Transport
for New South Wales

Woolgoolga to Ballina Pacific Highway Upgrade

Koala Population Monitoring Program Annual Report:

Year 7 spring 2023 – autumn 2024

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Woolgoolga to Ballina Pacific Highway Upgrade

Koala population monitoring program

Annual report year 7 (2023-24)

May 2025

Sandpiper
Ecological
Surveys

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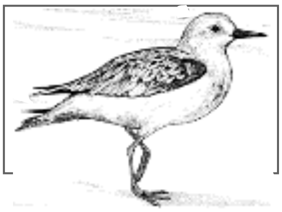

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Cover Photo: Koala recorded on transect in Broadwater National Park during spring 2023 monitoring

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 the approved Koala Management Plan. The Year 7 report (2023/24) presents results from biannual surveys, Bayesian modelling, and genetic monitoring to assess population trends and evaluate the effectiveness of mitigation measures.

Surveys were conducted in the Broadwater (Sections 8–9) and Bagotville (Section 10) focal areas during spring 2023 and autumn 2024. Density estimates indicate continued low koala density in the Broadwater study area, with 0.052 individuals/ha in Year 7, approximately 7% lower than in Year 6, and well below the baseline Bayesian estimate of 0.132 individuals/ha. Detections in Broadwater remained sparse and clustered within a few transects, increasing variability in density estimates and reducing statistical confidence. Whilst koala density remains below baseline levels, the steep declines recorded in earlier years appear to have stabilised. The rate of annual decline has decreased, shifting to a negligible decline at 1.2%. Bayesian analysis provides only limited evidence in favor of no trend over a continued decline.

The koala population in the Bagotville focal area continues to exhibit a stable population trend, with Year 7 Bayesian model averaged estimates of 0.083 individuals/ha. Extrapolated across approximately 2,135 hectares of preferred habitat, this equates to an estimated population of 177 individuals.

Genetic monitoring at Bagotville showed a positive trend in genetic diversity, with expected heterozygosity increasing from 0.672 in 2018 to 0.788 in 2024 and observed heterozygosity rising from 0.539 to 0.654. Allelic richness also improved, indicating a broadening gene pool. However, fluctuations in the inbreeding coefficient ($F_{(is)}$), which peaked at 0.205 in 2022 before declining to 0.165 in 2024, suggest possible localised inbreeding likely due to spatial clustering of koala records.

Power analysis confirmed that the monitoring program at Bagotville meets the target confidence level (>0.7), while Broadwater remains below this threshold due to the uneven distribution of detections. The concentration of records within a small number of transects reduces statistical power, limiting the ability to detect long-term trends with certainty. To improve statistical power and confidence in the population estimates it is recommended that survey coverage at Broadwater be expanded by adding 15–20 additional transects using a randomised grid approach

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 and 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 assistance with surveys within the Ngunya Jargoon IPA. The NSW National Parks and Wildlife Service provide access to Broadwater National Park and Ballina Shire Council provides permission to access Council land. 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.

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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 (*Phascolarctos cinereus)* population monitoring program in accordance with section 8.3 of the approved Koala Management Plan (KMP) (RMS version 4.4, July 2016). The population monitoring program was designed to guide and inform management of koalas along the highway upgrade. The koala is listed as Endangered by the NSW *Biodiversity Conservation Act 2016* (BC Act) and Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). The KMP identified critical focal populations of koala around Broadwater and Bagotville due to the high density of koala records during environmental impact assessments (RMS, 2016). Baseline data for the focal populations were derived from a combination of historical studies and targeted field surveys, including detailed investigations of the Bagotville population (Phillips and Chang, 2013; Phillips et al., 2015; Ecosure, 2014, 2015).

A Population Viability Analysis (PVA) was conducted for the Bagotville population to meet Commonwealth Conditions of Approval (CoA 5 and CoA 7). Under scenario 6 the PVA projected a 41% decline in the Bagotville koala population over 15 years, with significant declines expected over 50 years unless key threatening processes are managed (Kavanagh, 2016). In contrast, the Broadwater population, which was not included in the PVA, is being assessed for statistically measurable declines over 15 years compared to baseline survey values (RMS, 2016). Monitoring these two focal populations is essential to determine the success of mitigation measures implemented along the highway upgrade such as the installation of exclusion fencing, habitat restoration through koala tree plantations, and the construction of underpass crossing structures.

The primary aims of the monitoring program are to:

- Monitor trends in the size of koala populations surrounding the alignment at Broadwater (Sections 8-9) and Coolgardie-Bagotville (Section 10-11; hereafter referred to as Bagotville).
- Evaluate the effectiveness of mitigation measures implemented in Sections 1-11 of the upgrade for koalas.

The following report focuses on population monitoring and road mortality surveys only. Monitoring of connectivity structures is addressed by Sandpiper Ecological (2025b). That report includes linkages between data presented here and use of underpasses by koalas.

1.2 Scope of work, program objectives and performance indicators

Monitoring requires using direct counts of koalas across various transect sites, using methods such as diurnal canopy searches and nocturnal spotlighting, consistent with those used in the baseline surveys. An initial power analysis was conducted to determine the sample size and survey effort required to detect changes in koala populations with confidence (Rhodes and Preece 2016). The analysis determined that to achieve 97% power to detect a 30% population change in 15 years, monthly surveys would be required within each focal population (Bagotville and Broadwater) over 15 years. This high sampling effort was driven by the patchy distribution of koalas and the typically sparse counts recorded in the focal populations (Rhodes and Preece 2016). However, this level of effort was deemed impractical due to logistical constraints. As a result, a more practical approach was implemented, achieving 70% power through biannual surveys conducted at 50 sites per population (RMS 2016).

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 2016) include:

1. Determine whether there is a statistically significant decline at year 15 compared with no decline in sections 8-9 (Broadwater)
2. Determine whether the corrective actions of the KMP have been triggered by estimated population trends in section 10 (Bagotville) in accordance with predictions of the PVA.
3. Provide information that supports a program review by TfNSW at years 5, 10 and 15 in accordance with the KMP.
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 thresholds (Table 1). In addition, scat sampling will be conducted every second year (i.e. years 1, 3, 5 etc.) in section 10 for the purposes of genetic analysis, to provide information on distribution, gene flow and relatedness of individuals across the study area.

Table 1: KMP performance thresholds and corrective actions relevant to this report.

Monitoring component	Performance threshold	Corrective actions
Koala population trends in Sections 10 and 8/9.	• 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.	• 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 roadkill 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.
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), to reduce koala mortality across the region.
Fauna exclusion fence	• No breaches in fauna exclusion fence.	• Check fauna exclusion fencing, and fauna crossing structures for damage/blockage and rectify.

1.3 Monitoring

Koala population monitoring for the Woolgoolga to Ballina Pacific Highway Upgrade has been conducted annually since 2017. Each monitoring year runs from the 1 September to 31 August during the following year, with population surveys undertaken biannually in spring/summer (September–December) and autumn/winter (April–August). The spring/summer period coincides with the peak breeding season, while the autumn/winter period allows for assessment of population movements and survival outside the reproductive season (RMS, 2016).

Baseline surveys were conducted prior to the commencement of the monitoring program to establish population densities and habitat use (Phillips and Chang, 2013; Phillips et al., 2015; Ecosure, 2014, 2015). The entire monitoring timeline to date is as follows:

- Broadwater baseline survey (sections 8/9) – spring 2014
- Bagotville baseline survey (section 10) – autumn 2015
- Year 1: 2017/18 (spring/summer 2017 – autumn/winter 2018)
- Year 2: 2018/19 (spring/summer 2018 – autumn/winter 2019)
- Year 3: 2019/20 (spring/summer 2019 – autumn/winter 2020)
- Year 4: 2020/21 (spring/summer 2020 – autumn/winter 2021)
- Year 5: 2021/22 (spring/summer 2021 – autumn/winter 2022)
- Year 6: 2022/23 (spring/summer 2022 – autumn/winter 2023)
- Year 7: 2023/24 (spring/summer 2023 – autumn/winter 2024)

This report presents the methods and findings of Year 7 (2023/24) population monitoring in Sections 8–10. It also includes findings from Year 3 of road mortality and exclusion fence monitoring in Sections 3–11. The report evaluates the monitoring objectives, compares the outcomes to established performance indicators, and assesses whether any thresholds have been exceeded, requiring 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 7 population monitoring report, monitoring activities were conducted in sections 3-11 (road mortality monitoring) and sections 8-11 (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 2 and 3. The Richmond River forms a major movement barrier to the west and north of sections 8-9 and between the Broadwater and Bagotville focal areas. In sections 10-11, the Bagotville focal population area extends 13.5 km north of the Richmond River and includes the localities of Bagotville and Coolgardie (Figure 1).

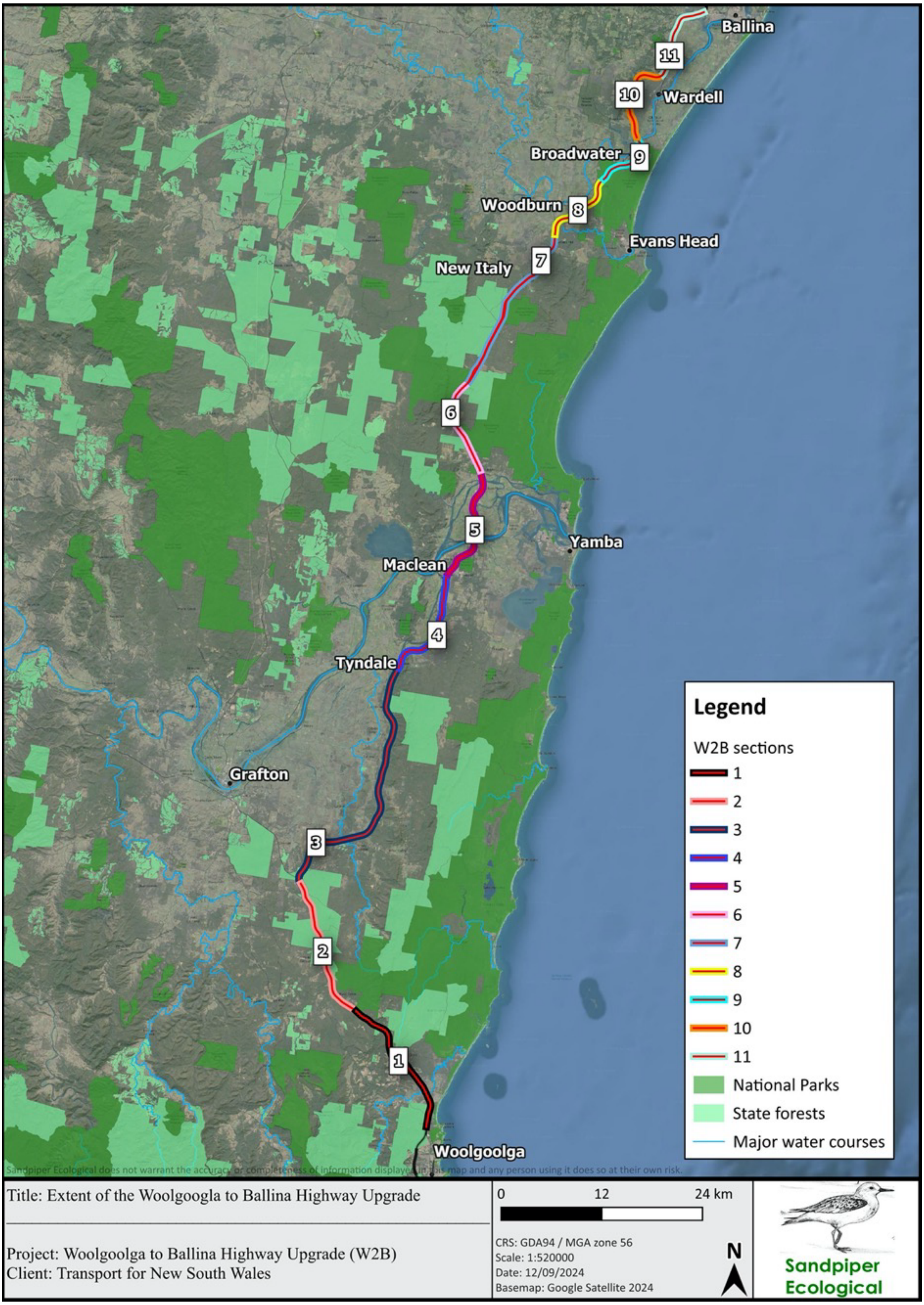

Figure 1: Sections 1-11 of the W2B Pacific Highway Upgrade.

3 Methods

3.1 Population surveys

Diurnal and nocturnal population surveys were conducted in sections 8–11 of the W2B upgrade during spring/summer 2023 and winter 2024. The spring/summer surveys took place between 27 November and 14 December 2023, while the winter surveys occurred between 11 June and 24 July 2024. Diurnal surveys were generally conducted between 0800 and 1700 hours, and nocturnal surveys between 1730 and 0100 hours. Surveys were undertaken during favourable weather conditions, avoiding rainfall or strong winds that could impair visibility.

In spring/summer 2023, 97 out of 100 sites were surveyed, 50 in Broadwater and 47 in Bagotville (Figures 3 and 4). During winter 2024, 96 transects were surveyed, with 50 in Broadwater and 46 in Bagotville. The unsurveyed transects in Bagotville were due to either landowner refusal (N44), unable to contact landowner, or site inaccessible.

Each site/transect was surveyed twice, once diurnally and once nocturnally during each sampling season (spring/summer 2023 and winter 2024) using the following methods:

1. Transect searches: Direct counts along a 250 m x 40 m transect (approximately 1 ha) were conducted by three observers walking 15 m apart—one on the centerline and one on each side (Figure 2). Observers used binoculars to search trees for koalas. Handheld spotlights and/or headtorches (700-1000 lumens) and binoculars were used during nocturnal surveys.

2. Radial searches: Direct counts within a 25 m radius of the midpoint of the belt transect (approximately 0.196 ha) were conducted by three observers, who slowly searched all trees. Radial searches were conducted concurrently with the transect searches (Figure 2).

In years 1 and 2, the same team completed diurnal surveys followed by nocturnal surveys on the same day. However, starting from year 3, diurnal and nocturnal surveys of the same transect were conducted on non-consecutive days (>24hrs apart) to ensure survey independence.

Koala locations were recorded using a hand-held GPS (Global Positioning System). Additional data collected included sex, age class, health status, behaviour, and other identifying features of individuals if present. For each tree where a koala was observed, the species and diameter at breast height were documented.

Figure 2: Example of transect survey design including centerline and radial search area.

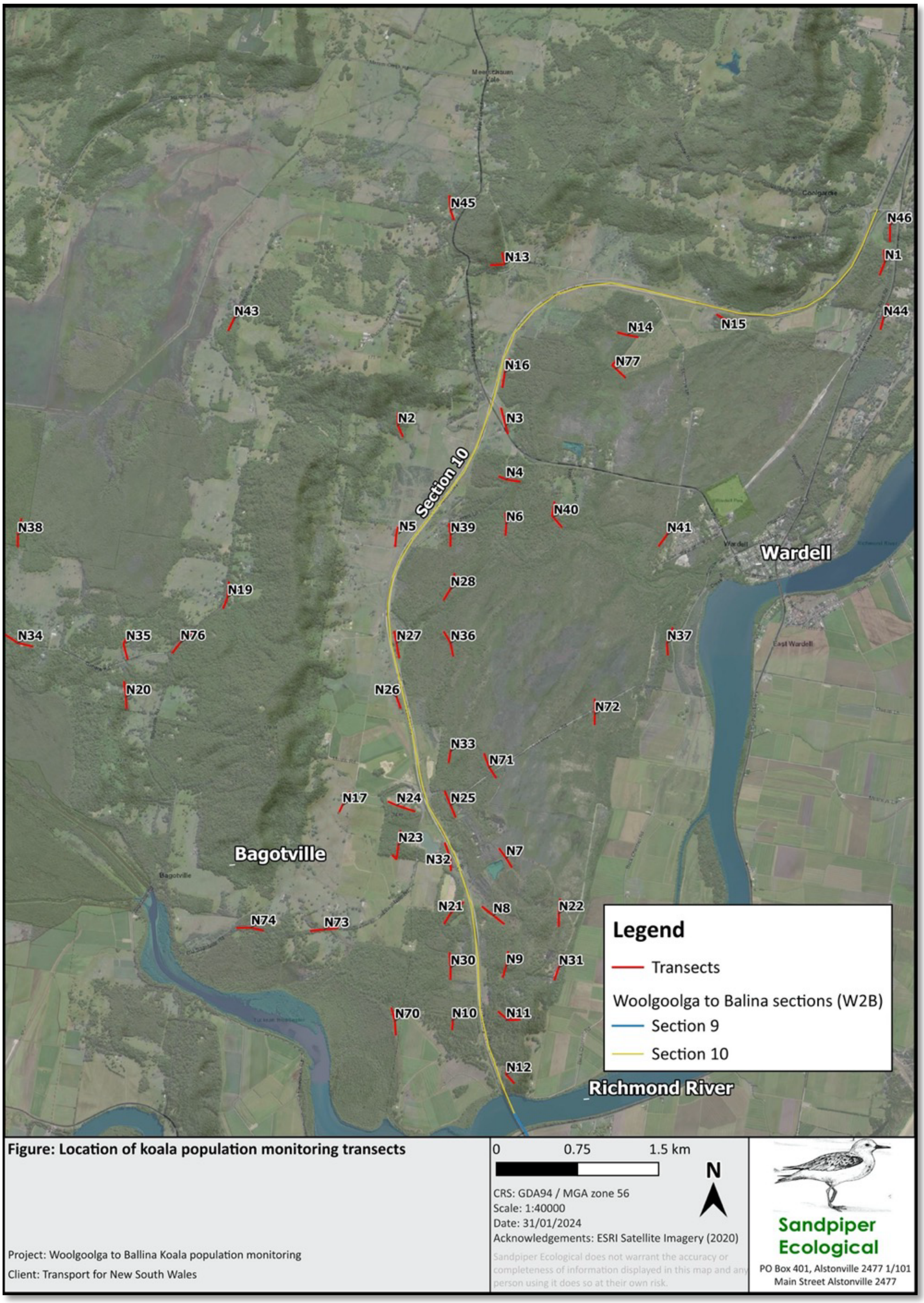

Figure 3: Location of sample sites within the Bagotville (Section 10) survey area.

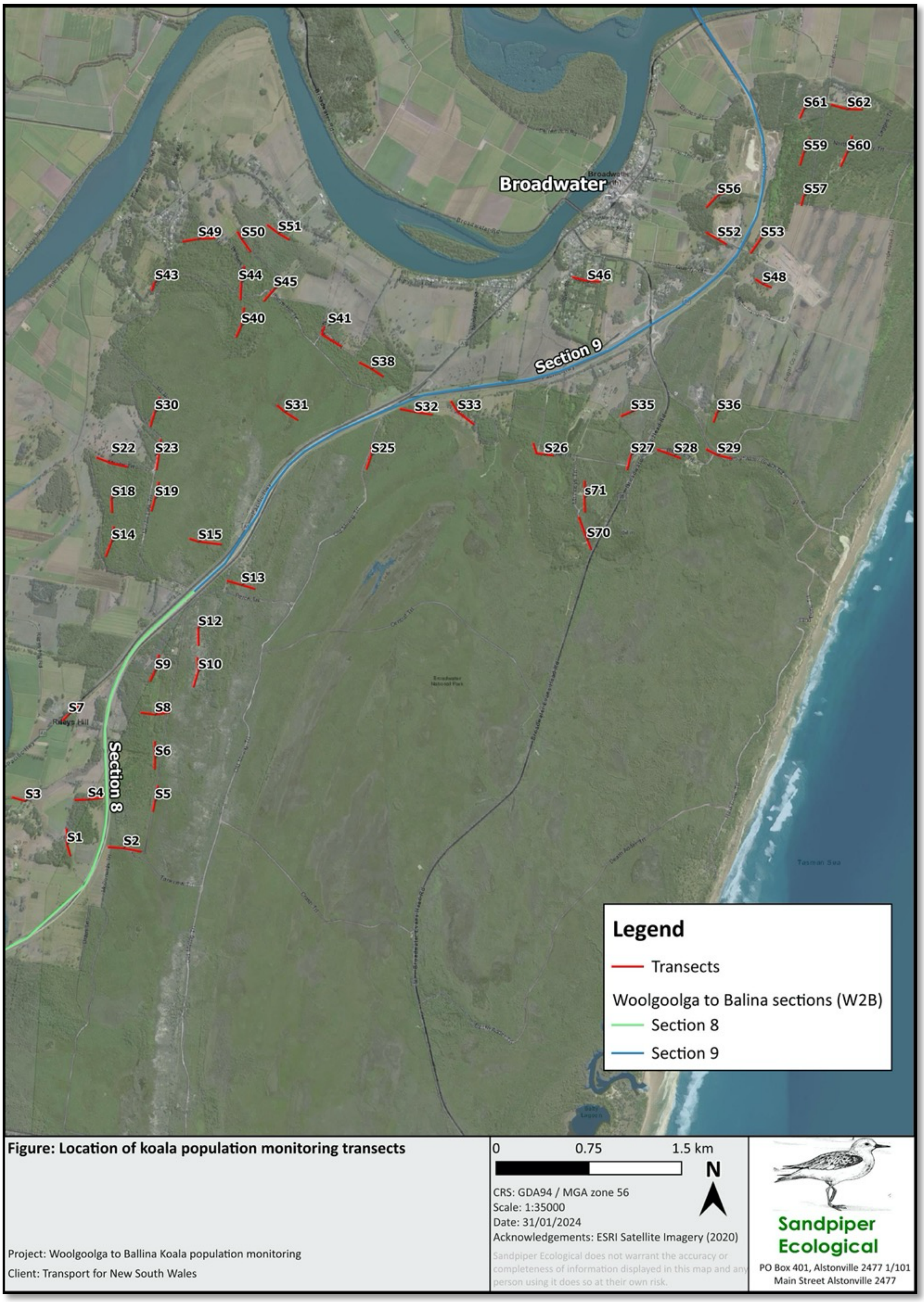

Figure 4: Location of sample sites within the Broadwater (Section 8/9) survey area.

3.2 Koala density and population size estimates

A report on statistical analysis of koala population data is provided by Rankin (2024) and included in Appendix A. Following is a summary of analysis methods.

3.2.1 Bayesian modelling

A Bayesian estimation approach was applied to estimate koala densities at Broadwater and Bagotville for Year 7, incorporating model-based uncertainty to refine population trend assessments. Unlike previous years, seasonal differences were not explicitly modelled, and data from spring and autumn surveys were pooled to provide a single density estimate for each site. The modelling process considered three core trend structures: a log-linear trend, no trend, and a model where each year had a unique density estimate. To account for potential overdispersion in count data, five different Negative Binomial priors were applied, ranging from high to low dispersion, with the latter approximating a Poisson distribution. These specifications resulted in a total of 280 models, which were weighted and averaged using posterior probabilities derived from the Watanabe-Akaike Information Criterion (WAIC) (Akaike 1998).

