2020 PORT STEPHENS KOALA POPULATION STUDY REPORT

Prepared by OWAD Environment
in collaboration with
WildDNA | Federation University Australia

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Project Summary: This report presents key results of investigations into the population structure, genetic diversity and disease status of Koalas sampled in the Port Stephens region of New South Wales. Individuals were non-invasively sampled by OWAD Environment in the region between November 2018 and May 2020. Genetic material was extracted from scats and analysed by WildDNA | Federation University Australia.

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Signed on behalf of OWAD Environment

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Date: 19 January 2021

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TERMS, ABBREVIATIONS AND DEFINITIONS

Allele	Each of two or more alternative forms of a gene that arise by mutation and are found at the same place on a chromosome. **Private alleles** are alleles that are found only in a single population among a broader collection of populations.
Chromosome	A thread-like strand of DNA that carries genes.
C. pecorum	*Chlamydia pecorum* is a bacterium from the family Chlamydiaceae. Chlamydiosis is considered the most important infectious disease of Koalas as it is the most common and the most pathogenic *Chlamydia* species infecting Koalas. In the Koala, *C. pecorum* can cause urinary tract disease, reproductive disease, infertility and death.
DNA	DNA (deoxyribonucleic acid) encodes genetic information. A DNA molecule is made up of a great number of smaller molecules called nucleotides. DNA governs the production of proteins and other molecules essential to cell function. Mammals generally have two sets of DNA, one half of the two sets is inherited from the individual's mother and the other from the father.
Epithelial cells	Epithelial cells are a type of cell that lines the surfaces of your body. They are found on your skin, blood vessels, urinary tract, gastrointestinal tract and organs.
Gene	The basic physical and functional unit of heredity. Genes are made up of DNA and act as instructions to make protein molecules.
Gene flow	The transfer of genetic material between groups of individuals.
Genotype	The genetic makeup of an organism or group of organisms with reference to a single trait, set of traits, or an entire complex of traits.
Haplotype	A group of alleles in an organism that are inherited together from a single parent.
KoRV-A	Koala retrovirus subtype A (the endogenous form). KoRV has been implicated in immunodeficiency disorders that leaves infected Koalas more susceptible to infectious disease and cancers. It is thought to be a recently introduced exogenous virus that is integrating into the Koala genome (becoming endogenous). The virus can be transmitted both horizontally (from animal to animal in the classic sense) and vertically (from parent to offspring as a gene).
LGA	Local Government Area
Locus	A locus (plural loci) is a fixed position on a chromosome, like the position of a gene or genetic marker (e.g. microsatellite).
Microsatellite	A stretch of short repeated DNA sequence at a particular position (locus) on a chromosome, that is passed down to an offspring by both parents equally. Microsatellites vary in the number of repeats within different individuals and can therefore be used for identifying unique individuals.
Mitochondrial DNA	Mitochondrial DNA, or mtDNA, is genetic material that is only maternally inherited. A copy of mtDNA is passed down from a mother to their offspring entirely unchanged; males cannot pass their mtDNA to their offspring although they inherit a copy of it from their mother. Analysing mtDNA informs on the long-term structure of a population, hence informs on its evolutionary history.
Pairwise relatedness	Estimates of relatedness between pairs of individuals using the dyadic maximum likelihood indicator. First degree relatives include an individual's offspring, parents or full siblings. Second degree relatives include grandparents, grandchildren, aunts/uncles, nephews/nieces or half-siblings.
Phylogenetic	Relating to the evolutionary development and diversification of a species.

1.0 EXECUTIVE SUMMARY

This report presents the key Koala genetics results from work conducted by OWAD Environment and WildDNA│Federation University Australia in the Port Stephens region of New South Wales between November 2018 and May 2020.

Koala genetic material was sourced non-invasively from Koala scats (faecal pellets) found by OWAD Environment's purpose-bred professional field detection dogs. The use of scat detection dogs allows vast areas to be quickly and effectively surveyed while concurrently sampling Koala DNA for population studies without causing stress or disturbance to free-ranging Koalas. Scats were also collected from several Koalas in care at the Port Stephens Koala Hospital for the genetic component of this study.

OWAD's detection dogs searched for evidence of Koala presence (scats, pap or live individuals) for a total of 137 km within Port Stephens LGA over the course of three rapid sampling events conducted in November 2018, July 2019 and May 2020. Koala presence was detected on all lands assessed within Port Stephens LGA except for Boomerang Park (Raymond Terrace, assessed in July 2019), the lands assessed in Medowie (May 2020) and the Campvale drain easement (assessed in May 2020).

Across the three sampling events, the detection dogs found naturally deposited Koala scats or pap at a total of 278 locations, of which 51 were collected by OWAD and submitted to WildDNA for genetic analysis. Additionally, OWAD collected nine scat samples from captive individuals in care at the Port Stephens Koala Hospital. Where sufficient DNA was available for analysis, samples were tested for the presence of KoRV-A and *Chlamydia pecorum*. 45 of the 60 samples (or 75%) provided a reliable DNA profile. DNA profile-matching revealed that the 45 profiles originate from 39 unique individuals, allowing preliminary assessment of population structure, connectivity and diversity within Port Stephens LGA. KoRV-A was detected in 100% of individuals (39/39), while *C. pecorum* was detected in 36% of individuals (14/39).

Phylogenetic analysis indicated that Koalas sampled in this study were historically connected. Contemporary population genetic analysis, however, indicates that Koalas from the Tilligerry and Tomaree Peninsulas (referred to as 'the peninsula') are now significantly different from those sampled further inland, suggesting that gene flow between peninsula and inland Koalas has been restricted over recent generations. Peninsula Koalas were also found to be less genetically diverse than inland Koalas, suggesting that peninsula Koalas may be losing genetic diversity due to a lack of successful migration from outside of the peninsula. Furthermore, fine-scale differentiation was detected in the inland group where three distinct genetic clusters were identified, suggesting that Koala movements are being restricted broadly throughout the region.

This study also identified an apparent decrease in Koala activity in two parklands which were sampled twice (Mambo Wetlands and Stoney Ridge Reserve, sampled in July 2019 and May 2020). This may suggest that Koalas in this part of the peninsula may have experienced a rapid decline between the two sampling events.

Future management of Koalas in the Port Stephens LGA should consider the impact habitat fragmentation is having on the population. Removal of key Koala habitat within the region has resulted in the genetic differentiation of Koalas on the peninsula from those inland. This study also suggests that Koala groups inland are also isolated from each other. Reconnecting Koalas within the region is critical to conserve genetic diversity, which underpins the health and viability of the population. Such measures should therefore be implemented as a priority.

2.0 ACKNOWLEDGEMENTS

WWF-Australia is thanked for providing funding assistance to enable the collection and analysis of further Koala genetic material in the Port Stephens region, and the preparation of this report.

Port Stephens Council and NSW Parks and Wildlife are thanked for facilitating access to public lands for this study, and for their active assistance during fieldwork to ensure the safety of the survey team.

The Port Stephens Koala Hospital is thanked for allowing the sampling of Koalas in their expert care, as well as for providing the rescue location of each Koala sampled.

The Hunter Water Corporation is thanked for allowing access to and assessment of lands around Grahamstown reservoir.

PM No1 Pty Ltd is acknowledged for its significant contribution in facilitating this study, by sharing biodiversity information and providing access to the Balickera site for investigations. PM No1 Pty Ltd initiated the investigation of Koala population structure in the Port Stephens region (i.e. collection and analysis of a significant portion of the Koala genetic material included in this study). Mark Aitkens from RPS Australia, engaged by PM No1 Pty Ltd, is thanked for arranging access to associated sampling sites at Karuah, Ferodale and several locations on the Tomaree and Tilligerry Peninsulas.

3.0 INTRODUCTION

3.1 PURPOSE OF THIS REPORT

This report presents the main Koala population survey and genetic structure results for the Port Stephens region of New South Wales, derived from a combination of several non-invasive Koala genetics sampling studies performed by OWAD Environment (OWAD) in the Port Stephens Local Government Area (LGA) in collaboration with WildDNA | Federation University Australia (WildDNA).

