.169	0.041	0.000	0.019	0.144	0.064	0.000	0.000	0.000	0.000	0.003	0.000	0.023	0.036	0.000	0.000	0.013	0.000	0.126	0.077	0.000	0.047	0.037	0.000
	BT_K10																				0.000	0.000	0.000	0.079	0.353	0.142	0.246	0.000	0.000	0.054	0.000	0.147	0.084	0.000	0.000	0.418	0.000	0.051	0.000	0.000	0.000	0.043	0.000	0.059	0.000	0.000	0.000	0.032	0.000	0.000	0.164	0.000	0.205	0.000	0.136
	BT_K11																					0.000	0.023	0.229	0.156	0.248	0.286	0.223	0.000	0.000	0.000	0.279	0.110	0.000	0.005	0.062	0.504	0.095	0.000	0.000	0.016	0.061	0.483	0.326	0.000	0.000	0.000	0.305	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	BT_K12																						0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.000	0.000	0.362	0.000	0.000	0.144	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	BT_K13																							0.000	0.156	0.257	0.243	0.077	0.215	0.000	0.000	0.247	0.255	0.000	0.034	0.119	0.206	0.000	0.000	0.000	0.090	0.126	0.137	0.053	0.000	0.000	0.000	0.120	0.000	0.147	0.155	0.000	0.409	0.012	0.000
	BT_K14																								0.000	0.101	0.225	0.014	0.000	0.006	0.000	0.155	0.117	0.000	0.131	0.420	0.000	0.157	0.026	0.000	0.000	0.040	0.000	0.053	0.000	0.000	0.000	0.022	0.000	0.088	0.000	0.000	0.060	0.000	0.000
	BT_K1																									0.000	0.622	0.236	0.048	0.000	0.000	0.895	0.304	0.000	0.000	0.258	0.227	0.056	0.000	0.000	0.000	0.198	0.214	0.205	0.112	0.000	0.011	0.187	0.000	0.136	0.150	0.000	0.043	0.000	0.183
	BT_K2																										0.000	0.143	0.011	0.000	0.000	0.609	0.180	0.000	0.000	0.349	0.116	0.199	0.000	0.000	0.000	0.168	0.090	0.070	0.111	0.000	0.116	0.078	0.000	0.217	0.027	0.000	0.008	0.000	0.031
	BT_K3																											0.000	0.038	0.000	0.000	0.319	0.000	0.000	0.046	0.158	0.261	0.221	0.107	0.000	0.014	0.000	0.264	0.151	0.000	0.000	0.038	0.104	0.000	0.000	0.000	0.000	0.021	0.000	0.004
	BT_K4																												0.000	0.000	0.000	0.060	0.046	0.000	0.000	0.138	0.000	0.000	0.034	0.000	0.164	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.076	0.000	0.062	0.000	0.000	0.142	0.000
	BT_K5																													0.000	0.000	0.000	0.180	0.000	0.299	0.065	0.048	0.000	0.150	0.000	0.082	0.181	0.060	0.006	0.027	0.000	0.174	0.022	0.000	0.112	0.000	0.000	0.035	0.120	0.190
	BT_K6																														0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.050	0.000
2020	BT_K7																															0.000	0.310	0.000	0.000	0.289	0.258	0.034	0.000	0.000	0.000	0.148	0.162	0.198	0.077	0.000	0.000	0.165	0.000	0.144	0.134	0.000	0.026	0.000	0.086
	BT_K8																																0.000	0.000	0.207	0.282	0.241	0.000	0.000	0.000	0.233	0.846	0.283	0.136	0.100	0.000	0.025	0.131	0.000	0.068	0.332	0.000	0.123	-0.075	0.134
	BT_K9																																	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.744	0.000	0.000	0.000	0.000	0.000	0.000
	SF_K10																																		0.000	0.224	0.247	0.145	0.370	0.000	0.065	0.129	0.161	0.028	0.072	0.000	0.016	0.000	0.000	0.115	0.000	0.000	0.000	0.068	0.041
	SF_K1																																			0.000	0.117	0.228	0.000	0.000	0.104	0.153	0.046	0.077	0.004	0.000	0.167	0.000	0.000	0.000	0.075	0.000	0.075	0.000	0.010
	SF_K2																																				0.000	0.181	0.116	0.000	0.084	0.177	0.770	0.416	0.000	0.000	0.064	0.362	0.000	0.125	0.049	0.000	0.000	0.000	0.000
	SF_K3																																					0.000	0.023	0.000	0.000	0.007	0.210	0.165	0.000	0.000	0.165	0.070	0.000	0.144	0.000	0.000	0.000	0.000	0.000
	SF_K4																																						0.000	0.000	0.000	0.000	0.287	0.000	0.049	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.495	0.000
	SF_K5																																							0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000
	SF_K6																																								0.000	0.171	0.000	0.000	0.024	0.000	0.103	0.000	0.000	0.000	0.049	0.000	0.000	0.000	0.000
	SF_K7																																									0.000	0.221	0.000	0.056	0.000	0.030	0.035	0.000	0.093	0.317	0.000	0.058	0.000	0.177
	SF_K8																																										0.000	0.365	0.000	0.000	0.051	0.479	0.000	0.017	0.034	0.000	0.000	0.000	0.069
	SF_K9																																											0.000	0.000	0.000	0.039	0.234	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	NSW10																																												0.000	0.000	0.126	0.000	0.041	0.000	0.000	0.000	0.000	0.000	0.000
	NSW12																																													0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	NSW1																																														0.000	0.191	0.000	0.030	0.040	0.000	0.054	0.000	0.089
	NSW2																																															0.000	0.000	0.050	0.259	0.000	0.163	0.190	0.046
	NSW3																																																0.000	0.000	0.000	0.000	0.008	0.000	0.000
2022	NSW4																																																	0.000	0.149	0.000	0.018	0.084	0.003
	NSW5																																																		0.000	0.000	0.218	0.222	0.076
	NSW6																																																			0.000	0.000	0.000	0.000
	NSW7																																																				0.000	0.058	0.063
	NSW8																																																					0.000	0.000
	NSW9																																																						0.000