Bayesian regression models were used to estimate population trends over time, smoothing natural variation in yearly counts and incorporating multiple sources of uncertainty. This approach avoids the limitations of a fixed-baseline method, which can be overly sensitive to random fluctuations in annual counts, particularly given the low density of koalas in the study areas. By recalibrating density estimates over time, Bayesian modelling provides a more robust representation of long-term trends.

3.2.2 Density and population estimates

At Bagotville, baseline density values were extrapolated across 2,152 hectares of preferred koala habitat before clearing and 2,135 hectares post-clearing (i.e. year 2 onwards). To align with the methodology used in the Population Viability Analysis (PVA) conducted by Kavanagh (2016), a correction factor of 0.204 was applied to population estimates to account for the unsampled 0–1-year-old age cohort. These adjusted estimates are referred to as revised population estimates. In contrast, the Broadwater population, which was not assessed under a PVA framework, was evaluated based on density estimates alone, with trends to be formally assessed at Year 15. The density estimates for Broadwater do not include the 0-1 year age cohort.

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 this report. Whereas population estimates are presented, determining population trends focused on comparison of density estimates rather than population estimates. Focusing on trends in density estimates 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, population trends are assessed according to density estimates and will be evaluated based on a statistically significant decline at year 15.

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 for DNA analysis from koalas observed in the Bagotville area during year 7 surveys. 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. Year 7 samples were compared to samples collected in years 1, 3 and 5. DNA analysis was undertaken by Dr L. Hulse, and the complete genetics report is presented in Appendix C.

3.4.2 DNA extraction and analysis

Genomic DNA was isolated using the QIAamp Fast DNA Stool Mini kit (QIAGEN) according to 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). All 22 faecal scat samples collected in 2024 were verified to contain koala genomic DNA.

3.4.3 Genotypes and samples

Genotypes across 32 microsatellite loci for 22 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 dataset to identify duplicate samples was performed using the software GENALEX version 6.5 (Peakall & Smouse, 2006). Duplicate genotypes from individuals have been removed from the dataset to prevent bias and ensure accurate genetic analysis.

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 Koala occupancy and distribution analysis

To analyse the distribution of koala records over time, QGIS software (version 3.30) was used to generate a heatmap. Koala locality data from Years 2–7 of monitoring was imported into QGIS from a CSV file. Year 1 data was excluded as clearing activities were ongoing throughout year 1, and the analysis aimed to assess changes in koala distribution in response to the upgrade and over time.

The heatmap was generated using the “Heatmap” tool under Vector Processing in QGIS. A dark rendering theme with a red-to-brown colour ramp was applied to highlight areas of higher koala record density. A radius of 700m was used to define the spatial extent of influence for each koala locality and only transect records were used. To examine temporal changes, records were grouped into three intervals: Years 2–3, 4–5, and 6–7.

To assess koala records in relation to plant community classes, NSW Plant Community Type (PCT) data was obtained from the SEED portal (DECCW 2020). This dataset was imported into QGIS and overlaid with koala records. Koala locations were then extracted based on vegetation classes to identify whether specific plant community classes were associated with koala occupancy.

3.6 Road mortality surveys and fauna fence inspections

Road-mortality surveys for the Woolgoolga to Ballina (W2B) project are required twice per year over a five-year period during the operational stage, specifically between July–August and October–November, as outlined in the KMP. Due to the staged opening of the W2B project, koala road-mortality monitoring has been completed for Wardell Road (Gubay Lane to Thurgates Lane – 1.54 km), the old Pacific Highway (Carlyle Street to Coolgardie interchange – 3.3 km), and Sections 1–2 (Arrawarra interchange to Glenugie) (Sandpiper Ecological 2021, 2024). During Year 7 (2023–2024), surveys occurred only in Sections 3–11, representing the third year of the five-year monitoring requirement for these sections. The two surveys as required by the KMP took place on 6 September 2023 and 17 November 2023.

During initial koala road-mortality monitoring for the W2B project, sections 1-2 were surveyed using pedestrian-based surveys. However, to mitigate safety risks, the approach was revised in year 3 of operation (2021), adopting vehicle-based surveys for monitoring in sections 1-11. 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. If any fauna was detected, the species or fauna group was recorded using the internal GPS of a smart device, and the waypoint was recorded in Australia Topo maps android application. Unidentified mammal carcasses were scored as either small (e.g., rodent, bat, small glider, brush-tailed phascogale), medium (i.e., echidna, long-nosed potoroo, rufous bettong, koala, bandicoot, cat, spotted-tail quoll, possum, large glider), 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).

The condition of the exclusion fence was assessed periodically during underpass monitoring and road-mortality surveys. Incidental observations were also recorded during population monitoring surveys. Any breaches or defects in the fauna fence were documented using GPS, and photographs were taken to report to TFNSW.

4 Results

4.1 Counts

4.1.1 Broadwater

During the year 7 monitoring period (2023 spring/summer and 2024 autumn/winter surveys), a total of 23 koalas were recorded within the Broadwater focal area (Table 2, Figure 5). Of these, 12 (52% of all koala records) were recorded within the transect or radial areas, and 11 (48%) were incidental records observed either just outside the transect or whilst moving between transects. Six koalas were detected within the belt transect and six were detected within the radial plots, representing the highest number of records within radial areas to date (Table 2). All the belt transect records occurred at night with three detections in both autumn and spring (Table 2). All radial detections occurred during the autumn surveys, with three recorded during diurnal surveys and three during nocturnal surveys. A total of seven males (30% of all records) and five females (22%) were recorded, while the sex of 11 individuals (48%) remained unconfirmed due to foliage obstruction or the koala being in an inaccessible location. The observed koalas were generally in good health, except for one individual displaying a stained and wet rump, likely indicative of a chlamydia infection (*Chlamydia pecorum*). Full details of Broadwater koala observations are provided in Table A1, Appendix A.

Table 2: Broadwater focal area koala observations – baseline to year 7 (2023/24). 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		Year 6		Year 7	
		Sp (52)	A (50)	Sp (50)	A (50)	Sp (50)	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	3	3	0	0
Nocturnal transect	NA	2	4	1	2	3	5	2	1	2	1	4	1	3	3
Diurnal radial	1	0	1	0	0	0	1	0	0	2	0	0	1	0	3
Nocturnal radial	NA	0	1	0	0	0	2	0	0	0	1	0	1	0	3
Incidental	1	2	8	11	3	6	4	2	2	6	3	2	4	5	6

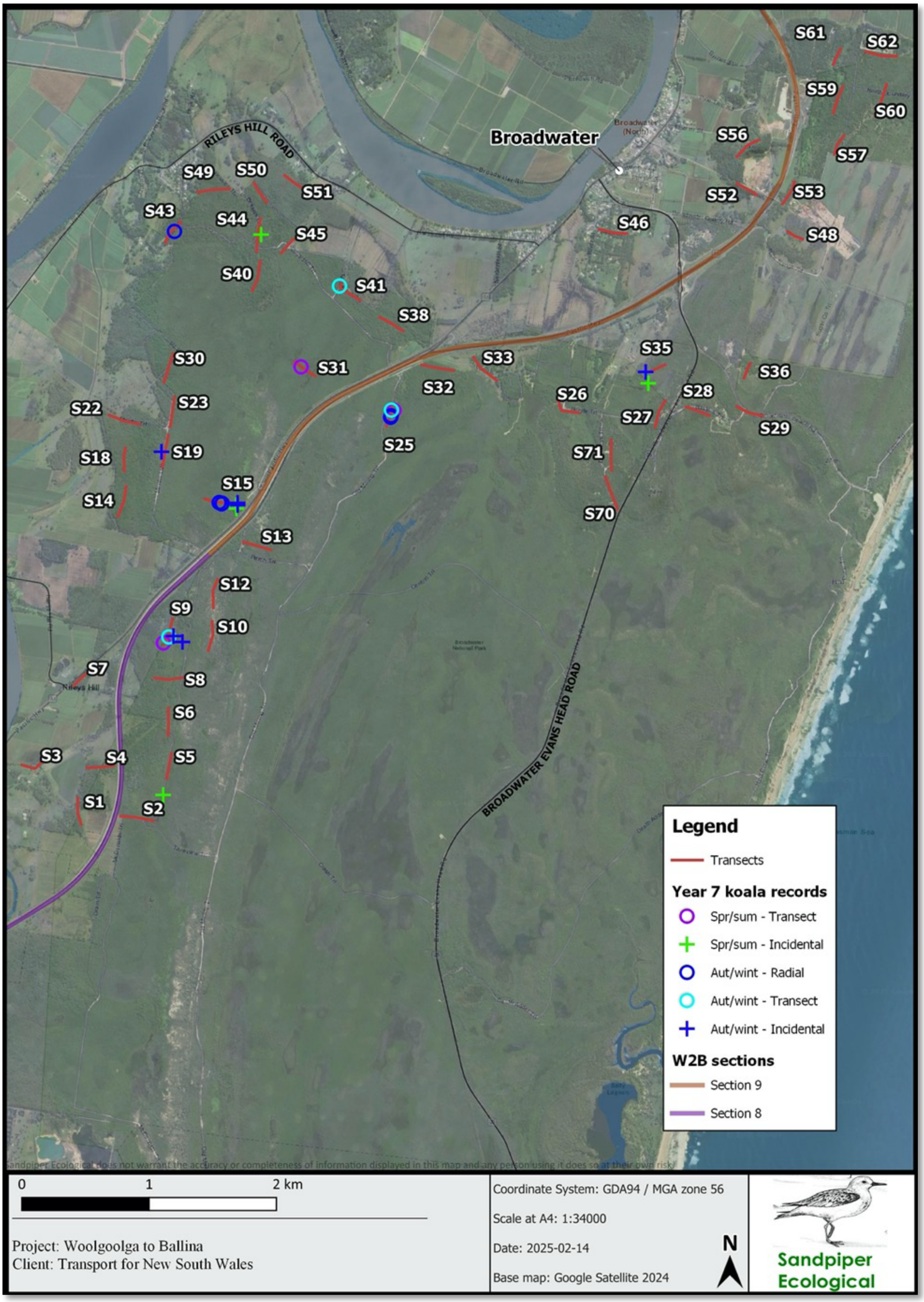

Figure 5: Broadwater survey sites and location of all koalas recorded during spring/summer 2023 and autumn/winter 2024 surveys.

4.1.2 Bagotville

During year 7, a total of 31 koalas were recorded within the Bagotville focal area (Table 3, Figure 6). Of these, 20 (65%) were recorded within the transect or radial areas, and 11 (35%) were incidental records observed either just outside the transects or whilst moving between transects. Fourteen koalas (45%) were detected on transects. Of these, 10 were observed during nocturnal surveys (five in spring and five in autumn), and four during diurnal surveys (one in spring and three in autumn) (Table 3). An additional six koalas (19%) were detected within radial plots, representing the highest number of records within radial areas to date (Table 3). Of these, three were observed during nocturnal surveys (two in spring and one in autumn), and three during diurnal surveys (two in spring and one in autumn). A total of 15 females (48%) and five males (16%) were recorded, while the sex of 11 individuals (35%) remained unconfirmed due to foliage obstruction or the koala being in an inaccessible location. The observed koalas were generally in good health, with four individuals displaying a slightly stained rump. Full details of Bagotville koala observations are provided in Table A1, appendix A.

Table 3: Bagotville focal area koala observations – baseline to year 7 (2023/24). Sp = spring; A = autumn. Number of sample sites shown in parentheses.

Time & type	Baseline (42)	Year 1		Year 2		Year 3		Year 4		Year 5		Year 6		Year 7	
		Sp (43)	A (50)	Sp (50)	A (50)	Sp (50)	A (50)	Sp (50)	A (49)	Sp (48)	A (46)	Sp (49)	A (47)	Sp (47)	A (46)
Diurnal transect	3	2	5	3	3	6	5	3	3	3	6	6	2	1	3
Nocturnal transect	NA	3	5	5	3	5	4	5	4	3	4	6	4	5	5
Diurnal radial	1	0	1	1	0	1	0	1	0	0	0	0	0	2	1
Nocturnal radial	NA	0	1	2	0	0	0	0	0	0	0	1	1	2	1
Incidental	5	5	8	4	3	6	3	4	4	4	6	4	5	3	8

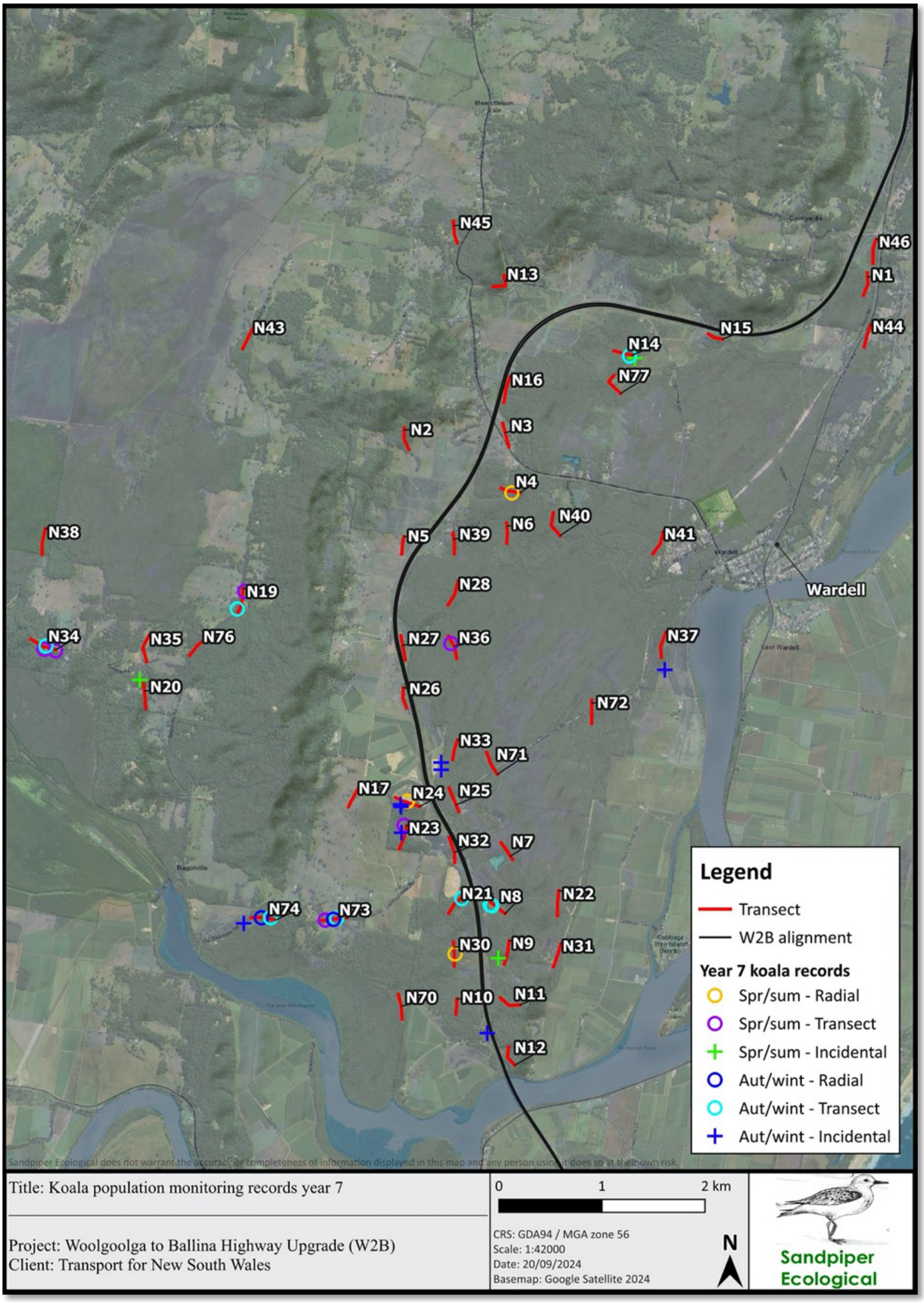

Figure 6: Bagotville survey sites and location of koalas recorded during spring/summer 2023 and autumn/winter 2024 surveys.

4.2 Distribution of records

4.2.1 Broadwater transects

Within the Broadwater study area, the distribution of koala records varied across transects and years, although detections were concentrated within a small number of sites (Figure 7, Table 4). During year 7 surveys, 12 koalas were recorded at six of the 50 transects surveyed (12% of all transects surveyed) (Figure 7). Most records occurred on transect S25 (4 records, 33% of all koala records on transect), followed by S15 (3 records, 25%) and S9 (2 records, 17%), which together accounted for 75% of all koala records during year 7 (Table 4).

Over the seven years of monitoring in Broadwater 78 koalas have been recorded on 20 of the 50 transects (40% of all transects) surveyed (Table 4). Koala detections were concentrated on a subset of transects, with S15 (17 records, 22% of all koala records), S38 (9 records, 12%), S32 (9 records, 12%), and S25 (8 records, 10%) accounting for 56% of all records. Several transects, including S8 and S44, recorded koalas intermittently, while others, such as S43 and S49, had only one or two records over the survey period. Some transects including S9, S19, and S41, recorded their first detections in years 5, 6, and 7 (Table 4). Transect S14 was not surveyed after the baseline period due to access constraints.

The number of transects that recorded koalas ranged from three (Year 2) to 8 per year (Year 3), with an approximate mean of 6 transects (12% of all transects) annually. These findings highlight the low frequency and patchy distribution of koala records, with detections concentrated at a small proportion of transects in any given year and high consistency in records at some sites between years.

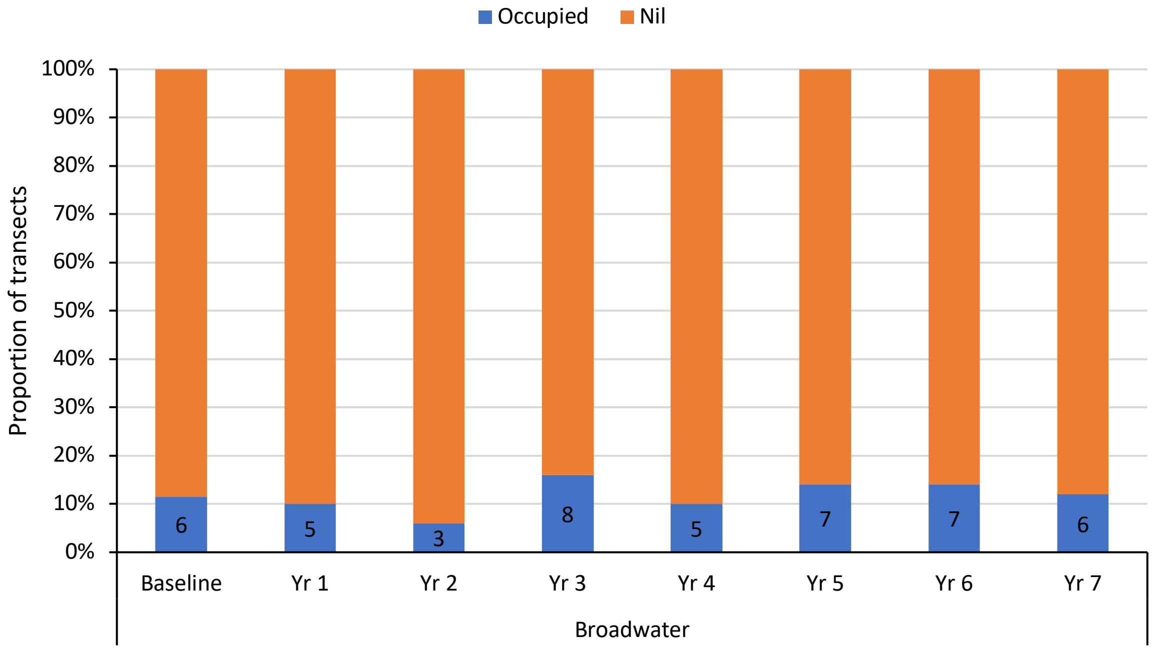

Figure 7: The number and proportion of transects occupied by koalas during population monitoring at Broadwater (Year 1-7). Occupied = recorded one or more koala records. Nil = no koala records.

Table 4: A comparison in the number of transects with koala records and the total koalas recorded on transects (including radial records) within each of the monitoring years at Broadwater.

Transect	Baseline	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Total
S15	1	2	-	4	1	2	4	3	17
S38	1	2	2	1	1	1	1	-	9
S32	3	2	-	1	3	-	-	-	9
S25	-	-	-	2	1	1	-	4	8
S08	-	2	1	-	-	-	3	-	6
S44	-	-	-	1	-	4	-	-	5
S09	-	-	-	-	-	-	2	2	4
S31	-	-	2	-	-	-	-	1	3
S26	-	1	-	-	-	-	1	-	2
S49	1	-	-	-	-	-	1	-	2
S10	-	-	-	1	-	1	-	-	2
S43	-	-	-	1	-	-	-	1	2
S50	-	-	-	-	-	2	-	-	2
S13	1	-	-	-	-	-	-	-	1
S14	1	-	-	-	-	-	-	-	1
S19	-	-	-	-	-	-	1	-	1
S30	-	-	-	-	1	-	-	-	1
S41	-	-	-	-	-	-	-	1	1
S46	-	-	-	-	-	1	-	-	1
S53	-	-	-	1	-	-	-	-	1

4.2.2 Bagotville transects

Koala records at Bagotville followed a similar pattern to those at Broadwater, with detections concentrated at certain transects (Figure 8, Table 5). However, Bagotville had a higher total number of records, with koalas detected on a greater proportion of transects (Table 5). In year 7, 20 koalas were recorded across 12 of 47 transects (26% of all transects survey), the highest proportion of transects with koala records to date (Figure 8, Table 5). Most detections were on N73 and N34 (three each), followed by N24, N08, N74, and N19 (two each). These six transects accounted for 70% of all koala records on transect in year 7 (Table 5, Figure 6).

During monitoring, 121 koalas were recorded on 29 of 50 transects (58% of transects), with the highest numbers from N73 (17 records, 14% of all records), N74 (14, 12%), and N34 (14, 12%) (Table 5). Some transects, such as N36 and N33 recorded koalas intermittently, while others, including N28 and N32, had only a few detections in isolated years. Several, such as N21 and N23, recorded their first detections between years 5 and 7.

Of the 29 transects with koala records, 17 (59%) recorded only one or two detections, while eight (28%) recorded between five and ten. Only four transects (14%) recorded more than ten, indicating that most detections were concentrated in a few locations (Table 5). The number of transects recording koalas annually ranged from four (Baseline) to 12 (year 7) with a mean of eight (16% of the 50 transects).