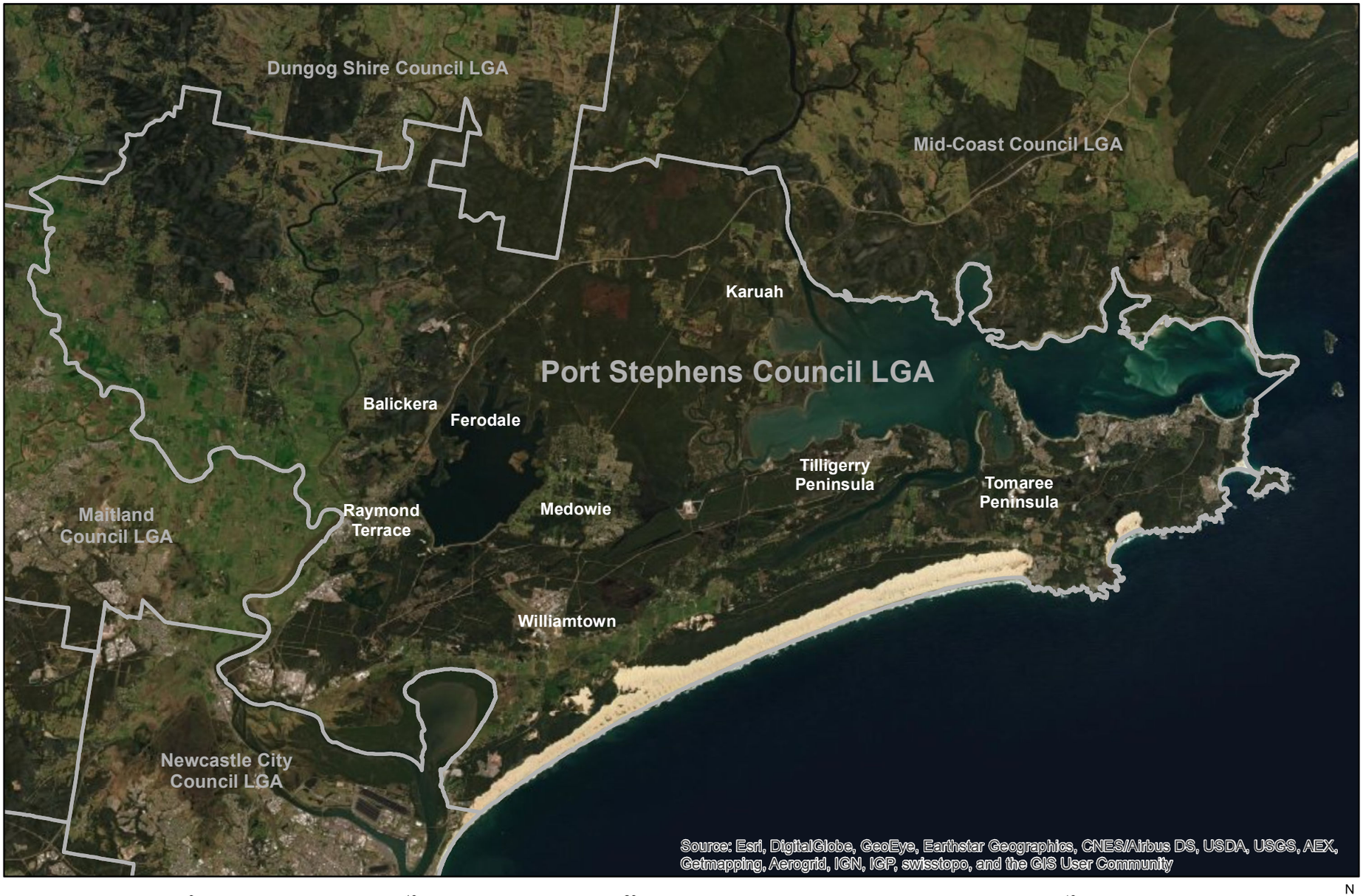
3.2 STUDY AREA

The study area is the LGA of Port Stephens (see **Figure 1**) which spans 979km^2 in the Hunter Region of New South Wales, Australia. It is located north of Newcastle and is adjacent to the Pacific Highway which runs through Raymond Terrace, the largest town in the LGA.

Lands surveyed within the study area can be classed into eight geographic locations:

- Tomaree Peninsula
- Tilligerry Peninsula
- Balickera
- Ferodale
- Karuah
- Medowie/Campvale
- Williamtown
- Raymond Terrace

The Tomaree Peninsula and Tilligerry Peninsula (referred to jointly as 'the peninsula') are situated in the east of the Port Stephens LGA where Koala habitat remained largely intact up until the 1940s, after which time removal of Koala habitat proceeded rapidly due to urbanisation (Knott *et al*. 1998). Land to the east of the peninsula was largely cleared throughout the 1800s (Knott *et al*. 1998), potentially reducing Koala movements from the peninsula to habitat patches further inland. In Karuah, north of the Tilligerry Peninsula, habitat connecting Karuah and Tilligerry is highly fragmented (Knott *et al*. 1998). Ferodale is bounded by the Grahamstown Dam to the east and the Pacific Highway to the west. Lands sampled in Balickera are located immediately west of the Pacific Highway and east of the Williams River, where Koala habitat is highly fragmented (Knott *et al*. 1998).



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LEGEND

Local Government Area boundaries

FIGURE 1: STUDY AREA

2020 Port Stephens Koala population study

OWAD ENVIRONMENT

4.0 FIELD SURVEY METHODOLOGY

This report utilises Koala genetic profiles obtained via several studies and several sampling events. Fieldwork for these studies was performed between November 2018 and May 2020.

4.1 SITE SELECTION AND SAMPLING DESIGNS

Each study had its own criteria for site selection. The lands sampled in these studies included private lands, Council lands, State Government lands, Council managed Crown land, and Hunter Water Corporation lands. A variety of sampling designs were used in these studies, included both probability and non-probability sampling techniques. Some sampling events utilised systematic sampling, some utilised targeted sampling, while others utilised convenience sampling and opportunistic sampling.

4.2 FIELD ASSESSMENT

4.2.1 Survey team, certifications and permits

The field assessments were conducted by OWAD Environment which includes Olivia Woosnam (senior Koala ecologist, Certified Environmental Practitioner, certified detection dog handler), Alex Dudkowski (field ecologist, Certified Environmental Practitioner, certified detection dog handler) and their two purpose-bred professional detection dogs certified for the detection of Koala scats. The dogs are Working English Springer Spaniels Wrangham Pink Knockout (aka 'Taz', famous for being Australia's very first certified Koala detection dog in 2015) and her cousin Wrangham Mistral Bowscale (aka 'Missy'). In the last five years, OWAD's detection dogs have searched for evidence of Koala presence over a total of more than 5,000km on applied Koala studies across Queensland and New South Wales. OWAD has to date submitted over 4,000 scats found by their detection dogs to WildDNA for testing, and to date 100% of scats have been confirmed as originating from Koala.

This field assessments were conducted under OWAD's Scientific Licence number SL101634 (issued by the NSW Government Department of Planning, Industry and Environment) and OWAD's Animal Research Authority and Animal Care and Ethics Committee Certificate of Approval number TRIM 18/567 (issued by the NSW Government Department of Primary Industries) for "Targeted fauna & flora species surveys using professional detection dogs".

4.2.2 Detection dog searches

One detection dog was handled at a time. The dog was led out of the work vehicle on leash. Once ready to begin searching, the dog was taken off the leash and when prompted by the handler, scanned the ground layer for Koala scats as well as above the ground for scats that may be above ground level (e.g. scats fallen on rocks, logs, bushes, or stuck in branches or behind bark along tree trunks).

The dogs worked independently and searched non-discriminatorily, following their trained search pattern. They were purposely not directed to any specific trees or tree species. The handler only gave the dog the initial general direction of the search. During searches, the dog was redirected, recalled or stopped at a distance using an Acme dog whistle, as required to keep the dog within the target lands or for safety reasons (e.g. to keep the dog safely away from traffic, stop the dog when encountering park users, prevent the dog from running into barbed wire, keep the dog away

from macropod mobs so as to not disturb them, etc.). The handler kept the dog within immediate sight at all times.

In order to minimise the risk of data loss in case of handheld GPS unit malfunction, the study team recorded the coordinates of all Koala scats found with two handheld GPS units (models Garmin Alpha 100 and Garmin GPSMap78). The detection dogs' search tracks were recorded with two Garmin T5 dog tracking collars (one for each dog) paired with the Alpha 320 unit and recorded the detection dogs' search tracks at a rate of one waypoint every 2.5 seconds. In order to further minimise the risk of data loss in case of equipment malfunction, OWAD carried one spare T5 collar and one additional handheld GPS unit paired with all three tracking collars. While working, the detection dogs wore a red 'detection dog' jacket with reflective stripe at all times.

When OWAD's detection dogs find Koala scats, they lie down with their nose on the scat and hold the indication until the handler give them a 'bridging cue'. If the scat is visually obstructed by leaf litter and/or dense ground vegetation, they use their paws and/or nose to expose the scat. In instances where the leaf litter or the low-lying vegetation is particularly thick, the handler prompts the dog to retrieve a scat with a 'soft mouth' and bring it up to the surface for the handler.

Left: 'Taz' in work gear − jacket, tracking collar and paired handheld GPS unit
Right: 'Missy' indicating on a Koala scat

The short video below (**Video 1**) shows an example of a search resulting in the detection dog finding fresh Koala scat, which was collected for laboratory testing.

Video 1: Overview of field methods (click to play)

4.2.2 Opportunistic searches

When the detection dogs were not actively searching (e.g. walking or driving between sample areas), the study team continued to pay attention to leaf litter and/or tree canopies in case fresh scats or live Koalas were spotted. Additionally, when coming across park users or Council workers, if needed and if appropriate the study team engaged in discussion to ask if a Koala was recently heard or sighted in the area.

4.2.3 Quality Assurance

Field Quality Assurance procedures

In all studies undertaken by OWAD, field quality assurance (QA) procedures are undertaken to ensure that the data collected in the field is representative of the true site conditions and is therefore valid for interpretation. QA procedures include the use of experienced Koala ecology expert staff, Certified Environmental Practitioners, purpose-bred field detection dogs professionally raised and trained for the task, certification of both the dogs and their handlers, the use of appropriate study designs and protocols, and the implementation of daily field quality control (QC) searches.

Field QC searches are performed each day on all projects performed by OWAD. Either the detection dog finds a naturally deposited target scat/or a live Koala within the first few minutes or hours of working each day, in which case there is no need for a third party to deposit a Koala scat for QC purposes. Or, if no naturally deposited scats/or no live Koalas are found within the first few minutes or hours of commencing work each day, then a third party (if available an

accompanying staff external to OWAD; or if not available, then the OWAD field assistant) randomly deposits target scats, ensuring the handler does not know when or where QC scats may have been placed. When scats are deposited for QC purposes, the field assistant starts a chronometer (without the handler knowing) when the dog/handler team is within approximately 100 metres from the QC scats, and records the time it takes the dog/handler team to find target scats (whether the QC scats or naturally deposited target scats, whichever are found first).

A QC search enables the assessment of the dog/handler team's ability to find a target in the specific conditions of a particular site at a particular time, within a maximum time of 5 minutes. This enables to ensure that there are no exceptional circumstances or factors that may be disabling the dog/handler team's ability to find targets (e.g. a scent that may be obscuring target odours for the dog; handler fatigue or distraction which may affect the handler's ability to correctly handle the dog, etc.). Crucially, the handler is never informed in advance where or when Koala scats are deposited for QC purposes. Not disclosing this information is crucial to ensuring there is no bias in how the handler handles the dog. It is only after the dog/handler team has found target scats during a QC search that the third party/or field assistant discloses that this was a QC search. At least one QC search per dog/handler team is performed on any given day, however the third party/or field assistant may perform more than one QC search per dog/handler team on any given day.