Appendix 2. Amplification of Y-linked DNA markers to determine koala gender. Lane 1, 1000 bp DNA Ladder; Lanes 3, 5, 9, 11 and 13: Male koala DNA isolated from koala faecal scats collected in 2022.



Appendix D - Road mortality surveys

Table C1: Details of road mortality surveys within sections 1-11 of the Pacific Motorway (PMW) and a section of Wardell Road and Old Pacific Highway, Wardell, in August 2021.

Road	Date	Start:End	Observers	Carriageway	Species/group	Easting	Northing
PMW	26/8/21	0930:1300	DR & TR	SB	Short-beaked Echidna	549637	6806889
PMW	26/8/21	0930:1300	DR & TR	SB	European hare	548785	6806757
PMW	26/8/21	0930:1300	DR & TR	SB	Short-beaked echidna	548173	6806574
PMW	26/8/21	0930:1300	DR & TR	SB	European hare	546202	6800490
PMW	26/8/21	0930:1300	DR & TR	SB	Bandicoot spp	542418	6798130
PMW	26/8/21	0930:1300	DR & TR	SB	European hare	543398	6791231
PMW	26/8/21	0930:1300	DR & TR	SB	Medium mammal	542757	6789683
PMW	26/8/21	0930:1300	DR & TR	SB	Large macropod	542468	6789512
PMW	26/8/21	0930:1300	DR & TR	SB	Large macropod	542256	6769379
PMW	26/8/21	0930:1300	DR & TR	SB	Medium mammal	542048	6789244
PMW	26/8/21	0930:1300	DR & TR	SB	Bird spp	541072	6789089
PMW	26/8/21	0930:1300	DR & TR	SB	Unid mammal	538136	6785997
PMW	26/8/21	0930:1300	DR & TR	SB	Unid mammal	533604	6782415
PMW	26/8/21	0930:1300	DR & TR	SB	Small mammal	520668	6758936
PMW	26/8/21	0930:1300	DR & TR	SB	European hare	520955	6758443
PMW	26/8/21	0930:1300	DR & TR	SB	Barn Owl	523424	6746376
PMW	26/8/21	0930:1300	DR & TR	SB	Bird spp	523394	6745985
PMW	26/8/21	0930:1300	DR & TR	SB	Unid mammal	523521	6745476
PMW	26/8/21	0930:1300	DR & TR	SB	White ibis	523395	6744127
PMW	26/8/21	0930:1300	DR & TR	SB	Unid mammal	520037	6739339
PMW	26/8/21	0930:1300	DR & TR	SB	Wallaby sp	519289	6734835
PMW	26/8/21	0930:1300	DR & TR	SB	Wallaby sp	518274	6732085
PMW	26/8/21	0930:1300	DR & TR	SB	Microbat	513152	6723714
PMW	26/8/21	0930:1300	DR & TR	SB	Unid mammal	512093	6715189
PMW	26/8/21	0930:1300	DR & TR	SB	Greater glider	502889	6704764
PMW	26/8/21	0930:1300	DR & TR	NB	Southern boobook	503833	6707307
PMW	26/8/21	0930:1300	DR & TR	NB	Laughing kookaburra	513667	6727850
PMW	26/8/21	0930:1300	DR & TR	NB	Red fox	514496	6729407
PMW	26/8/21	0930:1300	DR & TR	NB	Flying-fox spp	519146	6733969
PMW	26/8/21	0930:1300	DR & TR	NB	Medium mammal	520617	6740812
PMW	26/8/21	0930:1300	DR & TR	NB	Medium mammal	521305	6741785
PMW	26/8/21	0930:1300	DR & TR	NB	Bird spp	522129	6742539
PMW	26/8/21	0930:1300	DR & TR	NB	Small mammal	523389	6743599
PMW	26/8/21	0930:1300	DR & TR	NB	Duck	523409	6744575
PMW	26/8/21	0930:1300	DR & TR	NB	Duck	523416	6744722
PMW	26/8/21	0930:1300	DR & TR	NB	Little pied cormorant	523501	6749101
PMW	26/8/21	0930:1300	DR & TR	NB	Medium mammal	524316	6751819