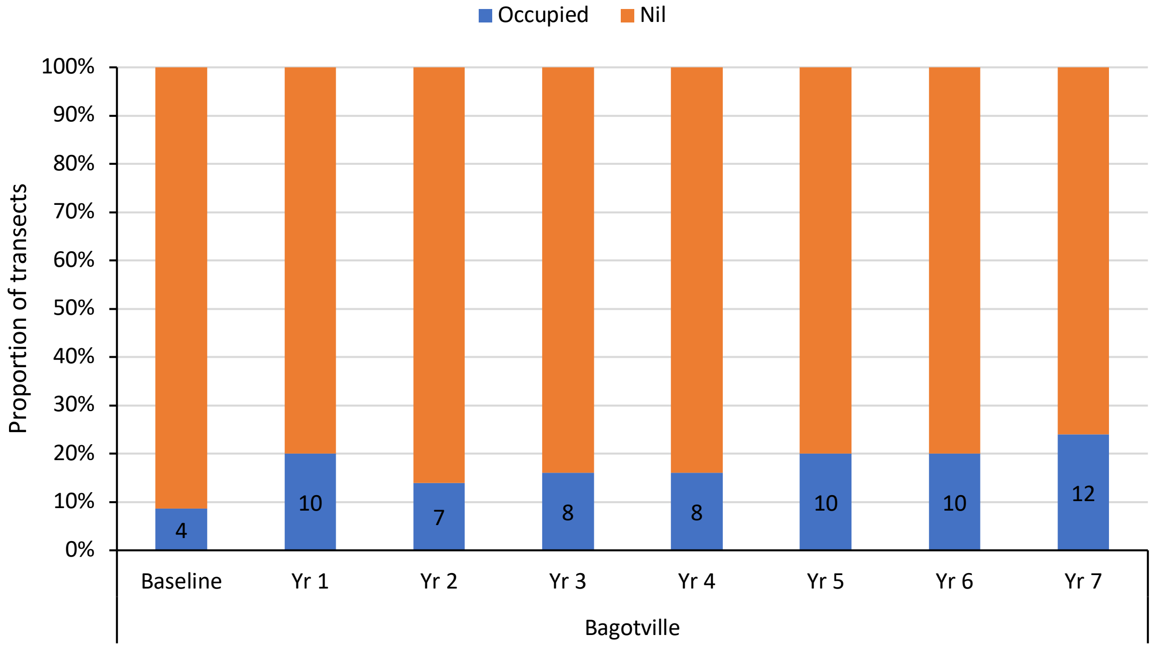

Figure 8: The number and proportion of transects occupied by koalas during population monitoring at Bagotville (Year 1-7).

Table 5: A comparison in the number of transects with koala records and the total koalas recorded on transects (including radial records) within each of the monitoring years at Bagotville.

Transect	Baseline	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Total
N73	-	1	1	4	1	2	5	3	17
N74	-	2	2	2	3	1	2	2	14
N34	-	1	1	4	3	1	1	3	14
N19	-	1	3	-	2	-	3	2	11
N20	-	-	-	3	2	1	3	-	9
N36	-	3	1	-	2	-	-	1	7
N33	-	2	1	1	-	2	-	-	6
N21	-	-	-	-	-	2	2	1	5
N23	-	-	-	-	-	2	1	1	4
N24	-	-	-	-	1	1	-	2	4
N28	-	2	-	-	-	2	-	-	4
N71	-	-	-	4	-	-	-	-	4
N04	-	-	-	-	-	-	1	1	2
N08	-	-	-	-	-	-	-	2	2
N09	-	1	-	-	1	-	-	-	2
N45	-	2	-	-	-	-	-	-	2
N32	-	-	-	-	-	2	-	-	2
N14	-	-	-	-	-	-	-	1	1
N30	-	-	-	-	-	-	-	1	1
N75	-	1	-	-	-	-	-	-	1
N40	-	-	1	-	-	-	-	-	1
N11	-	-	-	1	-	-	-	-	1
N77	-	-	-	1	-	-	-	-	1
N10	-	-	-	-	-	-	1	-	1
N72	-	-	-	-	-	-	1	-	1
N1	1	-	-	-	-	-	-	-	1
N18	1	-	-	-	-	-	-	-	1
N35	1	-	-	-	-	-	-	-	1
N3	1	-	-	-	-	-	-	-	1

4.2.3 Spatial distribution over time

During operational monitoring, koala records tended to be clustered in certain areas. A comparison of data from years 2-3, 4-5 and 6-7 identified several hotspots in Bagotville and Broadwater, although some variation was observed between the two periods (Figures 9 and 10).

In Broadwater, S15 (west of the highway) remained the most prominent hotspot during operational monitoring (Figure 9). During years 2-5, additional hotspots were evident at S32 (east of the highway) and S38 (west of the highway). Moving into years 6-7 records had disappeared from S32 and declined at S38. Consistent scattered detections were observed between S41 and S49, south of Rileys Hill Road, across both monitoring periods. Similarly, S8, S9, and S25 (east of the highway) showed relatively consistent records. In contrast, only a few or nil koalas were recorded between S1 and S4 and between S52 and S56 in Broadwater.

In Bagotville, detections remained consistently high at N74 and N73, west of the highway along Old Bagotville Road, forming a key hotspot area throughout operational monitoring. Koalas were also regularly recorded at N33, N34, N19, N20, and N36. During years 4–5 of monitoring, detections increased around N21 to N24, west of the highway. In contrast, few or no koalas were consistently recorded east of the highway between N15 and N46, or at N5 and N45, west of the highway.

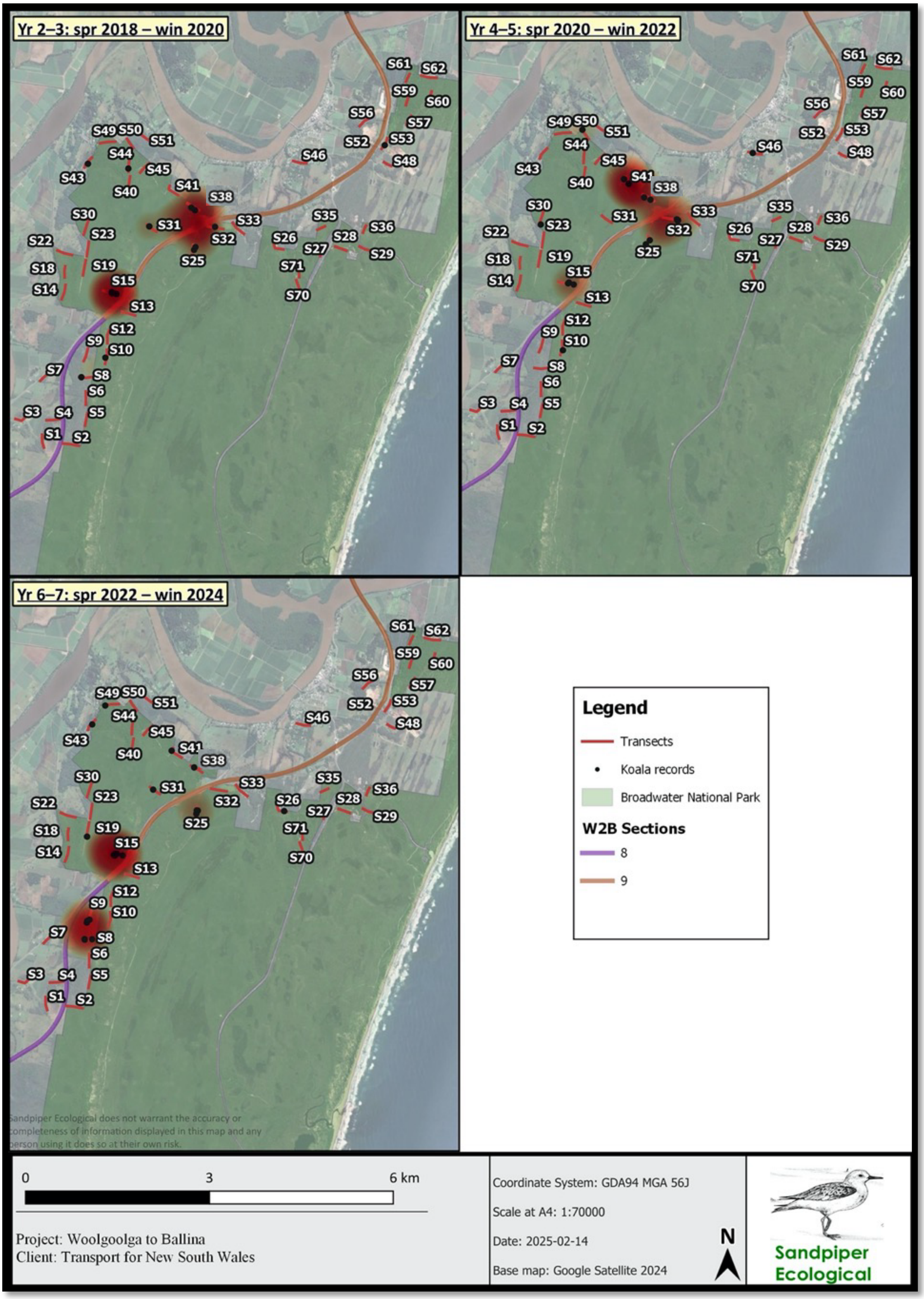

Figure 9: Heat maps showing the distribution and locations of koala records during years 2-3, 4-5 and 6-7 of operational phase monitoring at Broadwater.

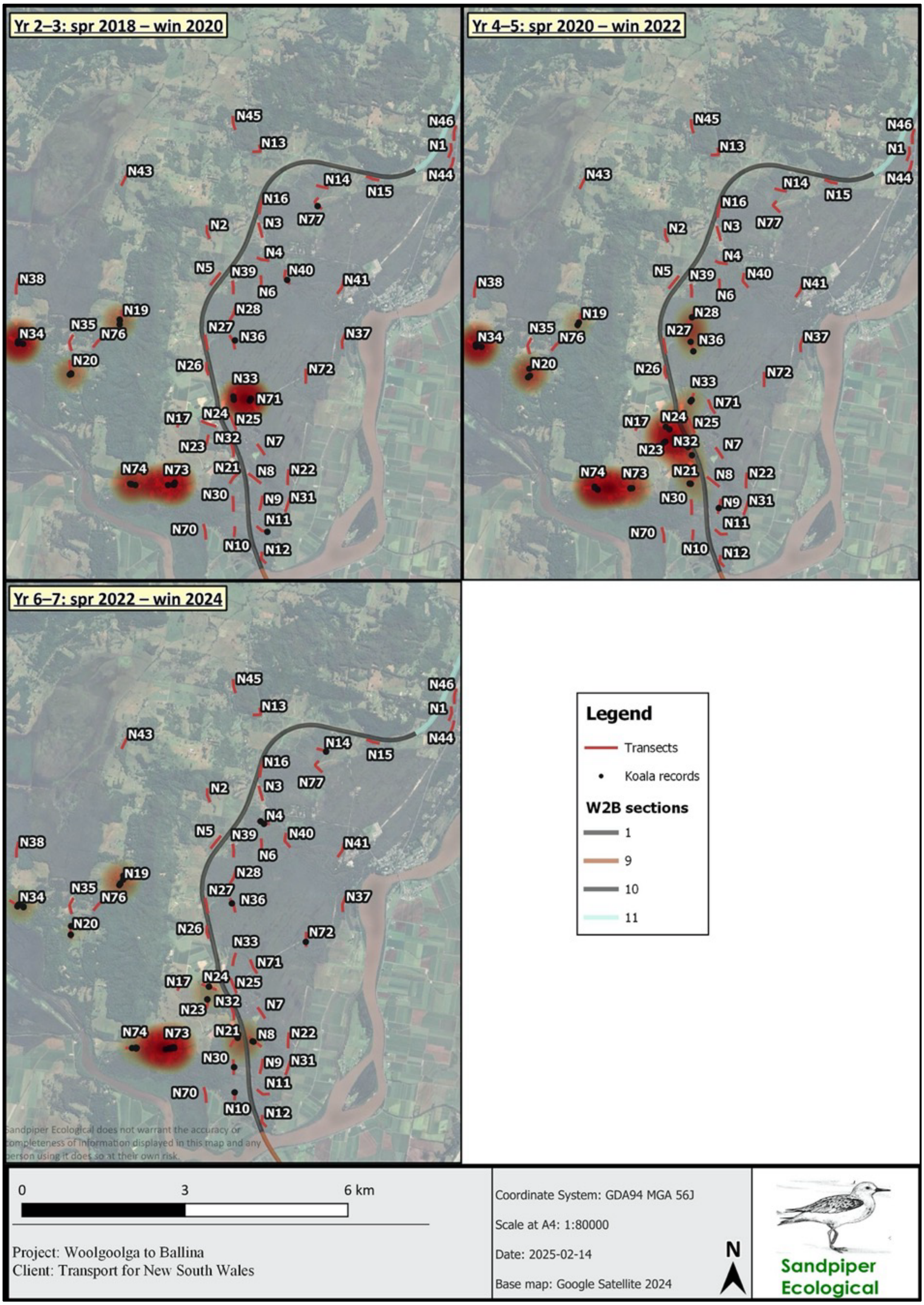

Figure 10: Heat maps showing the distribution and locations of koala records during years 2-3, 4-5 and 6-7 of operational phase monitoring at Bagotville

4.2.4 Vegetation preferences

Koalas have been recorded in multiple vegetation classes, with the highest proportion in coastal swamp forests (32.92%), followed by unclassified vegetation (18.50%), “other” vegetation classes (12.85%), coastal floodplain wetlands (10.97%), wallum sand heaths (10.34%), coastal headland heaths (8.15%), and coastal dune dry sclerophyll forests (6.27%) (Figure 11). The “other” category is a group of several vegetation classes, including rainforest, mangrove, and additional dry sclerophyll forests.

Unclassified vegetation accounted for 18.50% of koala observations overall, with a higher proportion in Bagotville (26.63%) than in Broadwater (7.41%). This difference was mainly due to roadside vegetation that remains unclassified at sites N73 and N74, where primary feed trees such as swamp mahogany (*Eucalyptus robusta*) and forest red gum (*Eucalyptus tereticornis*) are present. Coastal floodplain wetlands were recorded only in Bagotville, representing 10.97% of overall observations, with 19.02% in Bagotville and none in Broadwater. Coastal headland heaths contributed 8.15% of observations, with 16.30% in Broadwater and 2.17% in Bagotville. Coastal dune dry sclerophyll forests showed similar proportions in Broadwater (5.93%) and Bagotville (6.52%). These variations likely reflect differences in habitat availability between the two study areas.

Several vegetation classes contained primary feed trees, which appeared to influence habitat use by koalas (Figure 12). Overall, 65.8% of koalas were recorded in primary feed trees (i.e., forest red gum, swamp mahogany, tallowwood *E. microcorys*), with a higher proportion in Broadwater (71.9%) than in Bagotville (60.7%). Swamp mahogany accounted for 43.6% of records, with more in Broadwater (66.4%) than in Bagotville (24.3%). Forest red gum comprised 11.3% of records, with a greater proportion in Bagotville (16.2%) than in Broadwater (5.5%). Tallowwood was used exclusively in Bagotville, contributing 11.0% of observations. Non-primary feed tree species accounted for 34.2% of records and were more evenly distributed between Broadwater (28.1%) and Bagotville (39.3%). Differences in feed tree use between the two study areas likely reflect variations in the availability and distribution of the primary feed tree species.

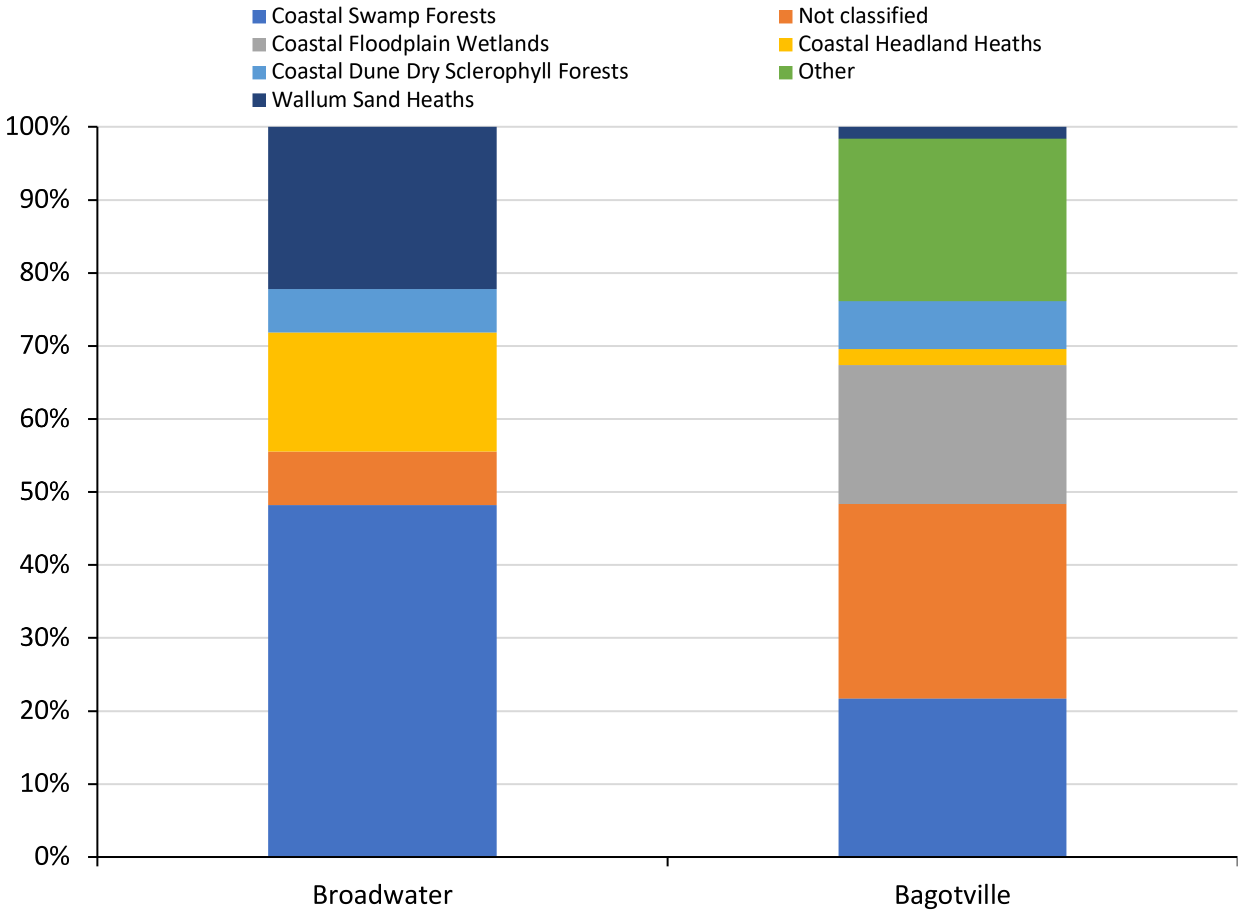

Figure 11: Proportion of koala records within each vegetation class for Broadwater and Bagotville, based on all recorded sightings from years 1 to 7.

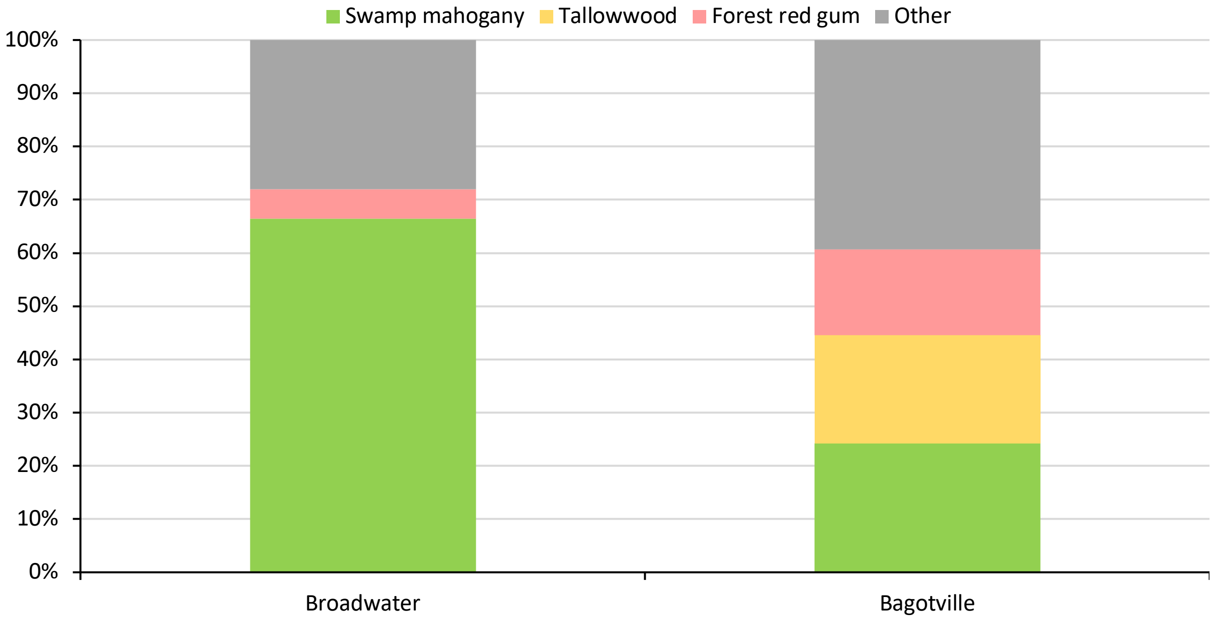

Figure 12: Proportion of koala records by tree species in Broadwater and Bagotville from years 1 to 7.

4.3 Observed density

4.3.1 Broadwater

A total of 12 koalas were recorded on transect at Broadwater during year 7 (spring 2023 and autumn 2024 surveys), resulting in an observed density of 0.052 koalas ha^{-1} (Figure 13). This represents a 7.1% reduction from year 6 and a 60.6% decline relative to the baseline survey (Figure 13). Koala density declined rapidly from 0.132 koalas ha^{-1} during the baseline to 0.022 koalas ha^{-1} in year 2, before fluctuating between 0.031 and 0.065 koalas ha^{-1} in subsequent years (Figure 13).