Field Quality Control search interpretation

- Should the dog/handler team find a deposited QC scat within 5 minutes, the field QC search is marked as successful, the time is recorded for record-keeping purposes and work continues. The coordinates of the QC scat are recorded for QA purposes, but results are not recorded as an actual result in the survey as this was not a naturally occurring scat.
- Should the dog/handler team find a naturally deposited target scat within 5 minutes after a QC search has started, the field QC search is marked as successful, the time is recorded for statistics purposes and work continues. The coordinates of the naturally deposited scat found are recorded as a result in the survey.
- Should the dog/handler team fail to find a target scat within 5 minutes after a QC search has commenced (whether the deposited QC scat or a naturally deposited target scat), the field assistant would immediately stop the handler and disclose that a QC search has failed[1]. In the event that a QC search were to fail, the survey team would cease work immediately to try and identify the reason for failure. Upon identification of the potential cause, a second QC search would be immediately conducted to confirm the reason for initial failure. Should the second QC search also fail, the study team would reassess the site conditions / the environmental conditions / the detection dog(s) / the handler(s) / the search protocol etc. If the cause for failure cannot be quickly identified and remediated, the study team would liaise with the client. No further survey work would be conducted until the reason(s) for failure is or are identified and remediated.

4.3 SELECTION OF SCATS FOR COLLECTION

Koalas typically produce 100 to 150 scats in each 24 hour period (Ellis *et al*. 1998). Depending on factors such as local weather events, ecosystems, microbacterial activity, insect predation, geography and moisture, Koala scats in Eastern Australia can maintain some structural integrity for up to 12 months or more (Witt and Pahl 1995, Cristescu 2011, Rhodes *et al*. 2011).

[1] This instance has never occurred to date.

Only scats deemed potentially viable for laboratory analysis were collected and submitted to WildDNA, meaning scats that met the following criteria:

- Deposited up to 12 weeks prior to field assessment
- Not damaged by rain/moisture/dew
- Not damaged by fire
- Not damaged by mould or fungi
- Not extensively preyed on by insects
- Not significantly covered in dust/dirt/debris
- Not significantly damaged by any other factor not listed above

Each time fresh scats were found a careful visual inspection was performed by the field survey team to assess whether they may fit the above criteria and be in sufficient condition to be viable for laboratory testing. Only scats deemed to have some potential of being viable were collected and submitted to WildDNA.

4.3.1 Distinguishing Koala scats vs. similar scats from other animals

A variety of species produce scats that can visually closely resemble Koala scats (e.g. some Possum and Glider species). However, OWAD's detection dogs discriminate between Koala scat and similar looking scats with 100% accuracy. Each time OWAD acquires a new dog from their expert trainer[2], OWAD thoroughly tests the dog before it is deployed on its first applied study. A new dog is not deployed for project work until it consistently performs to 100% target detection rate (i.e. does not miss a single target in a controlled environment) and 100% discrimination rate (i.e. never indicates on non-targets in both controlled and uncontrolled environments). These field trials are performed over several consecutive full days to replicate the demanding conditions of project work, and test the dog's physical endurance and mental focus to ensure it is able to work long hours over long consecutive days while maintaining 100% target detection rate and 100% discrimination rate.

OWAD's detection dogs' 100% discrimination rate is also backed by scientific evidence: OWAD has been regularly sending Koala scat or pap samples found by their detection dogs to WildDNA for testing since 2015. To date OWAD has sent WildDNA over 4,000 scats or pap samples found by their detection dogs, including some pap and scat samples of highly unusual shapes that experts would not typically associate with Koala. The origin of some of these samples has on several occasions been questioned by the geneticists upon receiving these, and understandably so. OWAD staff can occasionally themselves be surprised at the highly unusual appearance or even smell of some of the material indicated on by their dogs. However, what better way to scientifically measure the scent discrimination rate of their dogs, than to subject the material they indicate on to genetic testing by an external laboratory that has no vested interest in the performance of OWAD's detection dogs. Moreover, if any such material were ever found to not originate from Koala, OWAD would want to know immediately so as to quickly address and remediate the issue via targeted training sessions. However, to date 100% of all scat samples (>1,000) found by OWAD's detection dogs which have been genetically tested, have been confirmed as originating from Koala. The discrimination rate of OWAD's detection dogs is therefore maintained at 100% accuracy not only via ongoing training and reinforcement but is also

[2] Steve Austin CCPDT (certified by the Certification Council of Professional Dog Trainers)

scientifically proven to be 100% correct via extensive and ongoing genetic testing undertaken by a third party.

4.3.2 Identifying fresh scats

Fresh Koala scats are typically dark brown to black on the outside, have a shiny surface, bright green or yellow inside, and solid to the touch (or soft if extremely fresh e.g. deposited within a few hours). Occasionally, the surface of fresh scats can be dull or bright yellow, reddish or purple-blue.

Old scats typically have a duller surface, have less color contrast between the outside and the inside, and crumble under minimal compression. See **Plate 1** as an example.

Plate 1: 2-week old scats (left) and 10-months old scats (right) originating from the same Koala individual

4.3.3 Identifying scats from potentially distinct individuals

Typically, one individual produces scats that tend to be relatively consistent in both shape and size. There can be significant variations in the shape and/or size of scats produced by different individuals: they can be oblong or round in shape, can have a smooth or an irregular surface, can have ridges or lack ridges, and can range in size from approximately 5mm to 40mm. **Plate 2** is a typical example of the variations in Koala scat size and shape produced by four known distinct individuals. These variations were used by the study team in the field to infer whether a particular individual may have previously been sampled from a nearby location. Indeed, in instances where at one location there were fresh scats potentially originating from more than one individual, these variations in scat size/shape assisted the study team in estimating how many putative distinct individuals these may originate from, in order to sample each inferred individual in distinct scat collection kits.

Plate 2: Variations in scat size and shape originating from four known distinct Koalas

4.3.4 Burnt scats

Where bush fires occur, Koala scats can burn. This does not prevent the detection dogs from finding these scats, however burnt scats are not viable for analysis as the genetic material has been compromised. When working in areas where bushfires have occurred, only scats not directly damaged by fire may be viable for analysis. Depending on how long scats were subjected to fire, and the intensity of the fire, they can be either entirely burnt throughout or only the surface may be burnt/or partially burnt. See **Plate 3** below for an example of lightly burnt scats (left) and severely burnt scats (right) collected from two different fire grounds.

Plate 3: Burnt Koala scats

4.4 SCAT COLLECTION AND HANDLING PROTOCOLS

4.4.1 Collection and storage in the field

Potentially viable scats were collected from the environment using toothpicks, ensuring no direct contact with humans or the detection dogs, and minimal friction on the forest floor so as not to 'rub off' the genetic material. The toothpicks were then securely inserted into a piece of foam, and the foam placed and secured into a purpose-built collection kit (see **Plate 4**). Where possible, multiple scats were collected and placed in each kit. The reason for collecting more than one scat per presumed individual at each location, where possible, was to provide several chances of isolating DNA of the best quality and quantity obtainable to enable genetic profiling (see **Section 5** for details on scat analysis methods).

On each collection kit, a pre-affixed label was completed which included the following information:

- A unique sample code
- Site identifier and date collected
- Name of entity collecting the sample (OWAD Environment)
- Coordinates (UTM format, including UTM zone)

Each kit was then placed in a paper bag. Where the study team found fresh scats at one location potentially originating from multiple Koala individuals, these were placed into distinct collection kits.

Plate 4: Koala scat collection kit

While the survey team is in the field, the work vehicle can reach high temperatures and this can damage genetic material. Kits were therefore placed in a double insulated cool container with ice packs and desiccant sachets to control both the temperature and the ambient humidity within the container. This field storage method is also effective at containing the scent and prevents dissipation of the detection dogs' target odour in the vehicle. At the end of each fieldwork day, the collected kits were removed from the field container, transferred into a cardboard box with desiccant sachets and stored at ambient temperature in a cool room away from direct sunlight.

4.4.2 Sample submission protocol

At completion of a sampling event, or periodically during a sampling event, the samples were carefully packed in a cardboard parcel with desiccant sachets and posted to the WildDNA laboratory via overnight postage. Care was taken to not post any samples on a Friday or the eve of a public holiday. The parcel included a hard copy of the sample tracking sheet which contained the following information:

- List of sample codes contained in the parcel
- Coordinates of all sampling locations
- Field collection date for each sample
- Date posted to WildDNA
- Laboratory tests required

An electronic copy of the sample tracking sheet was also sent to WildDNA. Upon receiving the samples, WildDNA confirmed reception of the samples and that there was no loss of samples during postage.

4.5 FIELD DATA ENTRY

At completion of each survey day, the detection dogs' search tracks and all relevant coordinates were saved electronically. In order to minimise the risk of data loss, a copy of this data was saved daily in at least three devices (e.g. computer, external hard disk and USB key), with at least one of these devices kept in a different place to the other devices.

5.0 SCAT ANALYSIS METHODOLOGY

5.1 LABORATORY TEAM

The laboratory team at Federation University Australia who led and undertook the genetic testing for this project, were:

- Dr Fiona Hogan (Molecular Ecologist, WildDNA Director, lead project coordinator); and
- Dr Faye Wedrowicz (Molecular Ecologist, WildDNA Laboratory Manager)

Drs Fiona Hogan and Faye Wedrowicz have extensive experience in the isolation and analysis of DNA from non-invasive biological materials such as feathers and scats (Hogan *et al*. 2008; Wedrowicz *et al.* 2013). Drs Hogan and Wedrowicz developed and published the genetic methods used for this study (i.e. DNA profiling from a single Koala scat; Wedrowicz *et al*. 2013), detecting *C. pecorum* and KoRV-A in Koala scats (Wedrowicz *et al.* 2016), validating the use of DNA sourced from Koala scats for population genetic studies (Wedrowicz *et al*. 2017, Wedrowicz *et al*. 2018), and designing molecular markers to infer sex of South East Queensland Koalas using DNA isolated from Koala scats (Wedrowicz *et al.* 2018).