Road	Date	Start:End	Observers	Carriageway	Species/group	Easting	Northing
PMW	26/8/21	0930:1300	DR & TR	NB	European hare	524191	6752289
PMW	26/8/21	0930:1300	DR & TR	NB	Masked owl	523992	6752943
PMW	26/8/21	0930:1300	DR & TR	NB	Large macropod	526895	6771500
PMW	26/8/21	0930:1300	DR & TR	NB	Large macropod	529174	6775022
PMW	26/8/21	0930:1300	DR & TR	NB	Laughing kookaburra	531095	6776859
PMW	26/8/21	0930:1300	DR & TR	NB	Short-beaked Echidna	537133	6788449 6
PMW	26/8/21	0930:1300	DR & TR	NB	Tawny frogmouth	537476	6784598
PMW	26/8/21	0930:1300	DR & TR	NB	Swamp wallaby	537984	6785085
PMW	26/8/21	0930:1300	DR & TR	NB	Wallaby spp	541861	6789214
PMW	26/8/21	0930:1300	DR & TR	NB	Australian magpie	542090	6789309
PMW	26/8/21	0930:1300	DR & TR	NB	Purple swamphen	542284	6789420
PMW	26/8/21	0930:1300	DR & TR	NB	Small mammal	543432	6790821
PMW	26/8/21	0930:1300	DR & TR	NB	Swamp wallaby	545279	6799559
PMW	26/8/21	0930:1300	DR & TR	NB	Bird spp	546186	6800525
PMW	26/8/21	0930:1300	DR & TR	NB	Medium mammal	546342	6800886
PMW	26/8/21	0930:1300	DR & TR	NB	Medium mammal	546342	6800886
PMW	26/8/21	0930:1300	DR & TR	NB	Carpet python	548997	6806803
Wardell	26/8/21	1300:1305	DR & TR	WB	Nil		
Wardell	26/8/21	1305-1310	DR & TR	EB	Australian magpie	543368	6798153
Wardell	26/8/21	1305-1310	DR & TR	EB	Australian magpie	543370	6798158
Pacific Hway	26/8/21	1310:1320	DR & TR		Nil		

Table C2: Details of road mortality surveys within sections 1-11 of the Pacific Motorway (PMW) and a section of Wardell Road and Old Pacific Highway, Wardell, in November 2021.