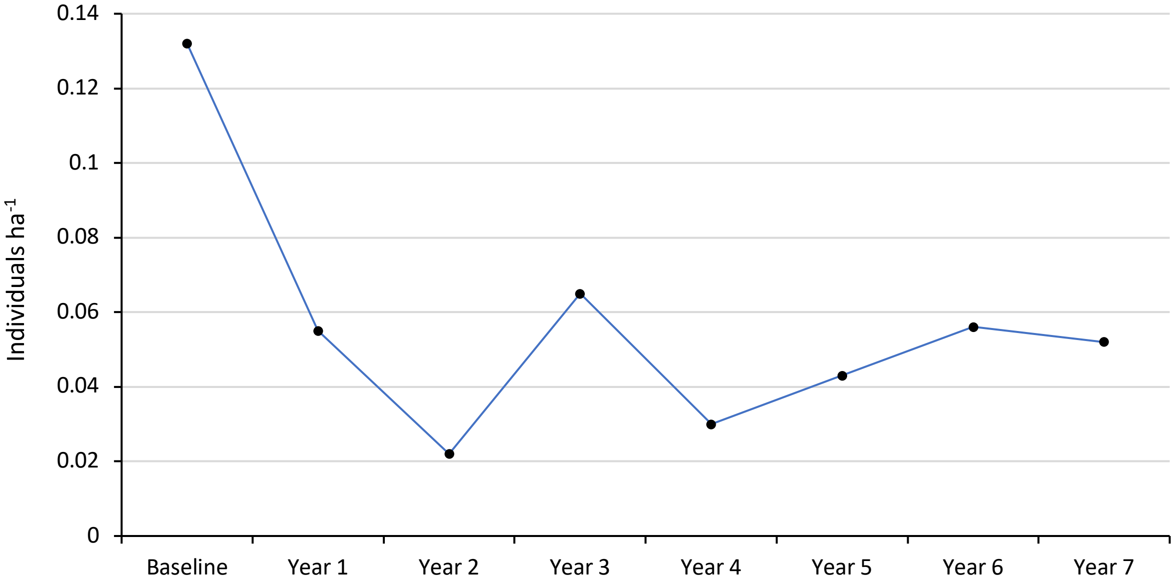

Figure 13: Comparison of koala density estimates in the Broadwater focal area from baseline to year 7 of operational monitoring.

4.3.2 Bagotville

A total of 20 koalas were recorded on transect at Bagotville during year 7, resulting in an observed density of 0.091 koalas ha^{-1} (Figure 14). This represents a 2.2% increase from year 6 and a 21.3% increase relative to the baseline survey. Koala density increased from 0.075 koalas ha^{-1} at baseline to a peak of 0.094 koalas ha^{-1} in year 3, before declining to 0.069 koalas ha^{-1} in year 4. In subsequent years, density fluctuated between 0.072. and 0.091 koalas ha^{-1}, with stabilisation occurring at density levels like or higher than baseline.

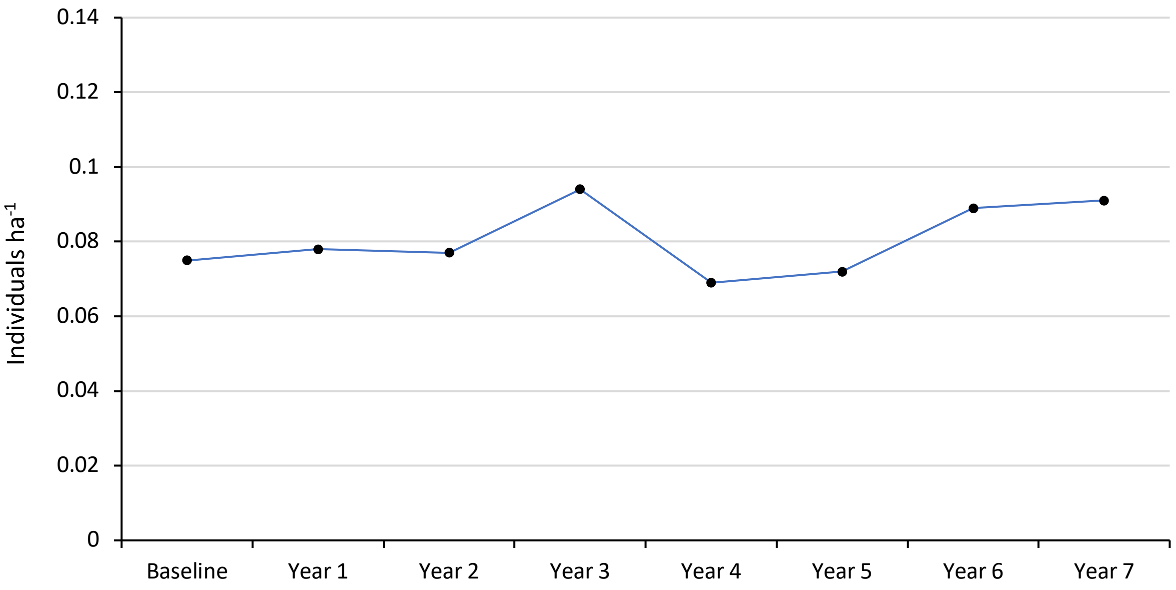

Figure 14: Comparison of koala density estimates in the Bagotville focal area from baseline to Year 7 of operational monitoring.

4.4 Bayesian model-averaged density and population size

4.4.1 Broadwater

The Bayesian model-averaged density estimate for Broadwater in year 7 was 0.046 koalas ha^{-1} (SE: 0.008; 95% CI: 0.032–0.062), approximately 20% lower than the baseline estimate of 0.057 koalas ha^{-1} (SE: 0.011; 95% CI: 0.038–0.081) and like the year 6 estimate of 0.047 koalas ha^{-1} (Figure 15). Strongly overlapping confidence intervals (CI's) suggest no clear trend (Figure 15).

The log-linear model indicated an annual decline of -1.2% ($\beta = -0.012$; SE: 0.025; 95% CI: −0.079 to 0.021), with a posterior probability of decline at 0.625. A value close to 0.5 suggests no trend. This represents a moderation of the steeper declines observed in previous years, such as the -3.3% per year reported in year 5 ($\beta = -0.033$; SE: 0.041; 95% CI: −0.124 to 0.023) (See details in appendix B).

For year 7, the Bayes Factor was 0.694, indicating only slight evidence in favour of no trend over a continued decline. While koala densities remain below baseline levels, the steep declines recorded in earlier years appear to have stabilised. However, a negligible negative trend persists. For full details refer to Appendix B.

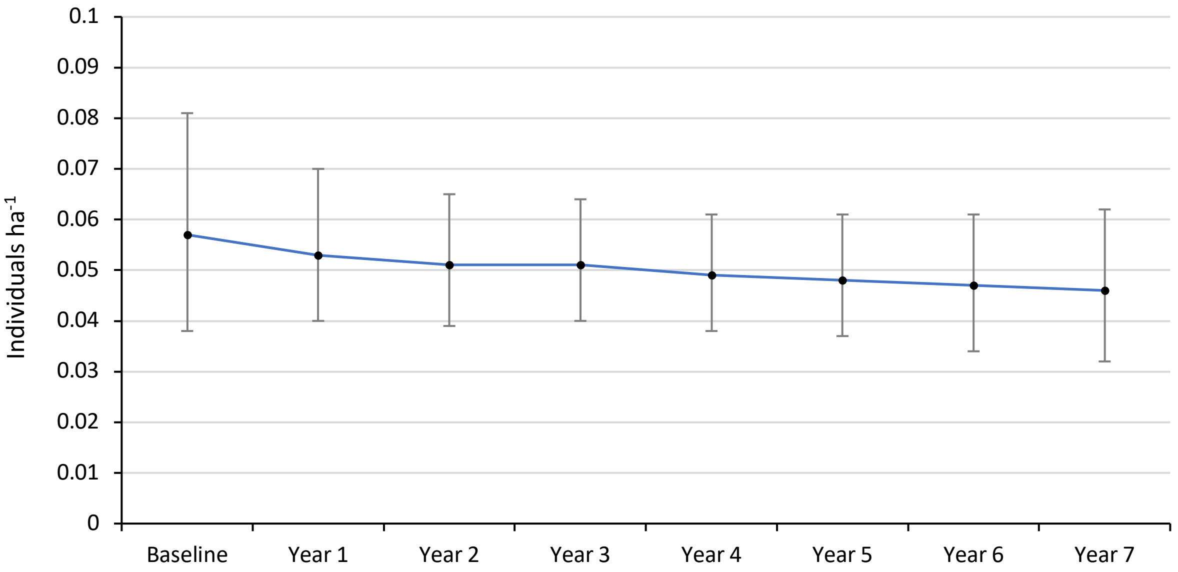

Figure 15: Comparison of Broadwater focal area (Bayesian) density estimates (± 95%CI) for the modeled baseline and monitoring years 1-6.

4.4.2 Bagotville

The Bayesian model-averaged density estimate for Bagotville in year 7 was 0.083 koalas ha^{-1} (SE: 0.012; 95% CI: 0.062–0.108), approximately 7.8% higher than the baseline estimate of 0.077 koalas ha^{-1} (SE: 0.012; 95% CI: 0.056–0.105) and like the year 6 estimate of 0.081 koalas ha^{-1} (Figure 16). The log-linear trend indicated a slight annual increase of +0.4% (β = 0.004; SE: 0.018; 95% CI: -0.029 to 0.052), up slightly from last year's trend estimate of 0.2% per year (β = 0.002/year). The Bayes Factor for year 7 was 0.537, providing only slight evidence against a definitive trend in either direction. These findings indicate that while no strong trend is evident, a marginal increasing trend persists overall. For full details refer to Appendix B.

The extrapolated population estimate for Year 7, based on Bayesian density, was 177 koalas (95% CI: 132–231) across 2,135 ha of preferred habitat (Figure 17). This reflects a gradual increase from the baseline estimate of 164 koalas (95% CI: 120–224) across 2,152 ha.

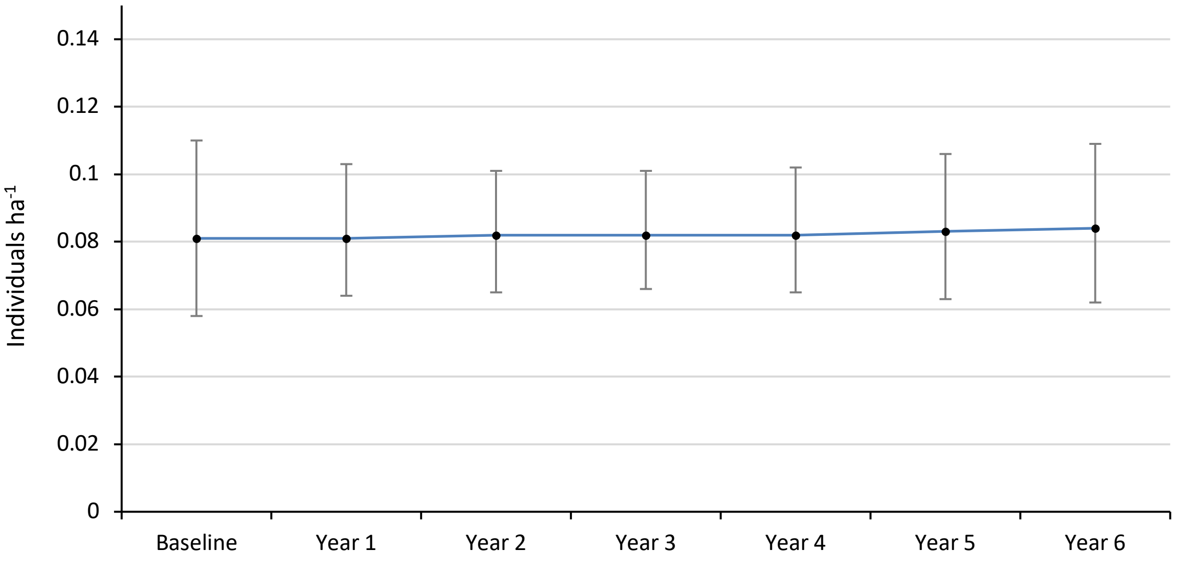

Figure 16: Comparison of Bagotville focal area (Bayesian) density estimates (± 95%CI) for the modeled baseline and monitoring years.

4.5 Power analysis

The year 7 power estimates for Broadwater and Bagotville, at a maximum Type I error rate of 0.30, were 0.650 and 0.728, respectively (Figure 18). Compared to year 6, when power was estimated at 0.647 for Broadwater and 0.732 for Bagotville, the year 7 values indicate a small increase at Broadwater and a small decrease at Bagotville. Both were slightly lower than the year 5 estimates of 0.657 for Broadwater and 0.734 for Bagotville.

When the significance level was increased to 0.35, the year 7 power estimates increased to 0.691 for Broadwater and 0.763 for Bagotville. Overall, power estimates at Bagotville exceed the 70% threshold, while those at Broadwater remain below the target level.

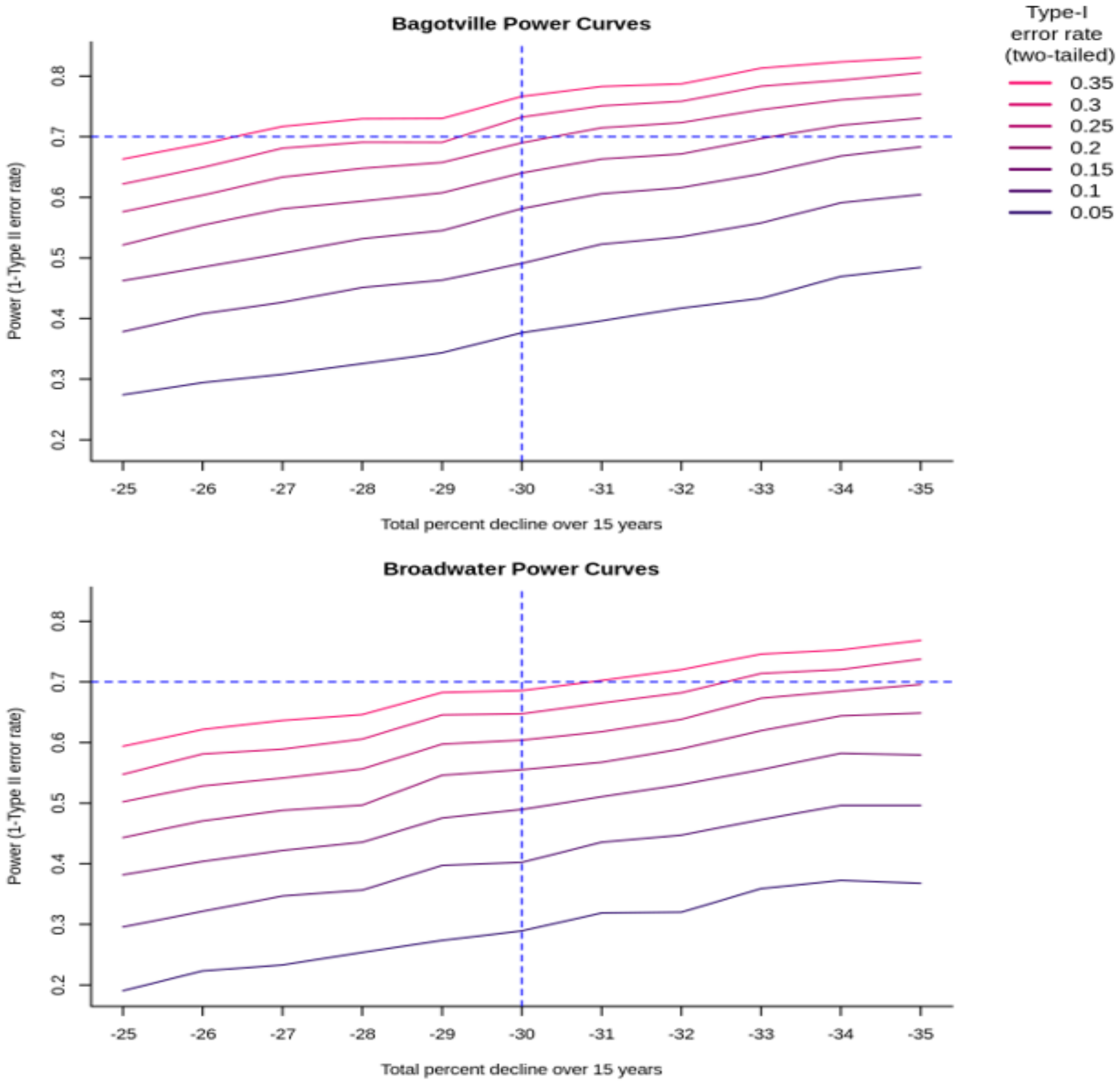

Figure 17: Statistical power to detect a 30% decline in baseline densities over a 15-year monitoring period for different maximum levels of Type-I errors (lines).

4.6 Genetic analysis

4.6.1 Genetic diversity

Genetic diversity was assessed using expected heterozygosity (H_e), allelic richness (A_r), and observed heterozygosity (H_o) across the 2018, 2020, 2022, and 2024 surveys (Table 6). The population exhibited low to moderate genetic diversity throughout the study period. In 2024, allelic richness (A_r) increased, indicating a higher mean number of alleles per locus (A_{mean}) compared to previous years, suggesting an expansion of the population's gene pool.

Expected heterozygosity (H_e) showed a steady increase from 0.672 in 2018 to 0.788 in 2024, indicating a progressive improvement in genetic diversity potential. Observed heterozygosity (H_o) also increased, from 0.539 in 2018 to 0.654 in 2024, aligning with trends in H_e and A_r (Table 6). An illustrative guide to classifying heterozygosity values may be defined as: Low Heterozygosity: 0.00–0.33; Moderate Heterozygosity: 0.34–0.66; and High Heterozygosity: 0.67–1.00.

The inbreeding coefficient (F_{is}), which quantifies genetic variance due to inbreeding, fluctuated over the study period. It decreased from 0.204 in 2018 to 0.114 in 2020, suggesting reduced inbreeding, but increased to 0.205 in 2022, indicating a return to moderate to high levels of inbreeding. By 2024, F_{is} decreased to 0.165, remaining within the moderate range (Table 6).

Overall, the increase in A_r, H_e, and H_o suggests a general improvement in genetic diversity from 2018 to 2024. However, fluctuations in F_{is} indicate that inbreeding remains a concern, with potential implications for the population's genetic health and adaptive capacity. For full details please refer to Appendix C.

Table 6: Genetic diversity statistics for the Bagotville population samples 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=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
2024 Survey	22	7.72	5.11	0.165	0.654	0.788

4.6.2 Pairwise genetic differentiation

Pairwise comparisons of genetic differentiation (F_{st}) between koalas east and west of the Pacific Highway across 2018, 2020, 2022, and 2024 indicate weak genetic differentiation ($F_{st} < 0.05$) within each survey year, suggesting genetic similarity between the populations on the east versus the west side of the highway.

However, moderate to strong genetic differentiation ($F_{st} = 0.05–0.25$) was observed between specific survey years, for instance:

- East sites in 2018 versus west sites in 2020

- West sites in 2020 versus east sites in 2022
- Both east and west sites in 2024 compared to all previous years

These findings indicate temporal shifts in genetic composition, with differences observed between some years, while genetic differentiation within years remained weak which is likely influenced by where scats are collected.

Table 7: Pairwise F_{st} Values for Bagotville in 2018, 2020, 2022, and 2024 for the east and west of the Pacific Highway. F_{st} values indicate the degree of genetic differentiation between koala populations. Values below 0.05 suggest weak differentiation, 0.05–0.15 indicate moderate differentiation, 0.15–0.25 represent strong differentiation, and values exceeding 0.25 denote very strong genetic differentiation.

Highway Side/Year	Overall West	West 2018	East 2020	West 2020	East 2022	West 2022	East 2024	West 2024
Overall east	0.0148							
West 2018		0.0366	0.0038	0.0871	0	0.0224	0.0948	0.0794
East 2018			0.0151	0.0115	0.0365	0	0.158	0.1279
East 2020				0.0383	0.0064	0.0186	0.1224	0.1057
West 2020					0.086	0.0346	0.1883	0.1583
East 2022						0	0.0947	0.0639
West 2022							0.1176	0.0931
East 2024								0.0056

4.6.3 Genetic relatedness

Genetic relatedness was assessed and compared across the 2018, 2020, 2022, and 2024 Bagotville koala populations. Figure 16 presents the mean relatedness (r) for each survey year and indicates that relatedness values in 2024 were more narrowly distributed than in previous years.

The mean relatedness values recorded were 0.128 in 2018, 0.174 in 2020, 0.121 in 2022, and 0.162 in 2024. The 2020 (0.174) and 2024 (0.162) surveys recorded mean relatedness values that exceeded the upper limit of the 95% confidence interval, suggesting that koalas in these years were more closely related than expected. This pattern may indicate an increase in familial relationships or a decline in genetic diversity due to limited new genetic input. The red lines in Figure 16 represent the upper (U) and lower (L) 95% confidence intervals expected for each population under the null hypothesis of no difference among years.

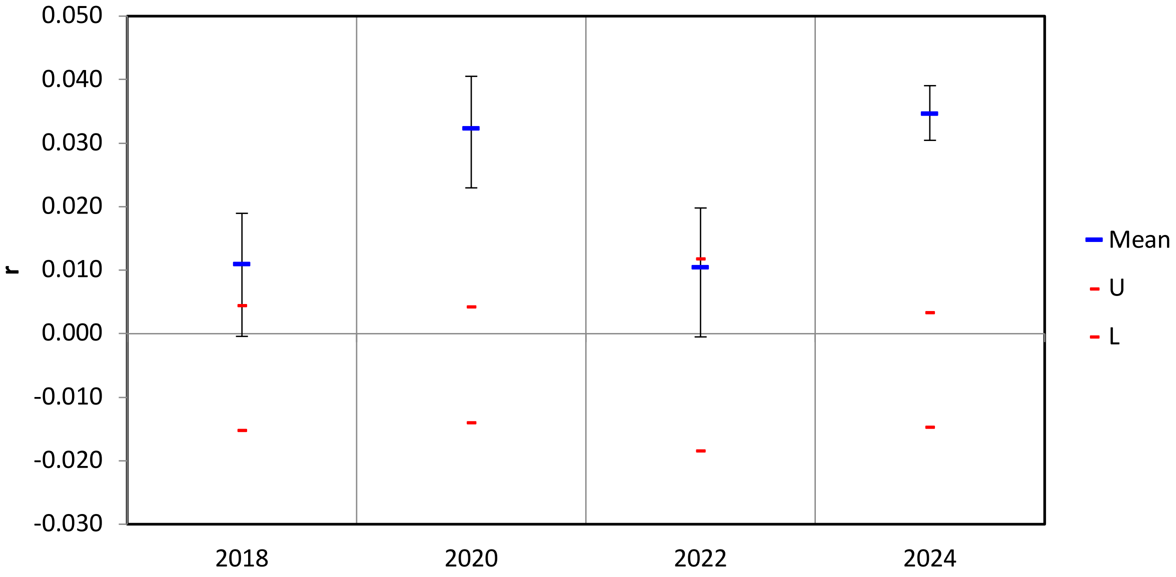

Figure 18: Mean genetic relatedness (r) for 2018, 2020, 2022 and 2024 koala site surveys.

4.6.4 Effective population size

Estimates of effective population size (N_e) for males and females from the 2018, 2020, 2022, and 2024 koala surveys are presented in Table 8. N_e represents the number of individuals contributing genetically to the next generation and is a key indicator of genetic diversity and population stability. The effective population size is the size of an idealised population that would experience the same rate of genetic drift as the real population. Whilst N_e is an important parameter it represents an ideal population where all individuals have an equal chance of reproducing, mating is random, and the population size remains constant over time. N_e is typically lower than the census population partly because chance events prevent some individuals from breeding and partly due to background selection. In this instance *Ne* is well below the census population size and is influenced by the scat collection method and clumped distribution of koalas within the study area.