Further, OWAD has subcontracted WildDNA on numerous applied Koala studies since 2016, and WildDNA has consistently produced excellent scat analysis results.

5.2 SCAT TESTING PROTOCOL

5.2.1 Surface wash

Scats received by WildDNA were processed upon arrival. Epithelial cells on the outer surface of each scat were removed using the washing technique described in Wedrowicz *et al.* (2013). Surface washes were stored frozen (-20°C) until all scats collected by the field survey team were received and processed.

5.2.2 DNA isolation and Quality Control

Genomic DNA was isolated from epithelial cells (surface washes) as per the methods described in Wedrowicz *et al*. (2013) using the DNeasy Plant Pro Kit (Qiagen). Each DNA sample was tested to determine the total quantity of DNA isolated (i.e. concentration, containing both Koala and non-Koala DNA) and the quality of the target DNA isolated (i.e. amplification of a Koala specific locus using the polymerase chain reaction (PCR)). This process is referred herein as 'Quality Control' or 'QC'. Samples were scored as 'pass', 'low quality pass' or 'fail' after QC. Samples failed QC where amplification of a Koala specific genetic marker failed for two replicates. Samples were classed as low-quality passes where PCR products were faint, or DNA concentration was low. Only the DNA isolate of the highest DNA concentration and of a 'pass' or 'low quality pass' standard from each kit was used for downstream genetic analysis. The scat washing process, DNA isolation and QC were conducted by WildDNA.

5.2.3 Genetic tests

All genetic tests for this study were conducted by the Australian Genome Research Facility (AGRF), under the instruction of WildDNA. AGRF is accredited by the National Association of Testing Authorities (NATA) Australia in the field of Biological Testing and operates in compliance with the international standard ISO/IEC 17025:2005.

Genetic testing involved four types of analyses:

Sex

Sex was determined by targeting the X and Y chromosomes using molecular markers specially designed for sexing Koalas (Wedrowicz *et al*. 2018).

DNA profiling

Samples were genotyped for 12 Koala-specific microsatellite markers which provided a unique DNA profile. A reliable DNA profile, derived from replicate analysis, allowed distinct individuals to be identified 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). Further details of the DNA profiling process are described in Wedrowicz *et al*. (2013) and Wedrowicz *et al.* (2018).

Pathogen detection

Samples were tested for the presence of *C. pecorum* and KoRV-A using target-specific molecular markers as described in Wedrowicz *et al.* (2016).

Mitochondrial DNA sequencing

mtDNA sequencing was undertaken using markers and methods reported in Fowler *et al*. (2000) and Wedrowicz *et al*. (2018) respectively.

5.3 ANALYSIS OF GENETIC DATA

Genotypic data produced by AGRF were used by WildDNA to generate a unique DNA profile, which was used to genetically tag individual Koalas. Genetic analyses were undertaken to provide preliminary information about Koala population structure, connectivity and genetic diversity in the Port Stephens LGA. Details of genetic analyses performed are described in more detail in **Section 7**.

6.0 SUMMARY OF RESULTS

6.1 SURVEY EFFORT AND EVIDENCE OF KOALA PRESENCE FOUND

OWAD's detection dogs searched for evidence of Koala presence for a total of 137km within Port Stephens LGA over the course of three rapid sampling events conducted in November 2018, July 2019 and May 2020.

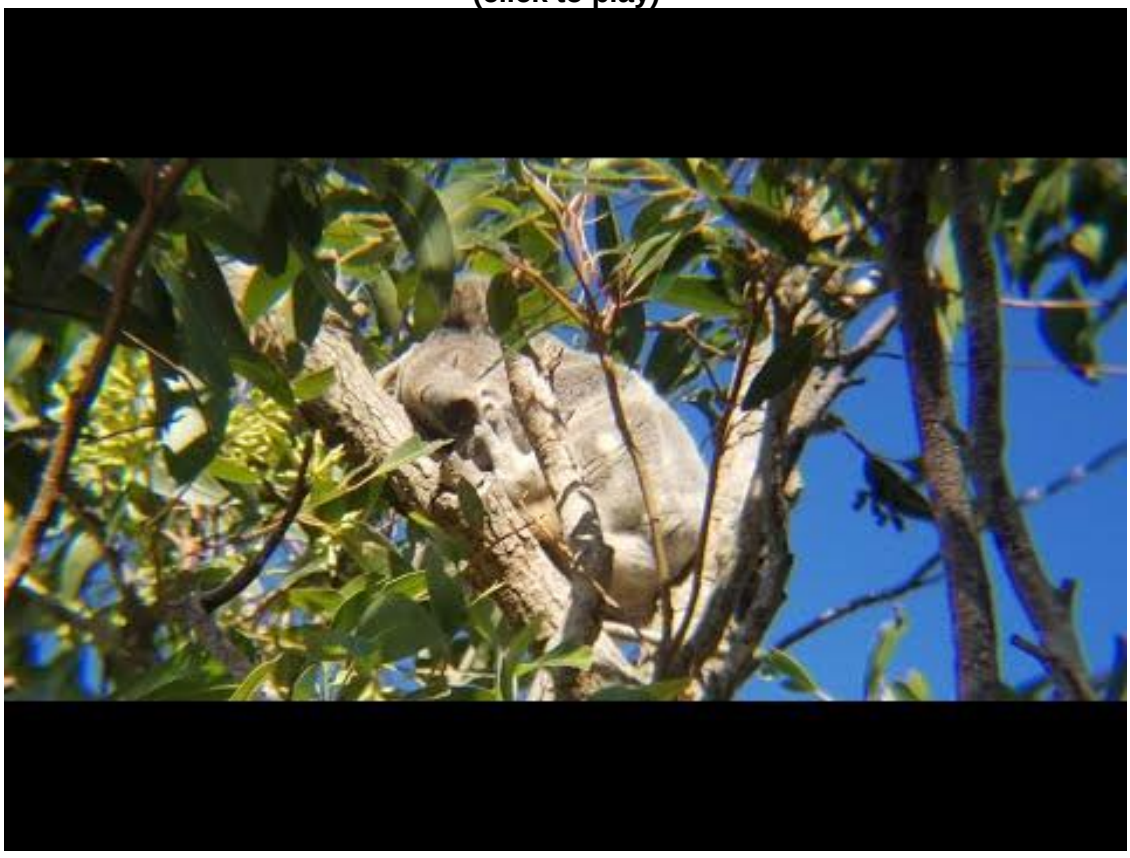
Over the three events, the detection dogs found naturally deposited Koala scats or pap at a total of 278 locations.

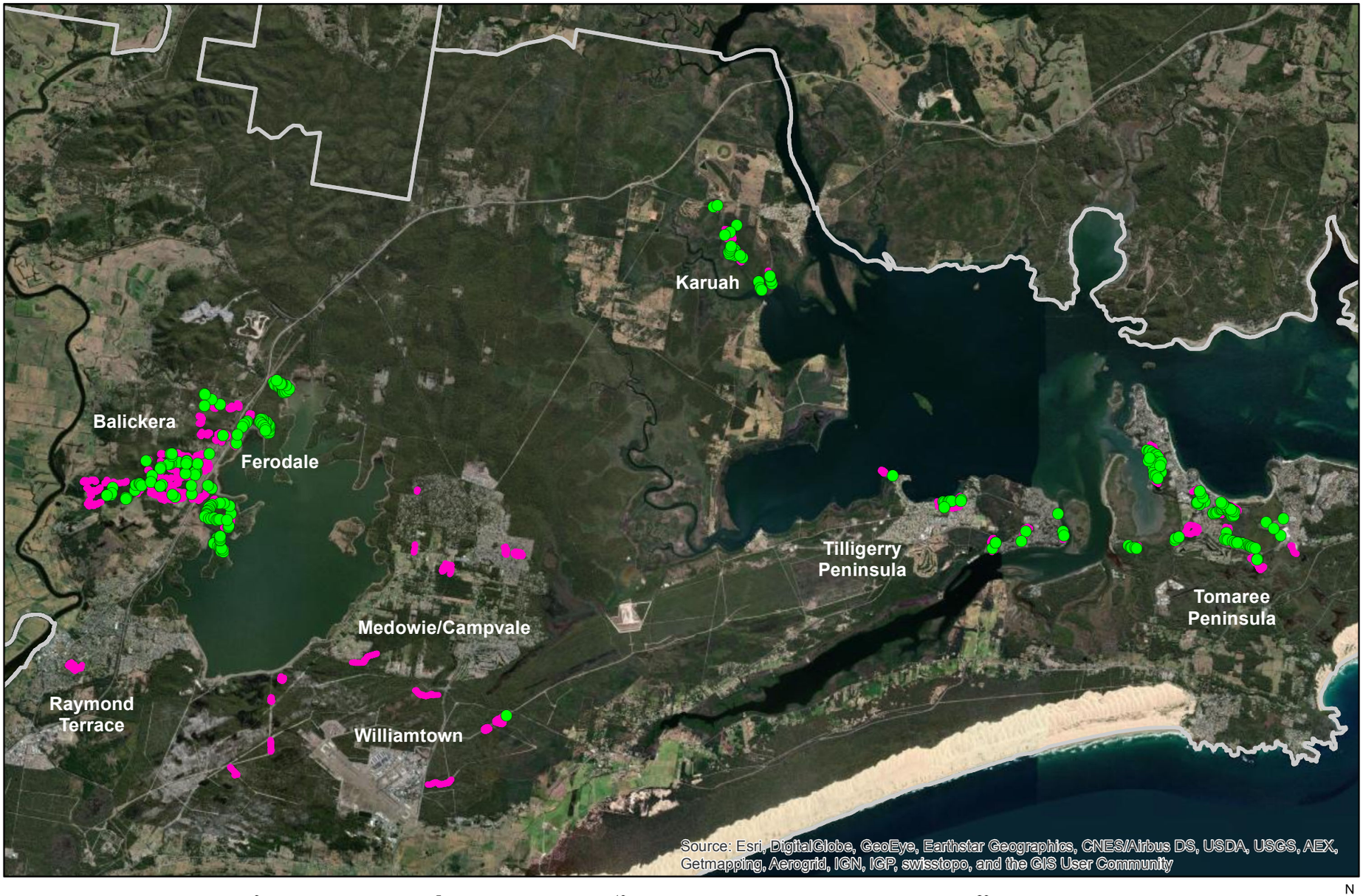
Evidence of Koala presence (scats, pap or live individuals) was detected on sites situated in six of the eight geographic locations investigated:

- Evidence of Koala presence was found on sites investigated in Tomaree Peninsula, Tilligerry Peninsula, Balickera, Ferodale, Karuah and Williamtown. We note here that in the lands assessed in Williamtown, only one old Koala scat (circa 12 months old) was found at one location.
- No evidence of Koala presence was detected in Raymond Terrace (one park assessed in July 2019) nor on the lands investigated in Medowie/Campvale (assessed in May 2020).

Figure 2 shows the detection dog search tracks and the 278 locations where evidence of Koala presence was detected during the three sampling events. **Video 2** below shows some footage taken during the May 2020 sampling event.

Video 2: Footage taken during the May 2020 sampling event in Port Stephens LGA (click to play)





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Revised on: 27/11/2020

LEGEND

- Local Government Area boundaries
- Detection dog search tracks (total 137km)
- Evidence of Koala presence detected (scats, pap or live individuals)

FIGURE 2: SEARCH EFFORT AND EVIDENCE OF KOALA PRESENCE DETECTED

2020 Port Stephens Koala population study

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6.2 FIELD QUALITY CONTROL RESULTS

All field QC searches performed during all three sampling events within Port Stephens LGA were successful. In other words, on all field QCs performed, the detection dogs found either a naturally deposited Koala scat or a Koala scat deposited for QC purposes within 5 minutes. As per OWAD's standard procedures, at least one QC was performed per dog/handler team per day. Up to six QCs were performed in one day during the 2018 sampling event, as unusual environmental circumstances occurred and worsened throughout the day (thick dust storm, smoke and high winds). These field QC results confirm that the dog/handler teams' detection abilities were not impeded by any factor on any of the three sampling events.

6.3 INDICATIVE KOALA ACTIVITY LEVELS DETECTED WITHIN THE LANDS ASSESSED

A rapid way of deriving indicative Koala activity levels, is to compare the number of 'finds' (i.e. locations where live Koalas, Koala scats and/or pap were found) per kilometer searched by the detection dogs within any given area. This provides a general indication of how much search effort had to be applied to detect evidence of Koala presence in any given area, and hence a sense of the general Koala activity level within the said area. The greater the value obtained, the higher the activity level detected. Conversely the smaller the value, the lower the activity level. These values are displayed in **Table 1** below, with lands sorted from highest to lowest activity level detected. Where Koala presence was detected, the highest activity level was found in Grahamstown Dam (centre) and the lowest activity level was found in Tilligerry State Conservation Area.

Table 1: Indicative Koala activity levels detected within Port Stephens LGA

Sampling period	Land name	Number of 'finds'	Search effort (kms)	Value (finds/km)
July 2019	Grahamstown Dam West (centre)	43	11.9	3.613
July 2019	Grahamstown Dam West (north)	39	13	3.000
May 2020	Tilligerry Habitat	8	3	2.667
July 2019	Karuah BioBank	24	9.2	2.609
May 2020 *	Mambo Wetlands *	10	4.1	2.439
July 2019	Wanda Wetlands	9	3.7	2.432
May 2020	Nyrang Reserve	3	1.3	2.308
May 2020	Port Stephens Drive	8	4.7	1.702
May 2020	John Parade/Gibbers Reserve	2	1.7	1.176
May 2020	Mungarra Reserve & Southern Foreshore	2	1.8	1.111
November 2018	Balickera	47	42.9	1.096
May 2020	Salamander Bay BioBank	2	3.6	0.555
May 2020 *	Stoney Ridge Reserve *	2	4	0.500
May 2020	Tilligerry State Conservation Area	1	10.3	0.097
July 2019	Boomerang Park	0	2	0
May 2020	Campvale Drain Easement	0	1.6	0
May 2020	Medowie Council depot, Kindlebark Oval and Medowie on-leash Dog Exercise Area	0	2.3	0

HIGH ↑ Koala activity level detected ↓ LOW

* Mambo Wetlands and Stoney Ridge Reserve were assessed on two occasions. Only the results of the May 2020 sampling event are presented in **Table 1** above for these two reserves. Further discussion about these two reserves is included in **Section 6.3.1.**

Please note:

- The values provided in **Table 1** are indicative only, and should not be viewed or interpreted as absolute;
- The indicative values obtained are only true for the time when each of the lands was sampled;

- Koala activity levels can vary over time either due to seasonal variations in habitat occupancy and/or due to variations in Koala numbers (i.e. Koala numbers increasing or decreasing over time) and/or due to changes in habitat availability and connectivity in the landscape;
- Koala distribution is typically not homogeneous across any given landscape and variations in survey design, search protocol and search effort intensity between sampling events and between sampling sites may skew the values obtained one way or the other;
- Koala activity values are not a valid indicator of Koala numbers, their genetic fitness, nor of their health status; and
- The indicative values provided in **Table 1** are to be viewed and interpreted in conjunction with the genetics results included in this report (see **Section 7**).

6.3.1 Changes in Koala activity levels observed in Mambo Wetlands and Stoney Ridge Reserve between July 2019 and May 2020

Stoney Ridge Reserve and Mambo Wetlands were sampled twice, once in July 2019 and a second time in May 2020. While on site re-surveying these two lands in 2020, the survey team noted that there appeared to be notably less evidence of Koala presence than what was observed in the field 10 months prior. For these two lands, the activity levels detected during both sampling events were compared. The values obtained, displayed in **Table 2** below, confirm a sharp decline in Koala activity detected on these two lands. Whether this decline is reflective of a decline in the number of individuals within these local landscapes, is at this stage unknown and would require repeat sampling to confirm.

Table 2: Indicative Koala activity levels detected in Mambo Wetlands and Stoney Ridge Reserve in July 2019 and May 2020

	July 2019			May 2020			Change
	Number of 'finds'	Search effort (kms)	Value (finds/km)	Number of 'finds'	Search effort (kms)	Value (finds/km)	
Mambo Wetlands	36	7km	5.143	10	4.1km	2.439	**- 2.704**
Stoney Ridge Reserve	21	6km	3.500	2	4km	0.500	**- 3.000**

6.4 SUMMARY OF KOALA GENETIC MATERIAL COLLECTED FOR THIS STUDY

A total of 60 Koala scat or pap samples were collected by OWAD and submitted to WildDNA for genetic analysis. These included:

- 51 samples collected by OWAD in the field within Port Stephens LGA during three sampling events (November 2018, July 2019 and May 2020); and
- 9 samples taken from captive individuals in care at the Port Stephens Koala Hospital. These individuals were rescued from within Port Stephens LGA.