Road	Date	Start:End	Observers	Carriageway	Species/group	Easting	Northing
PHW	10/11/21	10:00-15:00	AE/LA	SB	Australian Ibis	542190	6797821
PHW	10/11/21	10:00-15:00	AE/LA	SB	Australian Magpie	523532	6746606
PHW	10/11/21	10:00-15:00	AE/LA	NB	Australian Wood duck	520346	6740482
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	541712	6796508
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bandicoot spp.	543428	6790739
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bandicoot spp.	543173	6790079
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	543026	6789891
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	520845	6758545
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bandicoot spp.	524061	6752733
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	524338	6751622
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	523554	6749320
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	513327	6683776
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bandicoot spp.	513930	6682496
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bird spp.	543289	6791461
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bird spp.	539811	6788620

Road	Date	Start:End	Observers	Carriageway	Species/group	Easting	Northing
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bird spp.	538572	6787177
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bird spp.	537395	6784540
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bird spp.	533588	6782457
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bird spp.	524173	6752441
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bird spp.	523541	6746807
PHW	10/11/21	10:00-15:00	AE/LA	SB	Bird spp.	521243	6741709
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bird spp.	512040	6715030
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bird spp.	511938	6714406
PHW	10/11/21	10:00-15:00	AE/LA	NB	Bird spp.	506899	6708436
PHW	10/11/21	10:00-15:00	AE/LA	SB	Cane toad	547923	6806253
Wardell Road	10/11/21	10:00-15:00	AE/LA		Fox	543567	6798120
PHW	10/11/21	10:00-15:00	AE/LA	SB	Freshwater turtle spp.	532473	6778918
Wardell Road	10/11/21	10:00-15:00	AE/LA		Frog spp	542821	6798368
PHW	10/11/21	10:00-15:00	AE/LA	SB	Lace monitor	519394	6735121
PHW	10/11/21	10:00-15:00	AE/LA	SB	Macropod spp.	524472	6750903
PHW	10/11/21	10:00-15:00	AE/LA	SB	Macropod spp.	520081	6739828
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	547127	6804605
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	541960	6789252
PHW	10/11/21	10:00-15:00	AE/LA	SB	Medium mammal spp.	534221	6782719
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	528537	6774464
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	520933	6760716
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	523437	6745165
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	521132	6741347
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	519916	6738448
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	514932	6730577
PHW	10/11/21	10:00-15:00	AE/LA	SB	Medium mammal spp.	512024	6711658
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	502947	6703016
PHW	10/11/21	10:00-15:00	AE/LA	NB	Medium mammal spp.	511569	6686571
PHW	10/11/21	10:00-15:00	AE/LA	SB	Medium mammal spp.	516776	6679844
PHW	10/11/21	10:00-15:00	AE/LA	NB	Red-necked wallaby	520355	6740637
PHW	10/11/21	10:00-15:00	AE/LA	SB	Reptile spp.	538123	6786046
PHW	10/11/21	10:00-15:00	AE/LA	NB	Reptile spp.	520290	6740409
PHW	10/11/21	10:00-15:00	AE/LA	NB	Rodent spp.	523450	6745536
PHW	10/11/21	10:00-15:00	AE/LA	SB	Short-beaked echidna	542497	6793839
PHW	10/11/21	10:00-15:00	AE/LA	NB	Small bird spp.	530357	6775855
PHW	10/11/21	10:00-15:00	AE/LA	NB	Small bird spp.	512918	6718384
PHW	10/11/21	10:00-15:00	AE/LA	NB	Small mammal spp.	546709	6802511
Wardell Road	10/11/21	10:00-15:00	AE/LA		Small mammal spp.	542990	6798226
PHW	10/11/21	10:00-15:00	AE/LA	SB	Small mammal spp.	542425	6798158
PHW	10/11/21	10:00-15:00	AE/LA	SB	Small mammal spp.	530379	6775859
PHW	10/11/21	10:00-15:00	AE/LA	NB	Small mammal spp.	523408	6754164

Road	Date	Start:End	Observers	Carriageway	Species/group	Easting	Northing
PHW	10/11/21	10:00-15:00	AE/LA	NB	Small mammal spp.	520311	6740380
PHW	10/11/21	10:00-15:00	AE/LA	NB	Small mammal spp.	512849	6717997
PHW	10/11/21	10:00-15:00	AE/LA	SB	Snake spp.	546198	6800507
PHW	10/11/21	10:00-15:00	AE/LA	NB	Snake spp.	511771	6686437
PHW	10/11/21	10:00-15:00	AE/LA	NB	Swamp wallaby spp.	514375	6682261
PHW	10/11/21	10:00-15:00	AE/LA	SB	Toressian crow	510973	6687004
PHW	10/11/21	10:00-15:00	AE/LA	NB	Wallaby spp.	513318	6726785