For females, N_e estimates in 2018, 2020, and 2024 had relatively narrow confidence intervals, indicating lower levels of uncertainty. In contrast, male N_e estimates were infinite (∞) across all years, likely due to low genetic variation within the sampled population or limitations in the dataset rather than biological factors. Heterozygote excess (*D*) values were negative for all cohorts except for males in 2018, which had a positive value. This suggests a general heterozygote deficit and differences in allele frequencies between males and females, potentially indicating population structure or sampling effects (Table 8).

Table 8: Effective population size of males and females for koalas in the Bagotville study area. 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

2024	Female	17	19.6	16.9 – 22.3	-0.257
	Male	5	∞	13.4 – ∞	-0.059

4.6.5 Population structure

The genetic structure of the Bagotville population has remained stable, with two genetic clusters (K = 2) consistently identified in 2018, 2022, and 2024 (Figures 17–20). The 2020 survey was an exception, with three clusters (K = 3) detected (Figure 19), suggesting a temporary increase in genetic subdivision or gene flow. By 2022, the population had returned to a two-cluster structure, like 2018. The spatial distribution of genetic clusters (Figure 21 A–D) highlights regional variation. In 2020 (Figure 21B), genetic assignments were more mixed, supporting the observed increase in genetic subdivision. By 2022 and 2024 (Figures 21C and 21D), genetic structuring was more localized like in 2018. While the loss of a genetic cluster over a short timeframe is uncommon, it may result from genetic drift, fluctuations in population size, or restricted movement. Figure 21 A–D illustrates these spatial changes, with pie charts showing the proportional assignment of koalas to genetic clusters across survey years.

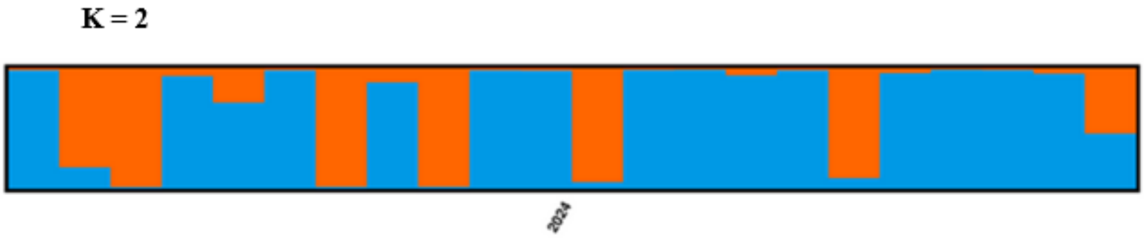

Figure 19: Population substructure of 2024 northern NSW koala populations using STRUCTURE based on 32 loci.

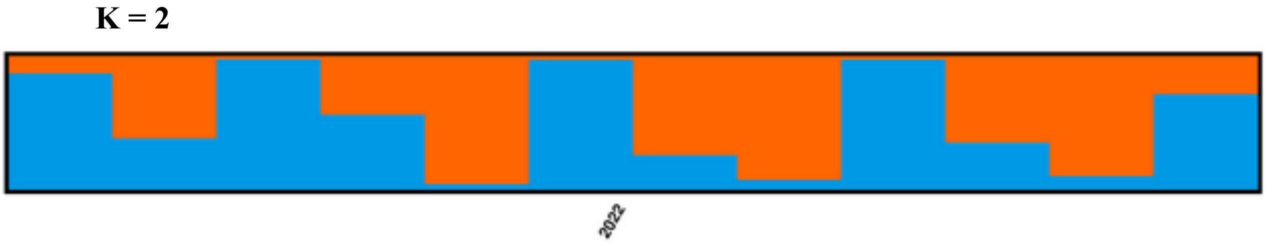

Figure 20: Population substructure of 2022 northern NSW koala populations using STRUCTURE based on 32 loci.

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

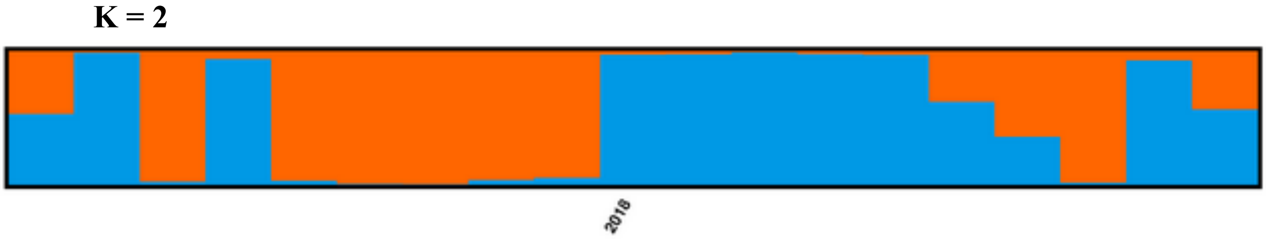

Figure 22: Population substructure of 2018 northern NSW koala populations using STRUCTURE based on 32 loci.

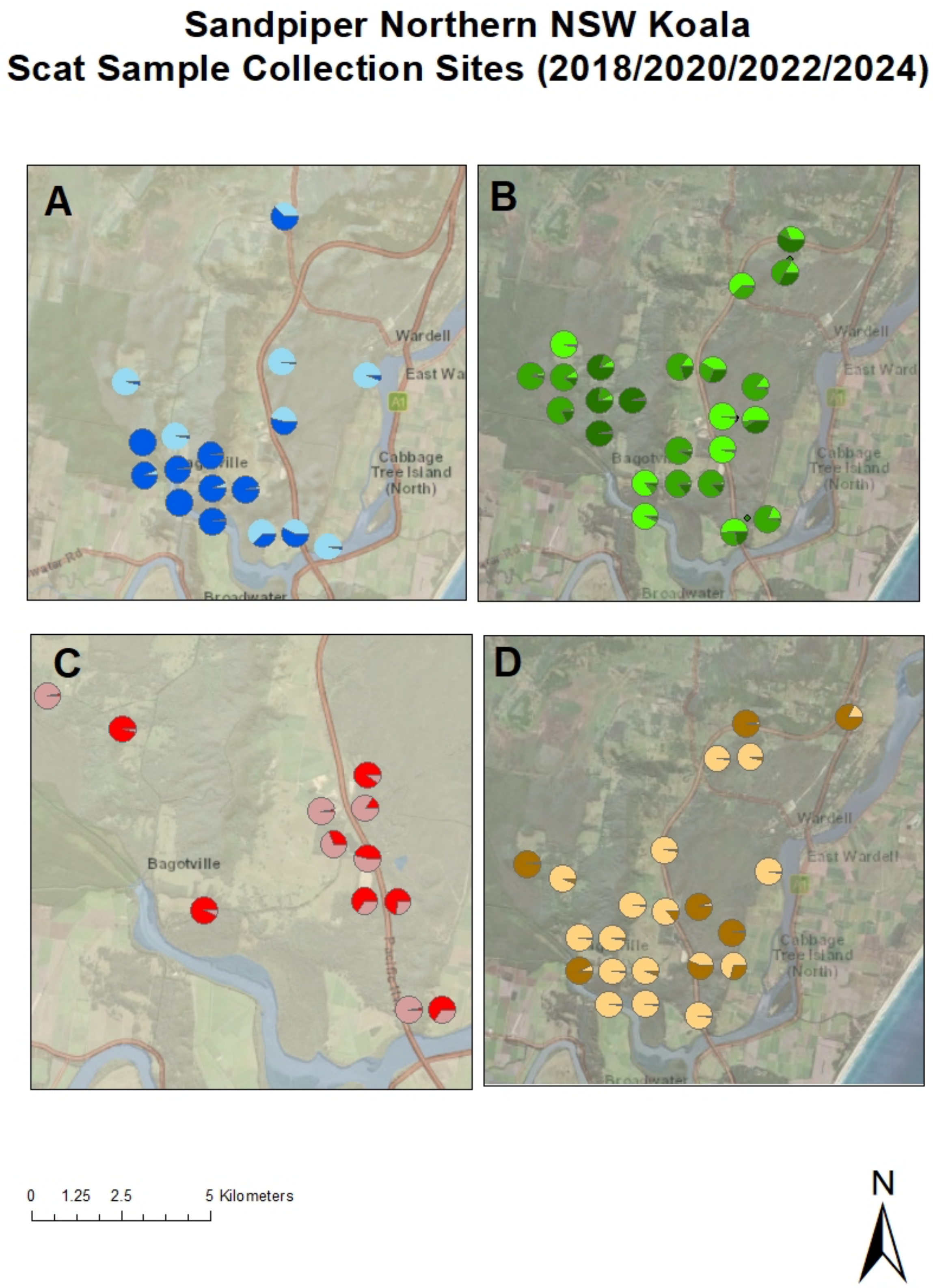

Figure 23: Inferred cluster assignments of (A) 2018 (K = 2); (B) 2020 (K = 3); (C) 2022 (K = 2) and (D) 2024 (K = 2) northern NSW koalas.

4.6.6 Movement of individuals

Genetic analysis allowed for the identification of individual koalas, enabling their movements to be tracked over time. Figure 15 shows the distribution and movement patterns of five koalas recorded on multiple occasions between 2018 and 2024. No individuals were re-recorded in 2024, and no individuals have been recorded crossing the Pacific Highway to date.

Individual 1 was recorded dispersing southward from its initial detection site, possibly as part of sub-adult home range establishment (Figure 15). However, this individual was initially in poor condition, and Friends of the Koala (FoK) were notified. Its subsequent movement is more likely related to rehabilitation and translocation as part of FoK procedures.

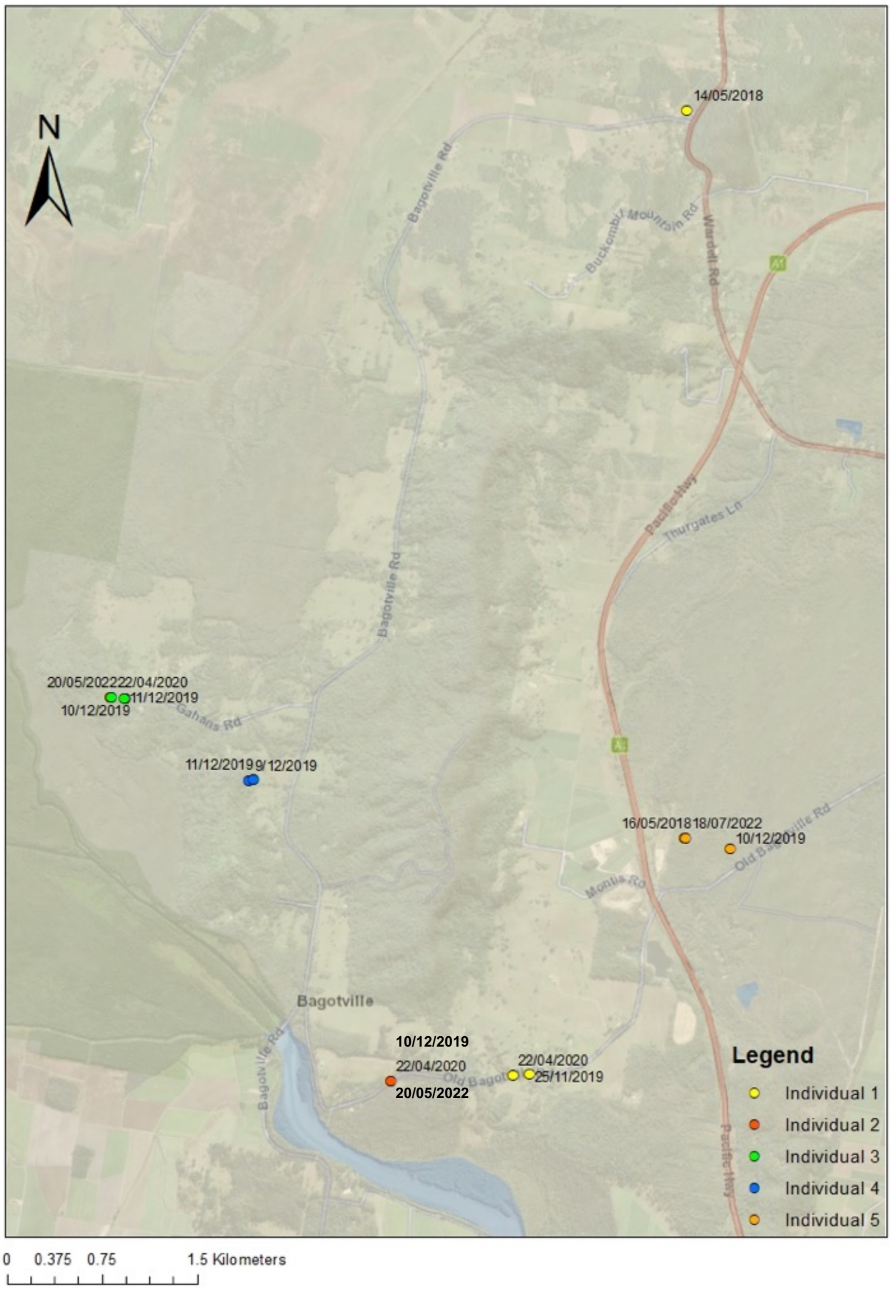

Figure 24: Distribution and movement of koalas over the survey period: 2018 - 2024. Each coloured circle denotes an individual koala.

4.7 Road mortality surveys and fauna fence condition

4.7.1 Road mortality

No koalas were recorded during road mortality surveys conducted in August and November 2023 along sections 1 to 11 (Appendix D). Surveys identified 129 road-killed fauna during this period. Accurate species identification during vehicle-based surveys is often difficult unless carcasses are fresh. Koalas fall within the medium mammal category, and 14 individuals in this category were recorded.

It is unlikely that koalas were included in the medium or unidentified mammal categories. Pelage colour is a key diagnostic feature in road mortality surveys, and mammals with grey pelage, which could include koalas, underwent closer inspection. Most of the medium and unidentified mammals recorded had dark pelage and were more likely bandicoots, short-eared brushtail possums, or swamp wallaby remains.

A total of 12 species were recorded during road mortality surveys, including seven bird species, two mammal species, one reptile species, and one amphibian species (Appendix D). An additional six fauna groups were identified. The overall road-kill rate for sections 1 to 11 was 0.47 individuals per kilometre (Table 6).

Additionally, no koala road strike mortalities were recorded by the TfNSW Roads Maintenance Division. Friends of the Koala (FOK) also reported no vehicle strikes within sections 3 to 11 during the Year 7 reporting period.

4.7.2 Fauna fence

No detectable breaches were observed in fauna fence on Wardell Road, old Pacific Highway or along sections 3-11 of the Pacific Highway during the year 7 reporting period.

5 Discussion

The Year 7 monitoring results provide important insights into the status of koala populations in the Broadwater and Bagotville focal areas along the W2B Pacific Highway upgrade. The following discussion interprets these findings in relation to the monitoring objectives and the broader management context outlined in the approved KMP.

5.1 Broadwater population trends

In Year 7, 12 koalas were recorded on transects at Broadwater, one fewer than the 13 recorded in Year 6. The observed density (number of koalas/total habitat area) for Year 7 was 0.052 koalas ha^{-1}, representing a 7.1% decrease from Year 6 and a 60.6% decline from baseline. Following an initial sharp decline from 0.132 koalas ha^{-1} at baseline to 0.055 in Year 1 and 0.022 in Year 2, observed densities increased to 0.065 koalas ha^{-1} in Year 3 and exhibited minor variations in years 4-7.

Bayesian model-averaged estimates, which are updated annually, provide a more reliable long-term assessment by incorporating new data and smoothing short-term variations. The Bayesian density estimate for Year 7 was 0.046 koalas ha^{-1} (SE = 0.008; 95% CI = 0.032–0.062), comparable to Year 6 (0.047 koalas ha^{-1}) and Year 5 (0.045 koalas ha^{-1}), and approximately 20% lower than the baseline estimate of 0.057 koalas ha^{-1} (SE: 0.011; 95% CI: 0.038–0.081). Bayesian modelling in Year 7 indicated a weak negative trend of -1.2% per year (β = -0.012; SE = 0.025; 95% CI = -0.079 to 0.021) with a posterior probability of decline at 0.625. The Bayes Factor (0.694) provides slight evidence supporting stability over continued decline. While koala densities remain below baseline levels, the substantial early declines appear to have stabilized, though a minor downward trend persists, with confidence intervals suggesting continued uncertainty.

Challenges

Monitoring at Broadwater is constrained by the limited number of transects detecting koalas and the uneven spatial distribution of records, which contribute to high variance in density estimates. This variability reduces the statistical power of analyses. In Year 7, 75% of detections were recorded at just three transects (S25, S15, and S9), a pattern consistent throughout the monitoring period. Despite being recorded at 10–12 active transects over the seven years of monitoring results show that the koala population in the study area is clustered around a small number of high value sites.

Unsurprisingly and despite minor improvements, statistical power remained low. In Year 7, power analysis estimated a detection power of 0.650 at a Type I error rate of 0.30, only slightly higher than Year 6 (0.647) but still below the 0.70 threshold required for robust trend detection. Adjusting the significance level to 0.35 increased power to 0.691, though this remains suboptimal. Raising the significance level to 0.35 increased statistical power to 0.691, but this remains relatively low. The reliance on a few high-use sites for detections reduces the robustness of trend analyses, as population estimates are heavily affected by fluctuations in these areas. Expanding the number of survey sites is necessary to enhance statistical power.

Influence of climate variability

Fluctuations in population estimates may be influenced by climate variability. Prolonged droughts and extreme heat wave events from 2017 to 2020, particularly the severe 2019 drought, likely contributed to physiological stress, impacting koala health and reproduction. Drought reduces habitat quality and food availability, as declining vegetation productivity leads to habitat degradation (Kotzur et al., 2024). Heat stress further exacerbates these pressures, affecting thermoregulation and increasing mortality rates despite behavioural adaptations (Mella et al., 2024). Additionally, heat stress has been linked to immune suppression, heightening susceptibility to chlamydia (Fernandez et al., 2024). The sharp declines observed in Years 1 and 2 coincide with these dry and hot conditions, providing potential evidence of a link between climate and a decline in the koala population or shift in distribution in response to changed weather conditions.

Well above-average rainfall from 2020 to 2022 resulted in widespread flooding, particularly in March 2022, when some transects remained inundated for weeks. Hotspot analysis indicates that koala detections declined in low-lying swamp areas, particularly at S31 (west of the highway) and S32 (east of the highway), in Years 5–7 post-flooding. Prolonged inundation may have temporarily reduced food availability and tree stability, affecting koala presence. However, while extreme rainfall events negatively impact koalas in flood-prone areas, moderate and consistent rainfall supports population stability. Increased precipitation improves the moisture content and nutritional quality of eucalyptus leaves, enhancing hydration and reducing physiological stress (Ellis et al., 2010; Davies et al., 2013; Seabrook et al., 2011). Furthermore, higher and more reliable rainfall correlates with improved koala condition and, therefore, likely fecundity (Whisson & Carlyon, 2010).

Implications

Koala habitat preferences influence population trend assessments, with occupancy linked to the distribution of preferred food tree species (McAlpine et al., 2023). Analysis from Years 1 to 7 shows that sites with abundant swamp mahogany consistently recorded higher koala detections. In Broadwater, 66% of koala records were from swamp mahogany trees in forested wetlands and wet heath areas, particularly around sites S25, S15, and S9. Further analysis of habitat data is likely to show relationship between koala occupancy and the presence of preferred food tree species within proximity to a transect.

Surveys at many transects yielded no koala detections, particularly between S1 and S7 on the eastern side of the highway and S52 to S56 on the western side. This pattern is likely due to the absence of primary feed trees and is supported by the results of thermal drone surveys which found no evidence of koalas in the aforementioned areas (Sandpiper Ecological 2023). As a result, population trend assessments rely heavily on a small number of high-use sites, limiting broader inferences about the overall population trend. The concentration of detections in these areas reduces the reliability of trend analyses, as fluctuations at these sites disproportionately affect population estimates and reduce statistical power.

In line with the KMP the monitoring design requires ongoing review to maintain power >70%, the threshold necessary to achieve a statistically significant result at 15 years. To achieve this, an additional 15–20 transects may need to be established in the Broadwater focal area (Dr. Rob Rankine, pers. comm., December 2024). Site selection should follow the randomised grid approach used in the baseline survey and target areas of suitable habitat such as the east of the alignment in Broadwater National Park.

5.2 Bagotville population trends and PVA predictions

In contrast to Broadwater, the Bagotville focal area exhibits more stable population trends despite highway construction, wildfires, droughts and floods as discussed for Broadwater and in the previous year 6 report (Sandpiper Ecological 2024). In Year 7, the Bayesian model-averaged density estimate was 0.083 koalas per hectare (SE = 0.012; 95% CI: 0.062–0.108), representing a 7.8% increase from the modelled Bayesian baseline density of 0.077 koalas per hectare. When extrapolated across the preferred koala habitat within Bagotville, this equates to an estimated total population of approximately 177 individuals, with a 95% confidence interval ranging from 132 to 230 individuals. Trend analysis using a log-linear model indicates a modest annual increase of 0.4% ($\beta = 0.004$; SE = 0.018; 95% CI: -0.029 to 0.052), and power analysis confirms that survey effort exceeds the 70% confidence threshold, supporting the reliability of detected trends.

Under the PVA, specifically Scenario 6, which assumes effective management actions, including a reduction in koala mortality by approximately four individuals per year (RMS 2016), the population was projected to be 234 individuals at Year 5 (CI = 195–276) and 219 individuals at Year 10 (CI = 147–272) (Figure 26). During the initial PVA, a correction factor of 0.204 was applied to account for the unsampled 0–1-year-old age class, which is often underrepresented in field surveys, particularly juveniles still dependent on their mothers (Kavanagh 2016). Based on the fecundity of breeding females aged 3–7 years, an estimated 40 individuals were added to the baseline population of 196, bringing the total to 236. This adjustment improved the representation of early age classes in the PVA model and should be considered when calculating population estimates in the Bayesian model to enable comparisons and track population trends as per the KMP requirements.

A key difference between the PVA and the Bayesian model lies in how each handles baseline population estimates. The original PVA uses a fixed baseline derived from extrapolated values, adjusted by a correction factor to account for underrepresented juveniles. In contrast, the Bayesian model updates its baseline as new data become available, making its estimates more responsive to recent observations. These differing baselines lead to inconsistencies when comparing absolute population estimates. However, when the focus shifts to relative change, such as percentage increase or decrease from each model's respective baseline, comparisons become more meaningful.