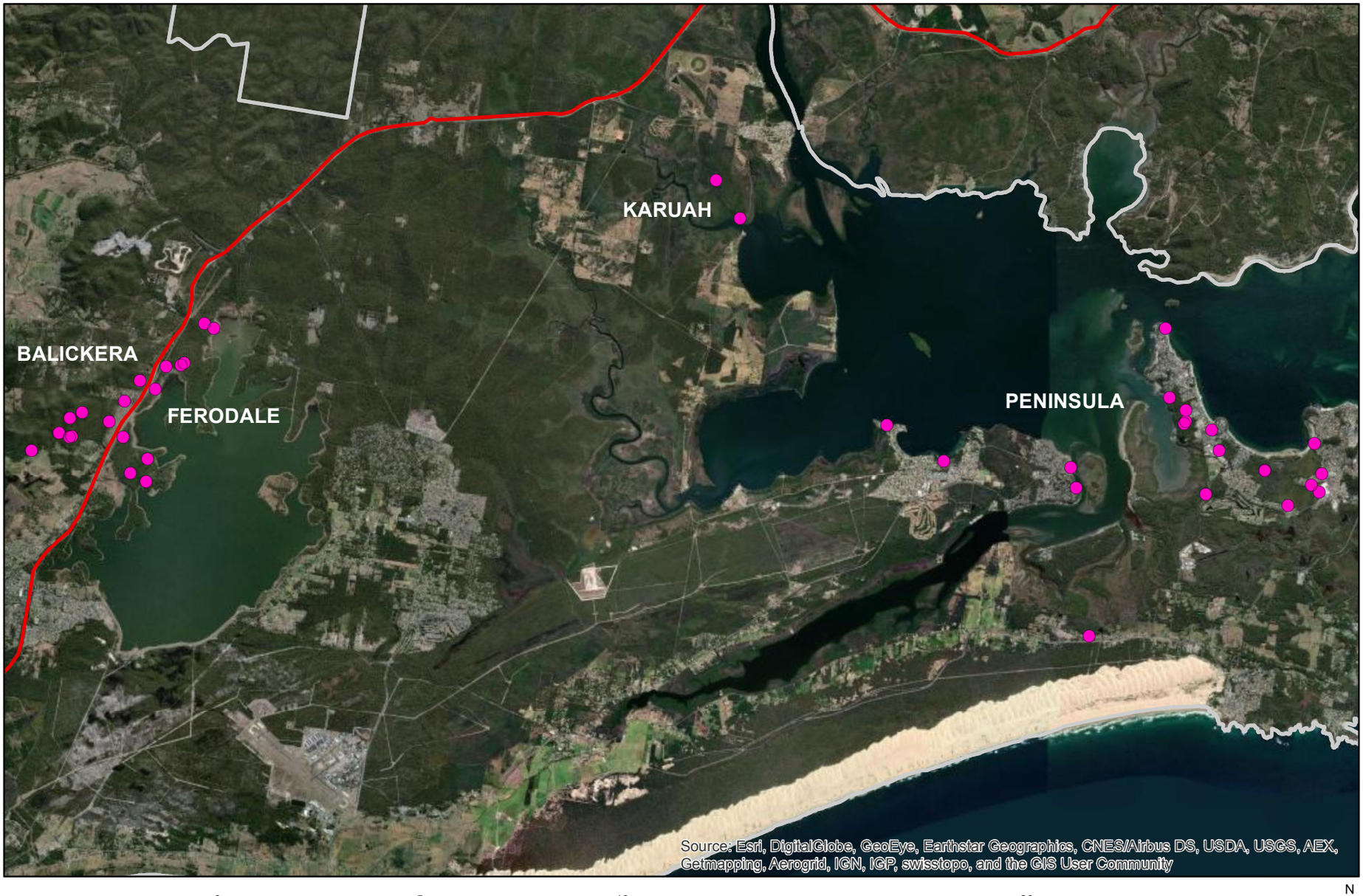
6.5 OVERVIEW OF LABORATORY TEST RESULTS

Of the 60 genetic samples submitted to WildDNA, 45 samples (or 75%) provided a reliable DNA profile. DNA profile-matching revealed that the 45 profiles obtained originate from a total of 39 distinct individuals, 19 females and 20 males. All of these individuals were sampled within Port Stephens LGA. Their sampling locations were classed into one of four geographic areas: Balickera, Karuah, Ferodale (centre and north Grahamstown Dam West sites) or Peninsula (including Tilligerry and Tomaree). 100% of individuals (39/39) returned a positive KoRV-A test, while 36% of individuals (14/39) returned a positive *C. pecorum* test. **Table 3** provides a summary

of the test results for the 39 unique individuals profiled from Port Stephens LGA. **Figure 3** shows their sampling locations. **Appendix 1** shows the test results for each individual profiled.

Table 3: Summary of Koala genetic profiling results, including sex and disease status

Geographic location	Number of unique individuals	Sex		*C. pecorum* detected	KoRV-A detected
		Female	Male		
Peninsula	16	8 (50%)	8 (50%)	6 (37%)	16 (100%)
Karuah	3	0 (0%)	3 (100%)	1 (33%)	3 (100%)
Ferodale	10	7 (70%)	3 (30%)	3 (30%)	10 (100%)
Balickera	10	4 (40%)	6 (60%)	4 (40%)	10 (100%)
Total	**39**	**19 (49%)**	**20 (51%)**	**14 (36%)**	**39 (100%)**



0 5 10 20 Kilometers

N

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LEGEND

- Local Government Area boundaries
- Pacific Highway
- Sampling locations of individuals genetically profiled

Note 1: At some locations more than one individual was sampled and profiled from the same location
Note 2: Some individuals were sampled from more than one location

FIGURE 3: SAMPLING LOCATIONS OF INDIVIDUALS PROFILED

2020 Port Stephens Koala population study

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7.0 KOALA POPULATION ANALYSIS

A population is defined as a group of individuals that live in the same geographic area and have the capability of randomly mating. A population may exist as either: (1) a single large population, (2) a metapopulation where there are multiple subpopulations with varying levels of genetic connectivity, or (3) as a small isolated population.

The loss of favourable habitat can have significant implications for the viability of vulnerable species, as inhospitable habitat can restrict animal movements (decrease mixing of genes) and pose increased risk to the survival of individuals. An understanding of how habitat fragmentation is affecting the movements of individuals, hence the population, is critical for the effective management, conservation or recovery of threatened species. Like most wild animals, Koalas are best conserved when populations are large, connected and individuals can safely disperse from their natal territories to secure their own territory, mate(s) and reproduce. When Koalas become isolated, there is a greater chance of inbreeding and genetic drift which can result in a reduction in genetic diversity and an overall decrease in 'genetic fitness', which can reduce chances of survival (e.g. compromised immune systems, reduced chance of adapting to climate change). Moreover, small isolated groups are at greater risk of being lost due to stochastic events such as wildfires.

Different regions within the genome can be targeted to define populations and provide information regarding how populations are connected across the landscape. In this study we sequenced the control region in the mitochondrial DNA (mtDNA) to assess historic population structure, and genotyped 12 microsatellite loci (hypervariable regions in the nuclear DNA) to infer the current population structure and connectivity.

Genetic data, mtDNA and microsatellite loci, from 39 individual Koalas were used to investigate historic and current population structure, respectively. Koalas sampled from Tilligerry and Tomaree are referred to as 'peninsula'. Koalas sampled from all other areas of Port Stephens LGA are referred to as 'inland'.

Population structure was investigated using the genetic software packages STRUCTURE (Pritchard *et al*. 2000) and GENELAND (Guillot *et al*. 2008). STRUCTURE analysis uses a Bayesian model-based clustering method where genotypic data are used to identify the most likely number of genetic clusters (K) in a sample set. The software proportionally assigns individuals to genetic clusters, providing a snapshot of current population structure. STRUCTURE is a robust test for inferring the presence of population structure within a species. Similarly, GENELAND also detects population structure using Bayesian modelling, but it additionally incorporates spatial coordinate data to estimate the number of genetic clusters in a sample set and can therefore be more powerful at delimiting populations at the landscape level (Blair *et al*. 2012).

7.1 CONTEMPORARY POPULATION STRUCTURE

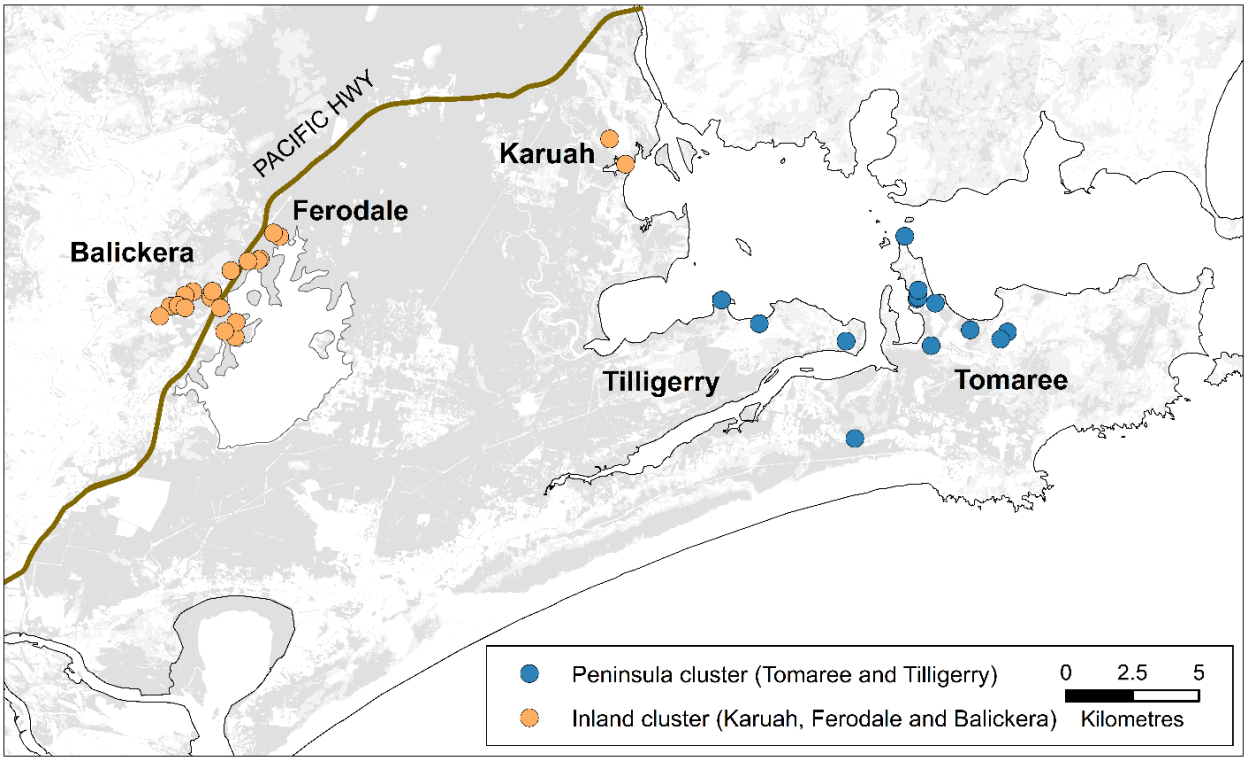
7.1.1 Broad population structure

Two genetically distinct Koala clusters were identified across the Port Stephens region.

Analysis of the current regional population structure was investigated using the STRUCTURE program. STRUCTURE indicated that there are two distinct genetic clusters of Koalas in the Port Stephens area, where Koalas on the peninsula (Tilligerry/Tomaree) were separated from inland Koalas (Balickera, Karuah and Ferodale).

See **Figure 4** for a spatial representation of the population structure results inferred by STRUCTURE.

Figure 4: Broad population structure inferred by STRUCTURE



Grey shading shows the predicted spatial distribution of Koala habitat using data obtained from the New South Wales Government Department of Planning, Industry and Environment (2019). Habitat suitability is estimated on a scale from 0 to 1, with higher values indicating a greater probability that habitat in a particular location is suitable for koalas. Two categories of Koala habitat suitability are shown on this map, 1) habitat suitability ranging from 0.75–1.0 (dark grey) and 2) habitat suitability ranging from 0.5–0.15. White regions indicate areas where the presence of habitat suitable for Koalas has a probability of less than 0.5.

7.1.2 Fine-scale population structure

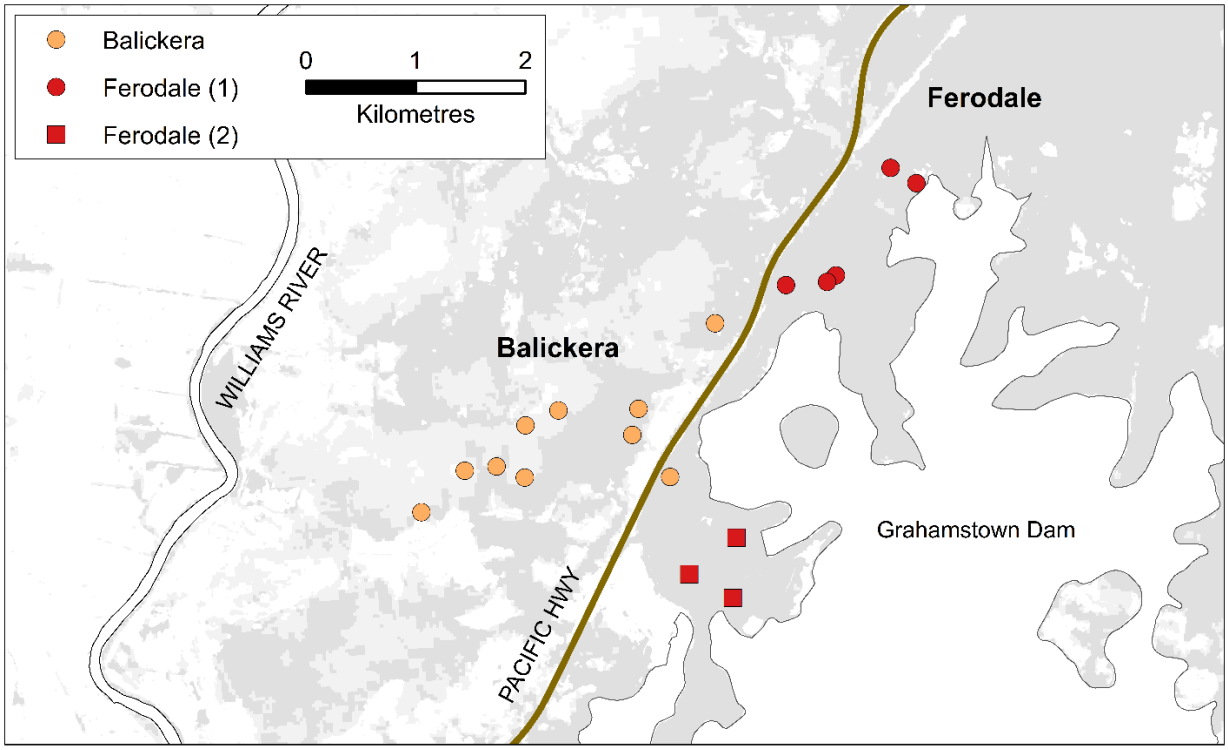
The Pacific Highway appears to be restricting Koala movements between the Balickera and Ferodale sites.

GENELAND was used to investigate gene flow between Koala individuals sampled in Balickera and in Ferodale, which are separated by the Pacific Highway.

Despite the distance between these two areas being minimal (i.e. 0.7 to 5.5 km), GENELAND indicated that there are three discrete Koala groups in this part of the study area, indicating that gene flow is limited between these geographic areas. It is noted that one individual sampled on the east side of the Pacific Highway in Ferodale, grouped with Koalas sampled in adjoining Balickera west of the highway. It is also noted that sample sizes are small in this study; a more comprehensive understanding of gene flow in this part of the study area would require further sampling.

Figure 5 provides a spatial representation of the genetic differentiation observed between Koalas sampled in Balickera and Ferodale.

Figure 5: Differentiation observed between Koalas sampled in Balickera and Ferodale



Grey shading shows the predicted spatial distribution of Koala habitat using data obtained from the New South Wales Government Department of Planning, Industry and Environment (2019). Habitat suitability is estimated on a scale from 0 to 1, with higher values indicating a greater probability that habitat in a particular location is suitable for koalas. Two categories of Koala habitat suitability are shown on this map, 1) habitat suitability ranging from 0.75–1.0 (dark grey) and 2) habitat suitability ranging from 0.5–0.15. White regions indicate areas where the presence of habitat suitable for Koalas has a probability of less than 0.5.

7.2 HISTORIC POPULATION STRUCTURE

Koalas across the Port Stephens region were historically connected.

Mitochondrial DNA (or mtDNA) is genetic material inherited maternally (from the mother). When investigating population structure, analysis of mtDNA sequence variation can provide information regarding historic population connectivity.

DNA sequencing of the mtDNA control region of Koalas sampled during this study were used to investigate historic connectivity of the Koalas across the Port Stephens region. Data from this analysis suggests that peninsula and inland Koalas were historically connected, as variations (haplotypes) were not unique to different population clusters. Rather, haplotypes were shared between both inland and peninsula Koalas (see **Appendix 2** for further detail).

7.3 GENETIC DIVERSITY

Koalas from the peninsula are less genetically diverse than Koalas from Balickera.

It is important for ecosystem health to conserve not only individual species, but biodiversity in general, including the gene pool − that is, the genetic diversity (i.e. variations in the DNA) that exists within each living organism. Genetic diversity measures the range of genetic characteristics (e.g. gene forms, alleles or genotypes) within a species or within a population. These characteristics can vary in response to environmental conditions (selection), mutation, chance effects (genetic drift) and breeding patterns.

Genetic problems can occur in small isolated populations, including inbreeding depression, genetic drift, erosion of genetic diversity and reduced ability to evolve and cope with environmental change (Frankham *et al*. 2017). If populations are totally isolated by habitat fragmentation, the genetic makeup of the isolated group will be determined by the genetic variation of that population alone rather than that of the wider population (Frankham *et al*. 2017).

Genetic diversity is important as it allows species to adapt to environmental changes, such as climate change, and may provide protection against emerging diseases. Genetic diversity is best conserved when populations are large and individuals can safely disperse from their natal territories to secure their own territory, mate(s) and reproduce. When groups of species become isolated, there is a greater chance of inbreeding and genetic drift, which can result in a reduction in genetic diversity and an overall decrease in 'genetic fitness'.

In this study, peninsula Koalas were found to have similar genetic diversity (the number of alleles and allelic richness) to Koalas at Ferodale, but less genetic diversity than Koalas at Balickera (**Table 4**). A reduction in genetic diversity is expected when a population becomes isolated, because individuals from the wider population are unable to move into an area and successfully reproduce. Barriers to movement are likely to occur when habitat patches become surrounded by an inhospitable matrix such as farmland or urban structures (including roads, fences and housing/buildings). Inbreeding can then become more common and the overall genetic fitness of the population can be compromised.

This appears to be the case here with Koalas in the Port Stephen region, where Koalas on the peninsula may be losing genetic diversity due to a lack of successful migration from outside of the peninsula. It is noted, however, that sample sizes for each of the four locations are small and

further sampling would be required to gain a greater insight into the genetic diversity of Koalas across the region.

Table 4: Genetic diversity by geographic location in Port Stephens LGA

	Peninsula	Balickera	Ferodale	Karuah
N	16	10	10	3
A	4.3	6.2	4.9	2.5
%	58	81	67	33
Ar*	3.7 (3.2 – 4.0)	5.2 (4.2 – 5.8)	4.4 (3.7 – 4.9)	–

N: Number of individuals sampled
A: Average number of alleles detected per locus
%: Percent of alleles in overall population found in each area
Ar: Allelic richness – mean number of alleles corrected for differences in sample size
*: Karuah was excluded from the calculation of allelic richness due to small sample size

8.0 HEALTH OF PORT STEPHENS KOALAS

8.1 CHLAMYDIA PECORUM

Chlamydia pecorum was detected in 14 of 39 individuals (or 36%) sampled within Port Stephens LGA, and in all four geographic locations where Koalas were profiled (Balickera 4/10 individuals, Ferodale 3/10, Karuah 1/3, Peninsula 6/16, see **Table 3** in **Section 6.5**).

This is comparable with the 40% *C. pecorum* prevalence found to date in Brisbane City Council LGA, South East Queensland (55/139 individuals tested as of November 2018, see OWAD Environment 2019).

It must be noted here that presence of *C. pecorum* does not necessarily mean that an individual has clinical signs. Clinical signs of disease due to *C. pecorum* infection may be induced by environmental stressors such as habitat loss, climate change, or modification of habitats (McAlpine *et al.* 2017).

It should also be noted that a negative result for *C. pecorum* from scat analysis does not necessarily mean that the individual does not carry the bacterium. Indeed, an individual could carry Chlamydia without shedding infectious particles (hence not detectable in scat).

8.2 KORV-A

Koala retrovirus subtype A, or KoRV-A, was detected in 100% (39/39) of the Port Stephens Koalas profiled and tested in this study. This result is in line with current data available for New South Wales and Queensland. To date WildDNA has tested over 300 wild Koalas sampled from various regions of New South Wales and Queensland, and to date all (100%) have tested positive for KoRV-A.

KoRV-A is known to be ubiquitous in most Queensland and New South Wales free-ranging Koala populations (Meers *et al.* 2014, Chappell *et al.* 2016), with all populations tested in these States to date showing a prevalence of 100%.