With this approach in mind and adding the 0.204 correction factor to the modelled Bayesian estimate the baseline estimate is 198 individuals, the year 5 estimate is 208 individuals (approximate CI = 167–254) and the year 7 estimate is 213 individuals (approximate CI = 159–278) which equates to an approximate 7.8% increase over the survey period. Comparatively the PVA predicted a 0.8% decline by Year 5 and approximate 7% decline by year 10 (Figure 26). The trends are divergent, with the Bayesian model demonstrating modest population growth and the PVA predicting a steady temporal decline. The modest population increase identified by the

Bayesian model is supported by the findings of EarthScapes (2024) who recorded a 3.9% increase in sites used by koalas from 2013 to 2023/24 in Ballina Shire. Several of the sites surveyed in that study overlap with the long-term monitoring transects.

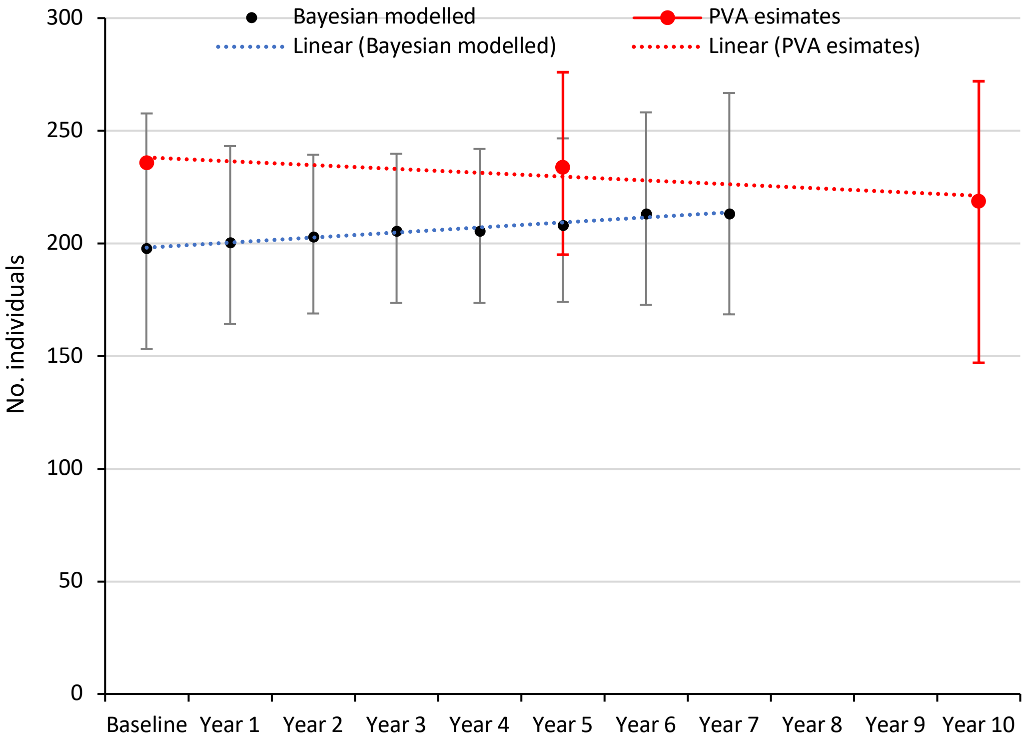

Figure 25: Comparison of Bagotville focal area population estimates (± 95% CI) from Bayesian modelling and PVA projections for the modelled baseline and monitoring years. Estimates are based on 2,152 ha of preferred koala habitat at baseline and 2,135 ha during monitoring years, as informed by the PVA (Kavanagh 2016). Note: This includes a correction factor of 0.204 for the unsampled 0-1 year cohort.

Genetics and implications

Genetic analysis of the Bagotville koala population indicates an overall increase in genetic diversity, though signs of localised inbreeding and relatedness are evident. Genetic diversity metrics show an increase in expected heterozygosity (H_e) from 0.672 to 0.788 and in observed heterozygosity (H_o) from 0.539 to 0.654, alongside greater allelic richness. These findings suggest an expanding gene pool despite habitat fragmentation and align with stable to slightly increasing population estimates, particularly the marginally higher density observed in Year 7.

Despite these positive trends, fluctuations in the inbreeding coefficient (F_{is}) indicate potential genetic structuring within the population. The F_{is} value decreased from 0.204 in 2018 to 0.114 in 2020, then increased to 0.205 in 2022 before slightly declining to 0.165 in 2024. This variability, coupled with elevated genetic relatedness in 2020 and 2024, suggests that localised inbreeding may be occurring in specific areas. The spatial clustering of koala detections supports this pattern, with consistently high koala detection rates at N74 and N73 (west of the highway along Old Bagotville Road), and at N33, N34, N19, N20, and N36. The tendency to collect scats from these areas may disproportionately influence genetic monitoring results, as sampling bias in clustered locations could overrepresent related individuals and skew assessments of population-wide genetic diversity.

Further analysis of pairwise genetic differentiation (F_ST) between koalas east and west of the Pacific Highway supports these trends. Within each survey year, weak genetic differentiation (F_ST < 0.05) suggests high genetic similarity across both sides of the highway. However, moderate to strong differentiation (F_ST = 0.05–0.25) was observed between survey years, particularly between east sites in 2018 and west sites in 2020, west sites in 2020 and east sites in 2022, and both east and west sites in 2024 compared to all previous years. These temporal shifts in genetic composition are likely driven by episodic environmental disturbances, (such as the wildfires, droughts and floods) as well uneven survival and reproduction, and localised demographic fluctuations.

Over a longer timescale, genetic divergence between koalas on either side of the highway may become more pronounced. Frere et al. (2023) found that a koala population subdivided by a highway could experience a 12–69% loss in genetic diversity over ten generations, with a minimum of eight dispersing individuals per generation required to maintain genetic connectivity. According to the NSW Scientific Committee (2022), koala generation length is estimated at 6–8 years, with wild individuals reaching 15 years for females and 12 years for males. To date, monitoring in Bagotville has not spanned a sufficient timeframe to determine whether genetic isolation is occurring. However, few confirmed crossings under the highway within the Bagotville study areas remains a concern (Sandpiper Ecological 2025), as prolonged habitat fragmentation could contribute to genetic divergence over time.

5.3 Road mortality

The overall 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, 0.29/km in year 5 and remained stable at 0.47/km in year 6 and year 7. 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.

Koala vehicle strikes were recorded in years 1, 2, and 3 on the old Pacific Highway or local roads near the upgrade alignment, as documented by Sandpiper Ecological (2019a, 2019b, 2020). In year 4, a single koala mortality was recorded near the Devils Pulpit rest area, with another individual koala vehicle strike occurring in section 1-11 during year 5. The year 5 road-mortality results in a road-mortality rate of 0.006 individuals/km/year, significantly lower than the 0.026 ind/km/year rate for the old Pacific Highway in S10, as cited by RMS (2016). Notably, year 6 and 7 experienced no koala road mortalities on sections 1-11, suggesting that the highway upgrade has effectively reduced koala vehicle strikes. It is important to note that both mortalities in years 4 and 5 occurred in areas with standard exclusion fencing, which lacked metal sheeting or a floppy top. Additionally, the incident in 2021 happened 200 meters south of a property access, possibly due to a gate left open, allowing koala access to the highway.

1. No injury to an individual koala as a result of vehicle strike across all upgraded sections.

a. No koalas were recorded during the 2023/24 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.

5.4 Fauna exclusion fence.

1. No breaches in fauna exclusion.

a. No breaches within the fauna fence were reported during year the year 7 reporting period.

6 Recommendations

1. An additional 15–20 transects should be surveyed within the Broadwater focal area to improve statistical power, ensuring robust detection of population trends in line with the KMP.
2. Consideration should be given to amending the existing scat collection method to ensure scats are collected from a broader area including around underpasses.

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Appendix A: Population survey koala detections year 7

Table B1: Details of koala observations during year 7 population monitoring in the Broadwater focal area.

Location	Transect	Day/ Night	Location	Tree species	DBH (cm)	Sex	Notes/Condition
Bagotville	N24	Day	Radial	Tallowwood	25	Male	Recorded in same tree as above - male stained rump
Bagotville	N24	Day	Radial	Tallowwood	25	Female	F healthy rump- male stained rump damp
Bagotville	N34	Night	Transect	Tallowwood	60	Male	Healthy eyeshine and rump; initially heard calling and found individual
Bagotville	N19	Night	Transect	Forest red gum	75	Female	Appeared healthy eyeshine- unsure of rump
Bagotville	N20	Night	Incidental	Unknown	No record	Female	Heard female calling off transect- waypoint approximate
Bagotville	N34	Day	Transect	Ironbark spp.	30	Female	Healthy
Bagotville	N73	Night	Transect	Swamp box	50	Female	Healthy eyeshine and rump; joey on back
Bagotville	N23	Night	Transect	Eucalypt spp.	18	Female	Healthy eyeshine and rump- coat; joey on back
Bagotville	N14	Night	Incidental	Swamp mahogany	15	Female	Healthy eyeshine and rump
Bagotville	N09	Night	Incidental	Eucalypt spp.	10	Female	Healthy eyeshine and rump
Bagotville	N36	Night	Transect	Scribbly gum	55	Female	Appeared healthy- slight stain on rump
Bagotville	N30	Night	Radial	Broad-leaved paperbark	30	Male	Healthy eyeshine and rump
Bagotville	N04	Night	Radial	Tallowwood	40	Unknown	Healthy eyeshine
Bagotville	N74	Day	Radial	Forest red gum	65	Female	Good- clear eyes- slight wet bottom
Bagotville	N74	Day	Incidental	Forest red gum	No record	Unknown	Healthy
Bagotville	N14	Night	Transect	Swamp mahogany	30	Unknown	Unknown
Bagotville	N21	Day	Transect	Pink bloodwood	35	Male	Healthy
Bagotville	N10	Night	Incidental	Tallowwood	65	Unknown	Healthy
Bagotville	N74	Night	Transect	Forest red gum	65	Female	Good- clear eyes- slight wet bottom
Bagotville	N73	Night	Transect	Swamp mahogany	50	Unknown	Clear eye shine
Bagotville	N73	Night	Radial	Swamp mahogany	55	Unknown	Clear eye shine
Bagotville	N34	Day	Transect	Tallowwood	55	Female	Healthy
Bagotville	N24	Night	Incidental	Tallowwood	No record	Unknown	Uk
Bagotville	N24	Night	Incidental	No record	No record	Unknown	NR
Bagotville	N19	Night	Transect	Flooded gum	120	Unknown	Healthy
Bagotville	N37	Day	Incidental	Forest red gum	25	Female	Slight brown bum
Bagotville	N08	Night	Transect	Swamp mahogany	12	Unknown	Healthy
Bagotville	N33	Night	Incidental	Swamp mahogany	45	Unknown	Healthy
Bagotville	N33	Night	Incidental	Swamp box	20	Female	Healthy
Bagotville	N23	Night	Incidental	Brushbox	10	Male	Healthy
Bagotville	N08	Day	Transect	White mahogany	25	Unknown	Healthy
Broadwater	S09	Night	Transect	Swamp box	15	Female	Healthy; joey on limb nearby
Broadwater	S05	Night	Incidental	Swamp mahogany	35	Male	Healthy

Broadwater	S44	Night	Incidental	Unknown	20	Unknown	Healthy- individual off radial by 2m
Broadwater	S35	Day	Incidental	Swamp mahogany	32	Female	Healthy
Broadwater	S31	Night	Transect	Swamp mahogany	17	Unknown	Healthy rump and eyeshine
Broadwater	S25	Night	Transect	Swamp mahogany	45	Unknown	Eyeshine bright rump obscured
Broadwater	S15	Night	Incidental	Swamp mahogany	46	Male	Off transect by 5 m
Broadwater	S15	Night	Incidental	Broad-leaved paperbark	25	Male	K1:Uk K2: Eyeshine bright- rump obscured
Broadwater	S15	Day	Radial	Leptospermum spp.	10	Male	Healthy
Broadwater	S19	Day	Incidental	Leptospermum spp.	10	Female	Healthy
Broadwater	S35	Day	Incidental	Swamp mahogany	No record	Female	NR
Broadwater	S15	Night	Radial	Swamp mahogany	35	Male	Healthy
Broadwater	S15	Night	Radial	Swamp mahogany	15	Unknown	Healthy
Broadwater	S15	Night	Incidental	Swamp mahogany	45	Male	Healthy
Broadwater	S15	Night	Incidental	Swamp mahogany	40	Unknown	Healthy
Broadwater	S25	Day	Radial	Swamp mahogany	30	Unknown	Dirty/damp rump
Broadwater	S25	Day	Radial	Swamp mahogany	30	Unknown	Healthy
Broadwater	S41	Night	Transect	Swamp mahogany	40	Unknown	Good
Broadwater	S43	Night	Radial	Leptospermum spp.	10	Unknown	Good
Broadwater	S25	Night	Transect	Swamp mahogany	35	Unknown	Healthy
Broadwater	S09	Day	Incidental	Swamp mahogany	10	Unknown	Healthy; brown belly
Broadwater	S09	Day	Incidental	Swamp mahogany	10	Female	Healthy
Broadwater	S09	Night	Transect	Swamp mahogany	30	Male	Healthy

Appendix B: Year 7 Monitoring Report: Updated Analysis of the W2B Koala Monitoring Programs in Bagotville and Broadwater, NSW, Australia.

By Robert W Rankin, Ph.D.

under contract by Rankin Holdings (1035179 Ontario Inc.)

Background

This report presents an updated Bayesian trend analysis and prospective power analysis for 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). New systematic surveys of koalas by human-observers were conducted during the spring of 2023 and autumn 2024 field seasons (hereafter referred to as "Year 7" data). The re-analyses incorporated data from the Baseline Year through to Year 7, using the totality of data from line-transects and radial search-plots conducted by human observers.

The purpose of the analysis was to evaluate the program's goal of detecting large declines in koala densities. Specifically, the program aims 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. To facilitate this goal, this report used Bayesian count-modelling, trend estimation, and model selection to statistically estimate the change in koala densities over time. To estimate statistical power, a simulation-based power-analysis was conducted using empirical values drawn from the Bayesian MCMC samples of trend and density parameters.

Methods

The Bayesian trend and prospective power analyses were conducted using R (R Core Team, 2016) and JAGS (Plummer 2007). Details about the methodology and Bayesian priors have been thoroughly described in previous reports (Sandpiper Ecological 2020, 2021, 2022, 2023) and will not be discussed further, aside from any changes in the present analyses. Readers are referred to last year's report for more details (Sandpiper Ecological 2023).

Of note, due to the expedited nature of this report, this year's objectives were limited to the Bayesian trend analysis, density estimation, and power analyses. This contrasts with prior years' reports that explored a variety of auxiliary hypotheses, such as estimating inflection points in trends ("change-point analysis"), testing for night-time vs. day-time differences in densities, exploring differences between frequentist AICc-based model-selection vs. Bayesian methods, and more. Such analyses were omitted from this report.

Bayesian Priors on Overdispersion

Previous analyses of koala-counts suggested that the Negative Binomial count distribution had more information-theoretic support as compared to a Poisson distribution (according to AICc model selection). The distinction is important and may impact statistical power. Consider that the Negative Binomial distribution allows for 'overdispersion' by accommodating wider probability tails, but at the cost of dampening systematic variation, including trends (i.e., incurring more Type-II errors). In contrast, the Poisson often underestimates overdispersion and may mistake count-variation for systematic variation like trends (i.e., incurring more Type-I errors). Because the sparse koala data has typically resulted in high amounts of overdispersion (i.e., a low θ parameter in the Negative Binomial process) and low power, it was recommended to update these analyses using a Poisson distribution for koala estimation. However, an external reviewer noted the lack-of-support for Poisson (by AICc) and instead plead to continue using the Negative Binomial.

As a compromise, this year's analysis continued to use the Negative Binomial count distribution (as recommended by the reviewer), but with slightly different priors on the overdispersion parameters. In particular, to gain some of the benefits of the Poisson, while allowing for data-driven overdispersion, if necessary. This set-up involved model selection among a suite of strong and weak priors on the overdispersion parameter, with goals including:

i. allowance for data-driven overdispersion (low θ values) or Poisson-like count variation (high θ values) given enough evidence in the data;

ii. a slight bias to Poisson-like count variation (high θ), if there was no evidence of overdispersion in the data; and

iii. restriction on overdispersion to be at-most 2.5x the variance of a Poisson (i.e., by truncating the permissible values of θ to a minimum of 5).

To achieve this set-up, we used a combination of parameter estimation and model-selection by WAIC (similar to previous years; Watanabe 2010), whereby different model-selection candidates had different prior values of the Gamma shape and rate hyper-parameters that governed the θ overdispersion variable. In addition, we used left-truncating of the Gamma distribution to ensure θ had a minimum value of 5. The candidate shape and rate values were: {(4, 0.08), (40, 0.8), (400, 8), (10000, 200)}.

Notice that: i) each shape-rate pair had an expected θ value of 50 (i.e., a Poisson-like distribution with little overdispersion, where $\mathbb{E}[\theta] \approx \mathrm{shape}/\mathrm{rate}$), and ii) the weak priors (shape=4 and rate=0.08) allowed for data-driven estimation of θ, while the strong priors (shape=10000 and rate=0.08) essentially fixed θ to a value of 50 (resulting in a Poisson-like distribution). Overall, given no evidence in the data, the model-selection bias was for a high value of θ (approximately little to no overdispersion).

This set-up was similar to previous years' set-up (Sandpiper Ecological 2021, 2022, 2023), but the previous rate and shape hyperparameters pairs were: {(5,1), (10,2), (20,4), (40,8), (500,100)}. Each had a expected value of θ=5, implying an overdispersion of 2.5x the variance of a Poisson. This meant that the overall bias was towards overdispersion (assuming no evidence in the data).

In summary, both set-ups allowed for data-driven estimation of θ if there was sufficient evidence in the data to do so. But, the Year 7 Gamma-priors had a bias towards low-overdispersion, while previous years' analyses had a bias towards overdispersion (~2.5x the Poisson variation).

Results

- Observed counts. 12 koalas were observed on-transect at Broadwater (Table 1), which was 1 less than last year, but higher than the counts in Year 5 and the Baseline survey (10 and 8 respectively). 20 koalas were observed at Bagotville, which was the same as in Year 6, and 4 more than observed in Year 5, and 12 more than observed during the Baseline survey.
- Observed densities. The observed density of koalas at Broadwater was 0.052 koala/ha (Table 1), which was 8% lower than the previous year, and down 39.3% since the Baseline (the trend in empirical counts vs. empirical densities differ due to the different total area surveyed in the Baseline as compared to the Year 7 survey, which influences the denominator of the density calculation). At Bagotville, the observed density was

0.091 koala/ha, the second highest in the series, representing a 2% increase from the previous year and up 21% since the Baseline.

- Broadwater statistical densities. The estimated Year 7 population density at Broadwater (pooled for both spring and autumn seasons) was 0.046 koalas/ha (SE: 0.008; CI: 0.032-0.062, see Table 2), nearly the same as the Year 6 estimate, but down 20% from the Baseline estimate (0.057 koalas/ha; SE: 0.011; CI: 0.038-0.081). The strongly overlapping credibility intervals suggest no meaningful trend. Table 3 shows the per-season density estimates as well.
- Bagotville statistical densities. The estimated Year 7 pooled population density at Bagotville was 0.083 koalas/year (SE: 0.012; CI: 0.062-0.108, see Table 2), nearly the same the Year 6 estimate, and an increase of 7% versus the Baseline estimate (0.077 koalas/ha; SE: 0.012; CI: 0.056-0.105). Table 3 shows the per-season density estimates.
- Broadwater trend estimate. The estimated log-linear trend at Broadwater was -1.2 %/year ($\hat{\beta}_t$= -0.012/year; SE: 0.025; 95%CI -0.079-0.021), with a 0.625 posterior probability of a decline (a value close to 0.5 would suggest no trend). This trend is shallower than last year's estimate of -1.8% ($\hat{\beta}_t$=-0.018 year; SE: 0.032; 95%CI -0.097-0.027; 0.668 posterior probability of a decline), suggesting continuing moderation of the trend estimate. For reference, the steepest decline was estimated to be -3.3%/year (-0.033/year; SE: 0.041; 95%CI -0.124-0.023) in the Year 5 report (Sandpiper Ecological, 2022).
- Broadwater trend detection. The trend at Broadwater had a posterior odds ratio (Bayes Factor) of 0.694, providing slight evidence in favour of 'no trend' (a value of 1 is the cut-off between evidence in favour of a trend vs. no-trend). This value was more pronounced than the Year 6 estimate of 1.001; such Bayes Factors have been consistently declining over the past couple of reports, suggesting moderation in the negative trend at Broadwater.
- Bagotville trend estimate. The estimated log-linear trend at Bagotville was 0.4%/year ($\hat{\beta}_t$= 0.004/year; SE: 0.018; 95%CI -0.029-0.052; 0.434 posterior probability of a decline), up slightly from last year's trend estimate of 0.2%/year ($\hat{\beta}_t$= 0.002/year).
- Bagotville trend detection. The trend at Bagotville had a posterior odds ratio 0.537, yielding slight evidence against the existence of a trend.
- Prospective power analysis. For a maximum Type-I error rate of 0.3, the estimated power for Bagotville and Broadwater were 0.728 and 0.650 respectively (Figure 1). The value for Broadwater was up slightly from the Year 6 estimate (0.647) but lower than the Year 5 (0.657) and Year 4 values (0.667). For a maximum Type-I error rate of 0.35, the estimated power at Bagotville and Broadwater were 0.763 and 0.691, respectively.
- Overdispersion estimation and model-selection. The model-averaged value of the overdispersion parameter (θ) was 48.83 (SE: 13.2; 95%CI: 18.7-79.2) with very wide credibility intervals. This value was very close to the expectation of the prior distributions (50), which nominally results in very little overdispersion. However, the top model 3 models by WAIC had Gamma shape and rate hyperparameters of (4, 0.08), (10000,200), and (400, 8), with nearly identical WAIC values of 1739.06, 1739.18, and 1739.22. This suggests a lot of 'multi-

model uncertainty' about what was the best prior distribution for θ, such that it was essentially being driven by our choice of priors, rather than strong evidence in the data.

Conclusions

This expedited report provides updated statistical estimates for the koala population densities and trends in sections 8/9 (Broadwater) and 10 (Bagotville) of the Koala Management Plan (RMS 2016), as well as a prospective power analyses, using data from the Baseline Year through to Year 7.

In nearly all statistical analyses, the estimates from Year 7 analyses are very similar to those from the Year 6 report (Sandpiper Ecological 2023), with the momentum from prior years continuing in the same manner. In other words, there is a very slight negative trend at Broadwater (-1.2 %/year), whose magnitude is dampening from more-extreme values estimated in prior years (Sandpiper Ecological 2021, 2022), and whose statistical confidence is becoming weaker and weaker (i.e., a low and declining Bayes Factor that is now in the range of 1 or below). The estimated koala population density at Broadwater is still 20% below the Baseline statistical estimate. The population at Bagotville seems to have had a slight but insignificant increase.

These statistical results at Broadwater seem to be driven by an underlying koala population that dropped and recovered sometime between Years 1 through 4 and may now be either stabilising or increasing slightly.

Similar to the momentum of trend and density estimates, the prospective power analysis continues to return similar results as the past few annual reports, with findings of adequate power at Bagotville, but slightly inadequate power at Broadwater. This was despite the use of a modified prior on the Negative Binomial overdispersion parameter $\theta \sim \Gamma(a,b)$ which reduced the amount of overdispersion assumed in the priors and theoretically should have increased the statistical power.

The future incorporation of a parallel dataset from drone platforms will likely increase the power in concert with the amount and quality of data. Future analysts may also want to explore a different count distribution, such as the Poisson or a Zero-Inflated Poisson (ZIP) distribution. The latter could be more appropriate than the Negative Binomial in situations where koala-occupancy is more sensitive to local environmental and habitat conditions, whereupon they attain an average density conditional on the local environment. In other words, one could model koala trends as a change in occupancy, whereupon the koalas have some target density that fluctuates around an average value according to a Poisson process.

After 7 years of data collection, the underpowered nature of either a Poisson-like distribution (as in this analysis) or an overdispersed Negative Binomial distribution (as in previous analyses) deserves more creative thinking about the most appropriate statistical model that can explain more heterogeneity in koala counts and distribution.

Table 1: Descriptive Statistics - Observed koala counts and observed densities by year

Location	Year	Koala Counts	Koala Obs Density
Bagotville	Baseline	4	0.075
Bagotville	1	17	0.078
Bagotville	2	18	0.077
Bagotville	3	22	0.094
Bagotville	4	16	0.069
Bagotville	5	16	0.072
Bagotville	6	20	0.089
Bagotville	7	20	0.091
Broadwater	Baseline	8	0.132
Broadwater	1	13	0.055
Broadwater	2	5	0.022
Broadwater	3	15	0.065
Broadwater	4	7	0.030
Broadwater	5	10	0.043
Broadwater	6	13	0.056
Broadwater	7	12	0.052

* Note: the densities are ‘micro average’ (i.e., the sum of counts divided by sum of areas) whereas in previous years we reported ‘macro averages’ (the counts divided by areas per transect, then averaged).

Table 2: Bayesian model-averaged estimates of koala densities, pooled for both spring and autumn seasons.

Year	**Broadwater**	**Bagotville**
Baseline	0.057 (SE: 0.011; CI: 0.038-0.081)	0.077 (SE: 0.012; CI: 0.056-0.105)
Year 1	0.053 (SE: 0.008; CI: 0.040-0.070)	0.078 (SE: 0.009; CI: 0.061-0.098)
Year 2	0.051 (SE: 0.007; CI: 0.039-0.065)	0.079 (SE: 0.008; CI: 0.063-0.096)
Year 3	0.051 (SE: 0.006; CI: 0.040-0.064)	0.080 (SE: 0.008; CI: 0.065-0.096)
Year 4	0.049 (SE: 0.006; CI: 0.038-0.061)	0.080 (SE: 0.008; CI: 0.065-0.097)
Year 5	0.048 (SE: 0.006; CI: 0.037-0.061)	0.081 (SE: 0.009; CI: 0.065-0.099)
Year 6	0.047 (SE: 0.007; CI: 0.034-0.061)	0.083 (SE: 0.010; CI: 0.064-0.104)
Year 7	0.046 (SE: 0.008; CI: 0.032-0.062)	0.083 (SE: 0.012; CI: 0.062-0.108)

Table 3: Bayesian model-averaged estimates of koala densities, for both spring and autumn seasons.

Location	**Broadwater**	**Bagotville**
Baseline	0.054 (SE: 0.010; CI: 0.039-0.077)	0.078 (SE: 0.010; CI: 0.058-0.098)
Year 1 Spring	0.052 (SE: 0.007; CI: 0.039-0.068)	0.079 (SE: 0.009; CI: 0.062-0.097)
Year 1 Autumn	0.052 (SE: 0.007; CI: 0.039-0.068)	0.079 (SE: 0.009; CI: 0.062-0.097)
Year 2 Spring	0.051 (SE: 0.007; CI: 0.039-0.064)	0.079 (SE: 0.008; CI: 0.064-0.096)
Year 2 Autumn	0.051 (SE: 0.006; CI: 0.039-0.064)	0.079 (SE: 0.008; CI: 0.064-0.096)
Year 3 Spring	0.050 (SE: 0.006; CI: 0.039-0.063)	0.080 (SE: 0.008; CI: 0.065-0.097)
Year 3 Autumn	0.050 (SE: 0.006; CI: 0.039-0.063)	0.080 (SE: 0.008; CI: 0.064-0.096)
Year 4 Spring	0.049 (SE: 0.006; CI: 0.038-0.062)	0.080 (SE: 0.008; CI: 0.065-0.097)
Year 4 Autumn	0.049 (SE: 0.006; CI: 0.038-0.062)	0.080 (SE: 0.008; CI: 0.064-0.097)
Year 5 Spring	0.049 (SE: 0.006; CI: 0.037-0.062)	0.080 (SE: 0.008; CI: 0.065-0.098)
Year 5 Autumn	0.049 (SE: 0.006; CI: 0.037-0.062)	0.080 (SE: 0.008; CI: 0.064-0.097)
Year 6 Spring	0.048 (SE: 0.007; CI: 0.036-0.062)	0.081 (SE: 0.009; CI: 0.064-0.100)
Year 6 Autumn	0.048 (SE: 0.007; CI: 0.036-0.062)	0.081 (SE: 0.009; CI: 0.064-0.100)
Year 7 Spring	0.048 (SE: 0.007; CI: 0.033-0.062)	0.081 (SE: 0.010; CI: 0.064-0.104)
Year 7 Autumn	0.047 (SE: 0.007; CI: 0.034-0.062)	0.081 (SE: 0.010; CI: 0.063-0.104)

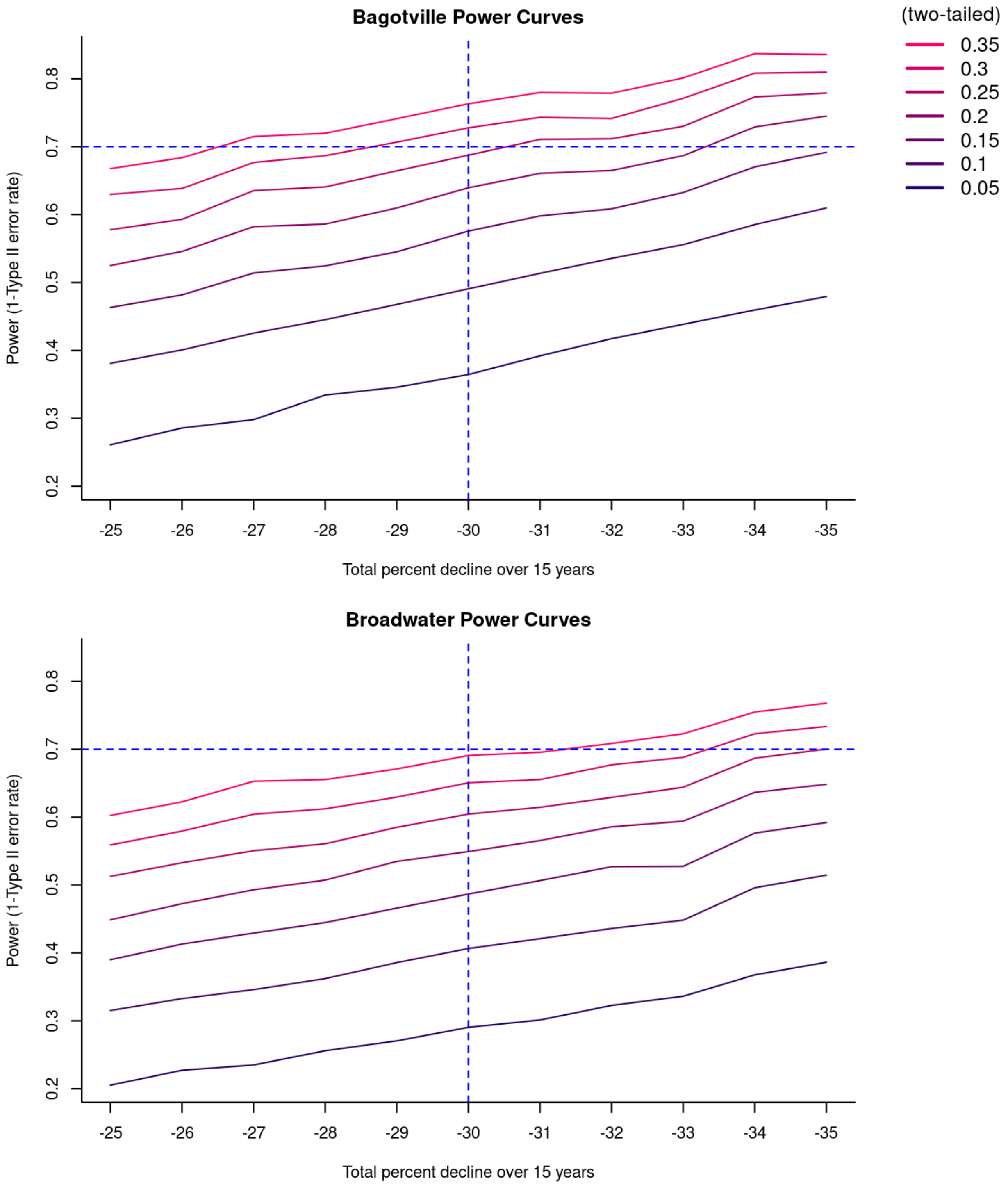

Figure 26: Statistical power to detect a -30% drop in baseline densities vs. Year 15 of the monitoring program, for different maximum levels of Type-I errors (lines)

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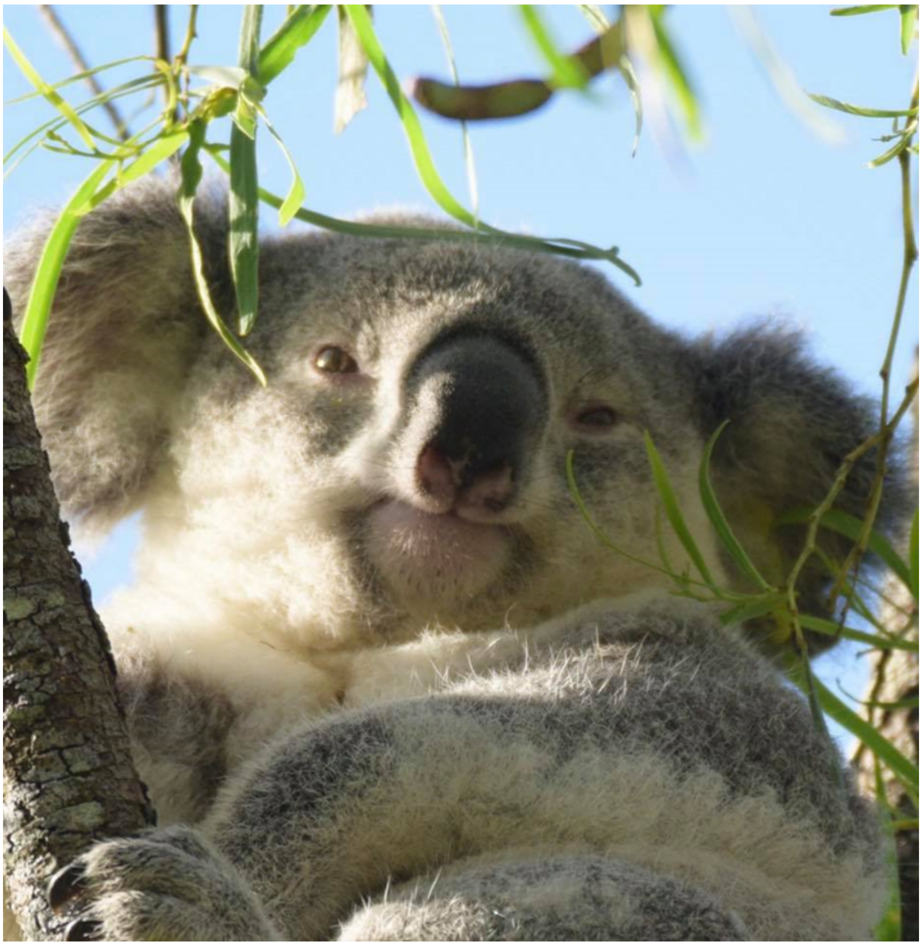
Appendix C – Genetics report

Final Report

Meta-Population Koala Genetics of Northern New South Wales

Prepared for Sandpiper Ecological Surveys Pty Ltd

January 2025



By Dr Lyndal Hulse BAppSc MScAg PhD

EXECUTIVE SUMMARY

This report presents the findings of a study into koala population genetics for 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 between 2018 - 2024. 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 based on 32 microsatellite genetic markers.

Analysis of repeated genotypes from scat samples collected in 2018, 2020, 2022, and 2024 identified duplicates with highly similar multilocus genotypes, confirming the presence of five unique individuals at the survey site from 2018 to 2022. However, these five individuals were not detected in the 2024 cohort.

Genetic analysis of the sampled population reveals an increase in diversity of the population, compared with previous genetic analysis surveys of the same site, with the 2024 population showing moderate to high genetic diversity with a gain of alleles over time. Inbreeding value of the population has decreased since the previous site survey in 2022, indicating geneflow is occurring.

Genetic differentiation between koala populations east and west of the A1 Pacific Highway was weak, suggesting ongoing gene flow and indicating that the highway has not significantly impeded movement. However, moderate to strong genetic differentiation was observed between the east and west sites in all survey years, suggesting changes in genetic diversity over time. This may

indicate a gradual reduction in gene flow between the subpopulations. If the trend of increasing genetic differentiation continues, it could lead to further declines in gene flow, ultimately suggesting that the Pacific Highway may act as a barrier to genetic exchange.

Genetic sub-structuring analysis identified two genetic clusters in the 2024, 2022, and 2018 surveys (K = 2), while the 2020 survey showed three clusters (K = 3), suggesting gene flow within the population. However, the 2022 and 2024 surveys indicate a reduction in the additional 2020 cluster. The disappearance of a genetic cluster over a short period is uncommon but can occur due to factors such as population decline from habitat loss, disease, predation, or anthropogenic pressures. Genetic drift, population bottlenecks, interbreeding, and sampling bias may also contribute to the observed loss or reduction of a genetic cluster.

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INTRODUCTION

Background

Koala faecal scat samples (N = 22) were provided by Sandpiper Ecological Surveys, collected between May and July 2024 from transects at a survey site in northern New South Wales. Genotype data from these samples were combined with population genetic data derived from scats collected in the same area during surveys conducted in 2018, 2020, and 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.

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.

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 January – May, 2018, November 2019 - May 2020, November 2021 – July 2022 and May – July 2024.

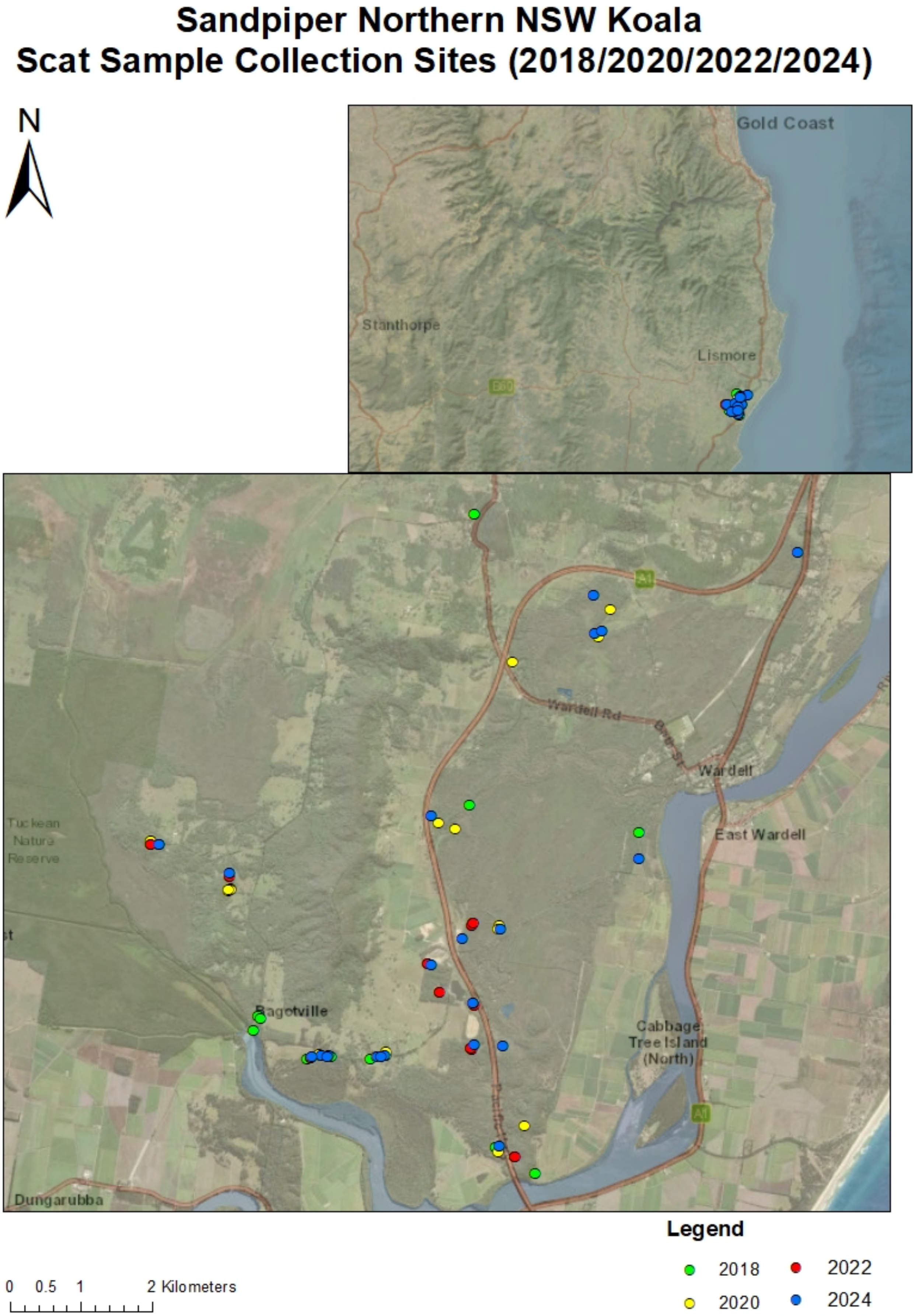

Figure 27 Northern NSW koala survey site with koala scat collection sites for 2018, 2020, 2022 and 2024.

SCAT ANALYSIS METHODOLOGY

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.

DNA Isolation

Genomic DNA was isolated using the QIAamp Fast DNA Stool Mini kit (QIAGEN) according to manufacturer's instructions. Each DNA isolate was tested for quality and concentration using spectrophotometry (Nanodrop, ThermoFisher Scientific, VIC, Australia). The successful isolation of koala genomic DNA, specifically *Phascolarctos cinereus* beta-actin mRNA from epithelial cells exfoliated onto the faecal scat surface, was confirmed using real-time PCR (Hulse et al., 2018). All faecal scats collected in 2024 were verified to contain koala genomic DNA.

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.

GENETIC ANALYSIS

Genotypes and Samples

Faecal scat DNA samples that confirmed positive for presence of koala DNA (PCR detection of koala beta-actin gene) were genotyped for 32 koala-specific microsatellite markers, providing a unique DNA profile per sample. There were no departures from Hardy Weinberg Equilibrium from the population, therefore a total of 32 loci were retained for analysis. This allowed for the identification of distinct individuals with a high degree of confidence (the probability that two individuals would share the same DNA profile by chance is less than 1 in 1,000,000,000). Analysis of repeated genotypes within the total dataset (scats collected during 2018, 2020, 2022 and 2024 surveys) to identify duplicate samples revealed samples with highly similar multilocus genotypes, suggesting these scats are from the same individual koala. Detection of repeated genotypes within the dataset to identify duplicate samples was performed using the software GENALEX version 6.5 (Peakall & Smouse, 2006). This analysis identified five individuals that remained within the survey site from 2018 to 2022, as presented in Table 1. Duplicate genotypes from individuals have been removed from the dataset to prevent bias and ensure accurate genetic analysis.

Table 9 Identification of unique individual koalas based on highly similar multilocus genotypes.

Individual #	Sample ID	Scat Collection Date
1	SF_K11	14/05/2018
	BT_K01	25/11/2019
	BT_K7	22/04/2020
2	SF_K05	10/12/2019
	BT_K9	22/04/2020
	NSW3	20/05/2022
3	SF_K7	11/12/2019
	BT_K8	22/04/2020
4	SF_K02	09/12/2019
	SF_K08	11/12/2019
5	BB_K01	16/05/2018
	SF-K04	10/12/2019
	NSW8	18/07/2022

Distribution and movement of koalas

Using genetic analysis, we were able to identify individual koalas, enabling us to track their movements over time. Figure 2 illustrates the distribution and movement patterns of five koalas during the survey period (2018–2024). Notably, most individuals did not cross the Pacific Highway. The analysis also indicates that Individual 1 dispersed southward from its original detection site, suggesting it may have been a sub-adult seeking to establish a home range; or due to translocation as part of an animal management decision.

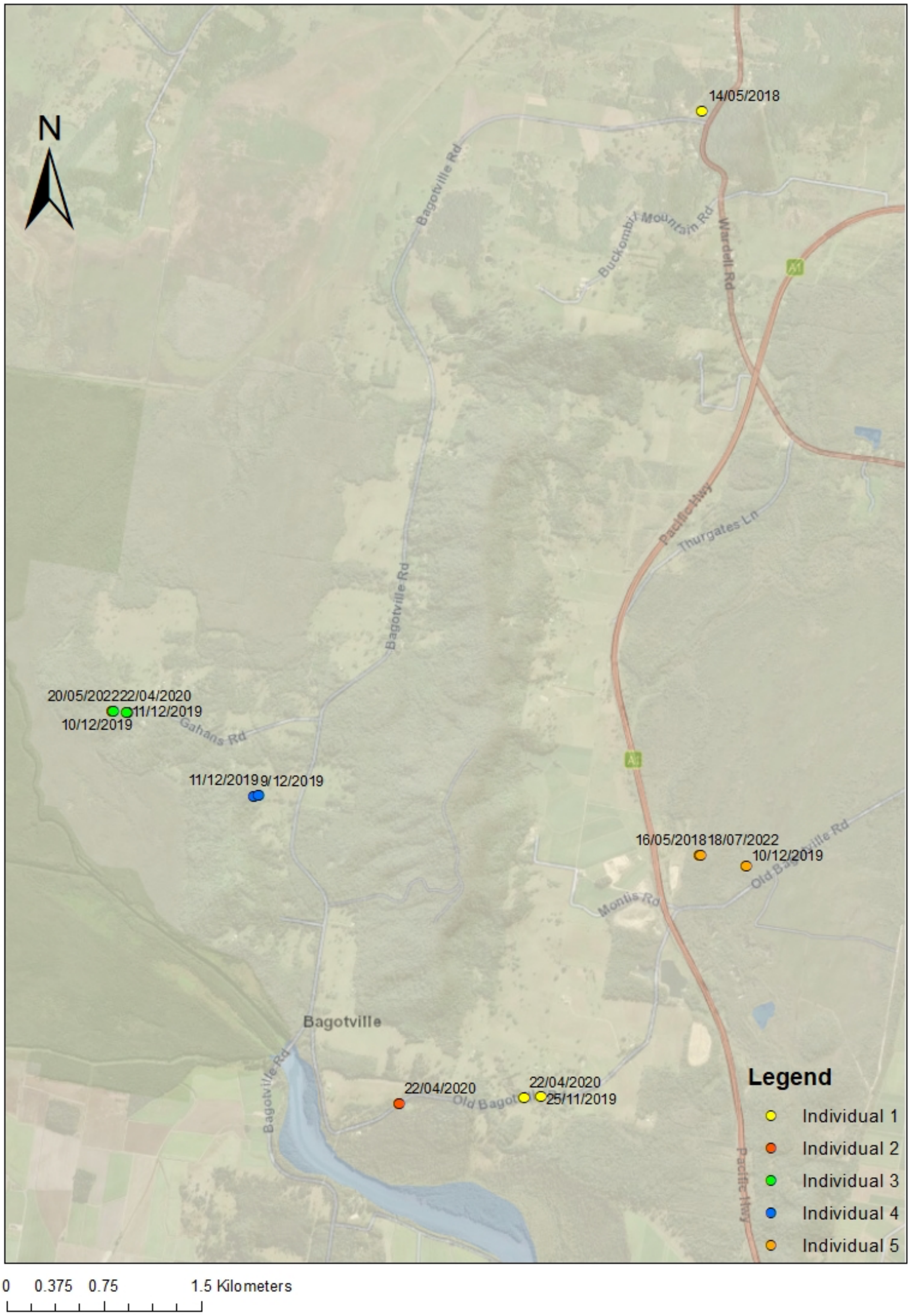

Figure 28 Distribution and movement of koalas over the survey period: 2018 - 2024. Each coloured circle denotes an individual koala.

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. An illustrative guide to classifying heterozygosity values may be defined as: Low Heterozygosity: 0.00–0.33; Moderate Heterozygosity: 0.34–0.66; and High Heterozygosity: 0.67–1.00.

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 GENALEX version 6.5 (Peakall & Smouse, 2006) 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, assessed through expected heterozygosity and allelic richness, was compared across the 2018, 2020, 2022, and 2024 surveys (Table 2). The analysis indicates that the local koala population has maintained low to moderate genetic diversity throughout this period. Notably, the 2024 survey shows an increase in allelic richness, reflected in a higher mean number of alleles per locus compared to the 2022 survey. This suggests that gene flow may be occurring within the population. The inbreeding coefficient (F_{IS}) values across the surveys ranged from 0.114 in 2020 to 0.205 in 2022, with a moderate value of 0.165 recorded in 2024. While the 2024 inbreeding coefficient represents a slight reduction compared to 2022, it still indicates a notable level of non-random mating and potential inbreeding within the population. This suggests the population may be experiencing genetic isolation, likely due to habitat fragmentation or limited gene flow. Although the decrease in F_{IS} by 2024 suggests some improvement, it remains critical to address these issues to prevent further accumulation of inbreeding. High inbreeding coefficients can reduce genetic diversity and increase the prevalence of deleterious alleles, potentially leading to inbreeding depression. For this koala population, such genetic constraints could compromise their resilience to environmental changes, disease, and other stressors. Conservation strategies should focus on increasing habitat connectivity to promote gene flow and mitigate inbreeding, ensuring the long-term genetic health and viability of the population.

The expected heterozygosity (He) values showed a consistent increase over the survey period, rising from 0.672 in 2018 to 0.788 in 2024. This upward trend suggests an improvement in the genetic diversity potential of the population, as higher He values indicate greater variability in the gene pool. Such diversity is critical for the population’s ability to adapt to environmental changes and resist diseases. However, while the increase in expected heterozygosity is a positive sign, the presence of high inbreeding coefficients suggests that this potential genetic diversity may not be fully realized within the population.

Individual heterozygosity has remained relatively stable across surveys, with no significant deviations from previous analyses. In the 2024 survey, individual heterozygosity ranged from 77.4% to 57.1%, with a median value of 67.2%. This marks an increase compared to the 2022 survey, where heterozygosity ranged from 78.0% to 37.5%, with a median of 56.3%. Similarly, in 2020, heterozygosity values ranged from 80.6% to 41.9%, with a median of 60.0%, while the 2018 survey showed a range of 75.0% to 35.5%, with a median of 53.1%. These findings indicate a gradual improvement in the median heterozygosity values over time, suggesting a potential increase in the genetic diversity within the population. Figures 3 - 6 presents the frequency distribution of heterozygosity of individual scat samples collected in 2024, 2022, 2020 and 2018, respectively.

Table 10 Genetic diversity statistics representing 2018, 2020, 2022 and 2024 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
2024 Survey	22	7.72	5.11	0.165	0.654	0.788

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 29 Frequency distribution of heterozygosity of individual scat samples collected in 2024.

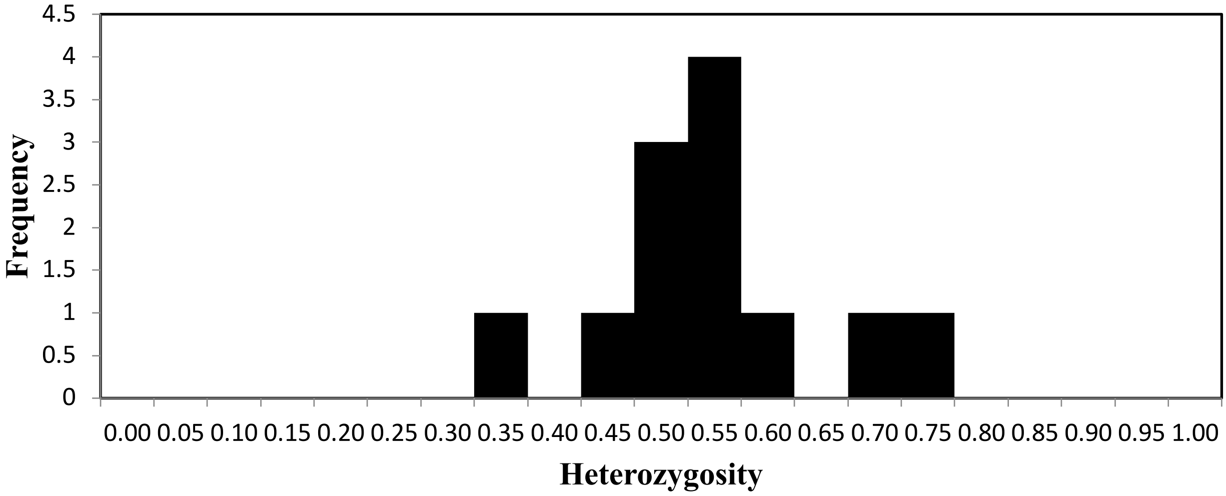

Figure 30 Frequency distribution of heterozygosity of individual scat samples collected in 2022.

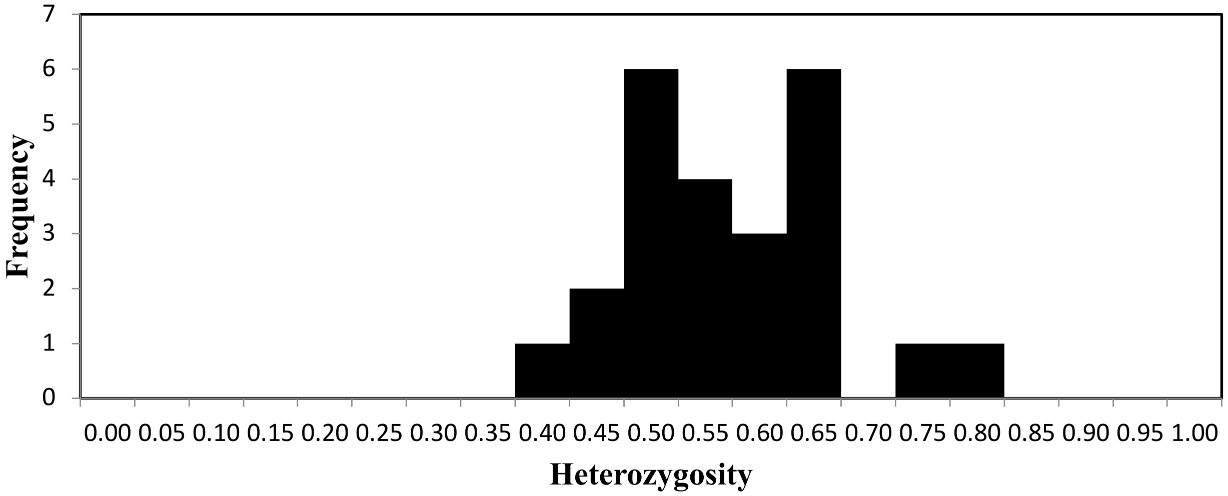

Figure 31 Frequency distribution of heterozygosity of individual scat samples collected in 2020.

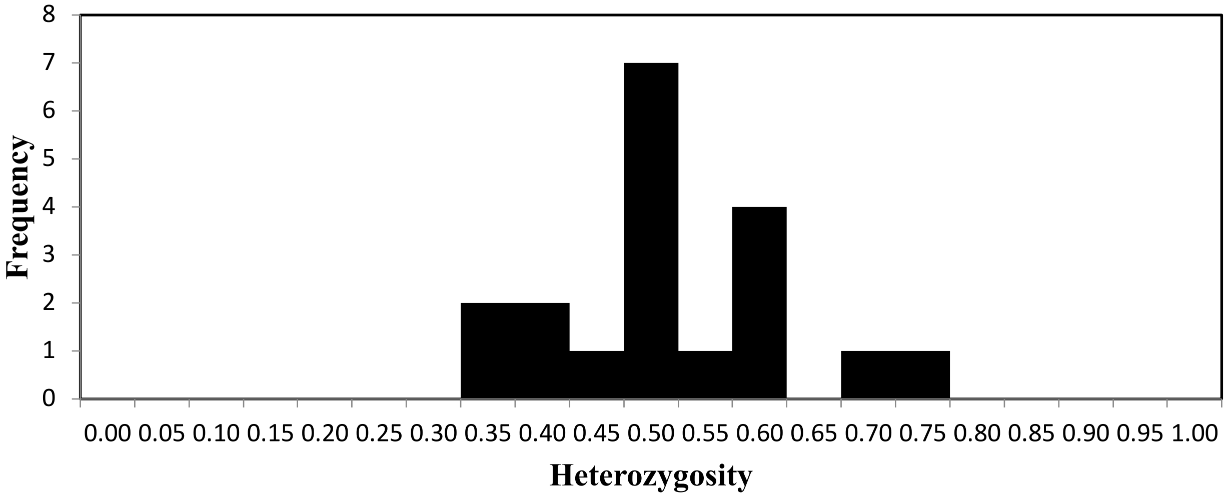

Figure 32 Frequency distribution of heterozygosity of individual scat samples collected in 2018.

Pairwise Genetic Differentiation (F_{ST})

Restrictions to gene flow among populations results in a genetic differentiation or divergence of the populations. Genetic differentiation (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; and values 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, 2022 and 2024, 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 the Pacific Highway has not posed a barrier for koala gene flow. However, there is moderate to strong genetic differentiation between both the east and west sites in 2018 and 2020 and 2024; and 2020 and 2022 and 2024 (highlighted yellow in Table 3). These findings suggest a change in genetic diversity over time, potentially indicating that the Pacific Highway may be contributing to a reduction in gene flow between the east and west koala sub-populations. If F_{ST} between the east and west koala populations continues to rise in future survey years, leading to greater genetic differentiation, it would suggest that the Pacific Highway may be acting as a barrier to gene flow between these populations.

Table 11 Pairwise F_{ST} values between 2024, 2022, 2020 and 2018 northern NSW koala surveys

	est of Pacific Highway
East of Pacific Highway	0.0148

	est of Pacific Highway 2018	st of Pacific Highway 2020	est of Pacific Highway 2020	st of Pacific Highway 2022	est of Pacific Highway 2022	st of Pacific Highway 2024	est of Pacific Highway 2024
East of Pacific Highway 2018	0.0366	0.0038	0.0871	0.0000	0.0224	0.0948	0.0794
West of Pacific Highway 2018		0.0151	0.0115	0.0365	0.0000	0.1580	0.1279
East of Pacific Highway 2020			0.0383	0.0064	0.0186	0.1224	0.1057
West of Pacific Highway 2020				0.0860	0.0346	0.1883	0.1583
East of Pacific Highway 2022					0.0000	0.0947	0.0639
West of Pacific Highway 2022						0.1176	0.0931
East of Pacific Highway 2024							0.0056

<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

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 & Smouse, 2006) using the Queller and Goodnight estimator of relatedness (Queller & Goodnight, 1989).

Genetic relatedness was estimated and compared across the 2018, 2020, 2022, and 2024 northern NSW koala populations. Figure 7 presents the average relatedness for each survey, showing a narrow distribution of relatedness values for koalas identified in the 2024 survey compared to the 2018, 2020, and 2022 surveys. Notably, the 2020 and 2024 surveys show a mean relatedness that exceeds the confidence interval, indicating that koalas in these years are significantly more closely related than expected. This may reflect an increase in familial relationships or a reduction in new genetic input, which could have implications for the population's long-term genetic health and adaptability.

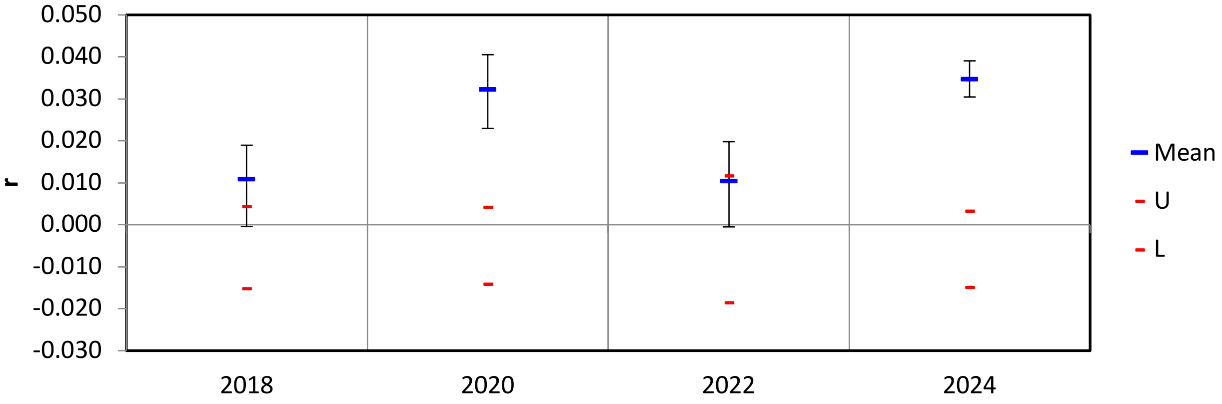

Figure 33 Mean genetic relatedness (r) for 2018, 2020, 2022 and 2024 koala site surveys.

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.

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 the 2024, 2022, and 2018 surveys, with the surveys comparable to each other (K = 2, Figures 8, 10 and 11, respectively) compared to genetic clusters at the 2020 survey site (K = 3, Figure 9). The identification of an additional genetic cluster at the 2020 survey site provides evidence of gene flow within the population. However, the 2022 and 2024 surveys reveal a reduction in this genetic cluster. The disappearance of a genetic cluster over a short period (e.g., two years) is uncommon but possible, depending on various biological and environmental factors. One potential cause is population decline, where a genetic cluster may vanish if individuals carrying those genetic signatures experience a sharp decline due to habitat loss, disease, predation, or anthropogenic pressures such as road mortality and land clearing. In small populations, genetic drift can also contribute to the loss of certain genetic clusters by chance. For example, a population bottleneck caused by events like drought or fire could eliminate specific genetic lineages. Additionally, extensive interbreeding between a distinct genetic cluster and another population may dilute unique genetic signatures, preventing them from forming a recognizable cluster in genetic analyses. Sampling bias is another important consideration, as changes in sample collection—such as reduced sampling from a particular area could create the false impression that a genetic cluster has disappeared when it is merely underrepresented.

Figure 12 A-D shows the location of each scat sample, represented by a pie chart that illustrates the proportional assignment of each individual to the genetic clusters identified in the STRUCTURE analysis.

K = 2

Figure 34 Population substructure of 2024 northern NSW koala populations using STRUCTURE based on 32 loci.

K = 2

Figure 35 Population substructure of 2022 northern NSW koala populations using STRUCTURE based on 32 loci.

K = 3

Figure 36 Population substructure of 2020 northern NSW koala populations using STRUCTURE based on 32 loci.

K = 2

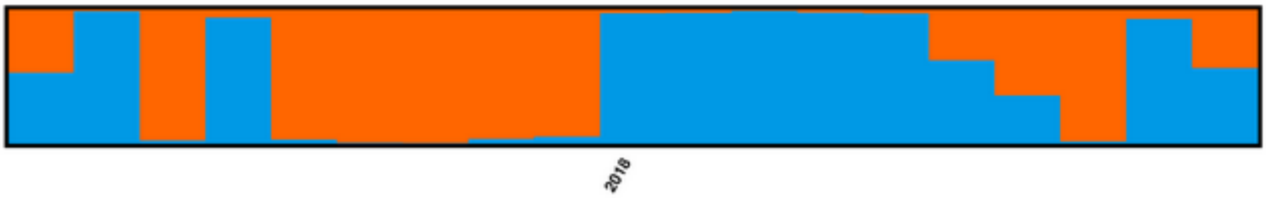

Figure 37 Population substructure of 2018 northern NSW koala populations using STRUCTURE based on 32 loci.

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

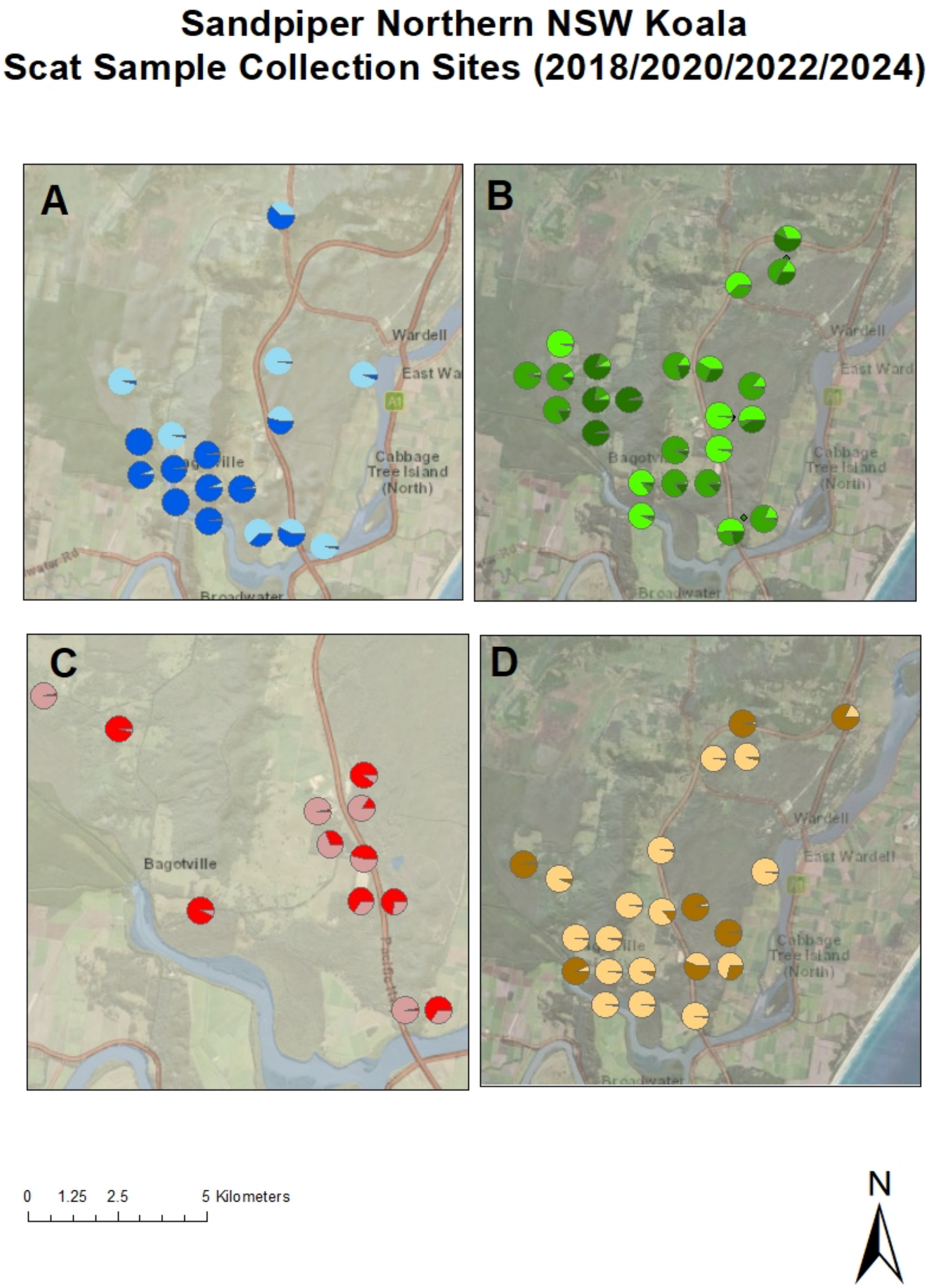

Figure 38 Inferred cluster assignments of (A) 2018 (K = 2); (B) 2020 (K = 3); (C) 2022 (K = 2) and (D) 2024 (K = 2) northern NSW koalas.

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 8 - 11), where clusters are shown by proportion of colour.

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). N_e was estimated using the molecular co-ancestry method of Nomura (2008), as implemented in NeEstimator V2.1 (Do et al., 2014).

Table 4 presents effective population size estimates of males and females from 2018, 2020, 2022 and 2024 koala surveys. Based on the sample sizes for both male and female koalas for all survey years, the confidence intervals for females in 2018, 2020 and 2024 are not notably wide indicating the N_e value is informative enough to predict the effective population size. However, the 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 is likely 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 12 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
2024	Female	17	19.6	16.9	22.3	-0.257
	Male	5	∞	13.4	∞	-0.059

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 D - Road mortality surveys

Table C1: Details of road mortality surveys within sections 3-11 of the Woolgoolga to Ballina Highway upgrade during August and November 2023.