The term 'Koala retrovirus' (KoRV) refers to a group of viruses specific to Koalas. In other mammals, retroviruses are linked to the development of cancer or immunosuppression (e.g. HIV is a retrovirus infecting humans). To date, the role of KoRV in Koala disease remains unclear. There is some evidence for association of KoRV infection with immune changes, infectious diseases, blood and bone marrow disorders (myelodysplasia) and neoplasia (e.g. lymphoma and leukemia, mesothelial and craniofacial tumours). Although the role of KoRV in causing disease remains under investigation, evidence is emerging for association of KoRV infection with immune changes or diseases.

9.0 CONCLUSION

This study has taken a contemporary scientific approach to investigating the Koala population of the Port Stephens region through the integration of two non-invasive methods: professional detection dogs which allow large areas to be surveyed quickly and in a non-biased manner, facilitating the efficient collection of Koala droppings (scats or pap); and scat genetic analysis, which can provide key genetic data from Koalas sampled.

The detection dogs searched a total of 137km within Port Stephens LGA, and evidence of Koala presence (scats, pap or live individuals) was found at 278 locations. DNA isolated from Koala scats or pap samples was used to provide the unique DNA profiles of Koalas sampled during this study. Analysis of the genetic data obtained indicated that, historically, Koalas in the region were previously connected but now appear disconnected. This result is not surprising as large amounts of habitat have been cleared throughout the region to allow for modern civilisation to prosper. Koalas in the region are now largely confined to smaller habitat patches which are surrounded by a matrix of inhospitable habitat such as farmland, roads and housing/buildings. The modified landscape has also introduced many threats which previously (before civilisation) were not an issue, such as roads (vehicle strikes), dog attacks and the introduction of novel diseases (e.g. *Chlamydia*).

Discrete genetic clusters of Koalas were revealed during this study, along with a reduction in genetic diversity in the group of Koalas sampled on the peninsula (compared to Koalas inland), highlighting the impact habitat loss has had on the Koala population within the Port Stephens region. In order to reinstate and conserve the genetic diversity of Koalas in the Port Stephens region, management actions should be directed first at reserving and adequately managing key habitats and, secondly, reconnecting groups of Koalas. Connectivity can be increased by protecting and safeguarding existing habitats, creating new corridors through revegetation efforts, and creating safe movement options across physical barriers (e.g. functional Koala crossing structures across roads). Where proposed developments are considered, tangible incentives must be provided to minimise habitat loss and increase gene flow in targeted areas.

Non-invasive methods, i.e. professional detection dogs combined with scat genetic analysis, provided the first information regarding the Koala population structure and genetic measures for the Port Stephens region. Non-invasive genetic sampling and analysis via Koala scat is a powerful and cost-effective way of monitoring any Koala population over time. It is increasingly used by various bodies, including government authorities, to manage and monitor Koala populations as it provides clear scientific indicators (i.e. laboratory analysis results) against which to measure the success of conservation measures and/or recovery actions. Repeat sampling and analysis of Koala scats in given areas also enable scientifically robust estimates of Koala numbers, as 'genetic tagging' via scat enables mark-recapture analysis (Marucco *et al.* 2011) – without the need for the vast resources typically associated with locating and capturing live Koalas to extract genetic material directly from them, and without causing capture-associated stress on the wild animals. It is highly recommended to continue to add to the data and information collected in this initial baseline for the Port Stephens region. Threatened species conservation and management, however, cannot be effective if confined to small regions and conducted in isolation. It is therefore highly recommended to expand the data (and associated understanding of population structure) to neighbouring Local Government Areas that may share populations with Port Stephens LGA in order to ensure a coordinated approach to the management, recovery and conservation of shared populations.

The summer wildfires of 2019/2020 demonstrated how large areas of habitat can be destroyed in a short period of time. During this event it is estimated that millions of hectares were burnt and

over 3 billion animals impacted. In New South Wales, it is possible that several thousands of Koalas perished, which could represent a sizable proportion of the State's remaining population. The Koala is particularly vulnerable in New South Wales due to continued habitat loss through land clearing, and climate change which is increasing the frequency, scale and intensity of major wildfire events.

It is therefore now critically important that a coordinated management and conservation approach for the Koala across the State is implemented. This involves not only conserving habitat for individual Koala groups, but also ensuring that the genetic integrity of the species is maintained. It is imperative to manage Koalas at the landscape level to ensure that groups are interconnected and populations are large, rather than small and isolated clusters. In this respect, planning and land use instruments, and any associated mapping, must (1) reflect the current distribution of the species across a given region, (2) identify key areas that have become or are at risk of becoming disconnected, and (3) identify opportunities to restore connectivity between isolated patches/isolated groups of individuals. Genetic data can be used to provide information on the distribution and connectivity of populations, and should be used to inform future management and conservation strategies. Scat genetic studies should be undertaken across the State to obtain a broad understanding of the Koala population structuring across New South Wales. Such baseline information is now critical to direct future management and conservation of the Koala in the State. Furthermore, scat genetic studies can be used to assess and monitor the effectiveness of management actions, identify where corrective actions are required, and guide future on-ground conservation efforts.

10. STUDY LIMITATIONS

10.1 GEOGRAPHIC LIMITATIONS

This study and all spatial and genetic results included in this report, and interpretation thereof, are limited to the sites and the geographic areas of Port Stephens LGA investigated as part of this study, and to the viable Koala genetic profiles obtained (and the sampling locations associated with these individuals). The spatial and genetics results included in this report cannot be extrapolated to any other site or any other geographic area not investigated as part of this study.

10.2 KOALA SCAT DETECTABILITY

The use of purpose-bred, expertly raised and trained professional detection dogs minimises the risk of not detecting Koala scats when they are present. Professional detection dogs and their handlers are extensively trained by a professional detection canine expert. The dogs are then continuously trained and developed by their designated handlers, and the handlers and the dogs obtain professional certification once fully operational. Before deploying a detection dog in the field, OWAD thoroughly tests each dog. A dog is not deployed for project work until it consistently performs to 100% target detection rate and 100% scent discrimination rate (i.e. never indicates on non-targets) in field trials performed over several consecutive full days in the field.

Even though the professional detection dogs used in this study can perceive the scent of Koala scats or pap long after these have decomposed, they are purposely trained to not indicate on *target scent* alone. Instead, they are intentionally trained to indicate on and retrieve only *target objects*. This ensures that they do not indicate on Koala scats or pap that are so old that they have lost all structural integrity (hence no longer recognisable by humans), and that OWAD's findings are contemporary and inform only about current or recent Koala presence; not historical distribution. In this study, this corresponded to Koala scats or pap deposited up to a maximum of approximately 12 months prior to field survey.

10.3 SCAT ANALYSIS LIMITATIONS

10.3.1 Scat condition

The key limitation of current methods for extracting genetic material from Koala scats is that the scats need to be relatively fresh (12 weeks or less) and be in good physical condition. Wet or damp conditions and other environmental factors (e.g. mechanical abrasion, insect predation, etc.) may affect the quality and quantity of the target DNA (i.e. Koala, KoRV-A and *C. pecorum*) available for isolation.

10.3.2 Personnel collecting scats

There are several prerequisites in order for the personnel collecting scats to provide the laboratory with reasonably viable Koala genetic material for analysis purposes:

Ability to identify and differentiate Koala scats from other animal scats

OWAD personnel is highly experienced in identifying Koala droppings (scats or pap) and differentiating these from similar looking droppings originating from other animals (e.g. Possum species). Additionally, their professional detection canines receive extensive and ongoing discrimination training, and never indicate on any similar

looking droppings that don't originate from Koala[3]. Their discrimination rate is closely monitored all year round and is furthermore regularly put to the test by submitting samples to WildDNA. To date more than 4,000 Koala droppings have been submitted by OWAD to WildDNA for testing. To date, 100% of scats or pap samples tested have been confirmed as originating from Koala.

Appropriate timing for scat collection

Non-invasive Koala genetic sampling is ideally best conducted during dry periods in order to maximise viability of the genetic material. Unfortunately, two of the three sampling events were conducted in less than favourable conditions, but OWAD had to push ahead due a number of constraints. During the May 2020 sampling a significant rainfall event occurred. OWAD temporarily suspended fieldwork and resumed a few days after the rain eased.

Appropriate collection, storage and postage of scats

Field personnel followed the scat collection and storage procedures detailed in **Section 4.5**. Scat collection, storage and packing of the samples for postage was done with great care to minimise damage to genetic material from the time of collection in the field to reception by the WildDNA laboratory. In this study there was no loss of genetic material during postage and handling.

Ability of personnel to estimate the age and condition of Koala scats

Assessing the 'freshness' of scats and the ability to collect scats which are most likely to be suitable for genetic analysis can be challenging. OWAD is highly experienced in estimating the age and condition of Koala scats and prior to conducting this study had extensive previous experience performing numerous similar studies. In a number of instances in this study, scats were intentionally collected and sent to WildDNA despite OWAD estimating those scats might be of limited quality and possibly unviable for analysis. This was done in instances where these scats may be the 'best on offer' in a given area (i.e. limited Koala activity hence few fresh scats on offer), or when the location of these scats was of particular relevance for this study. Some of these degraded samples indeed failed to provide a reliable genetic profile, while others succeeded in providing a reliable genetic profile.

Ability to distinguish scats from putative distinct individuals

At each location where fresh scats are found, field personnel must be sufficiently experienced to make an educated guess as to whether these may originate from distinct individuals, as scats from distinct individuals should be placed in distinct collection kits. This instance occurred on three occasions during this study, with two presumed distinct individuals sampled under the same tree on three occasions (confirmed as six distinct individuals via genetic analysis).

[3] Note, both detection dogs used in this study also have the scat of three Quoll species as targets. However humans can easily differentiate Quoll scat from Koala droppings.

10.3.3 Chlamydia pecorum detection

A positive *C. pecorum* result from scat analysis indicates that the individual carries the bacterium, however this does not necessarily mean that the individual displays clinical symptoms of the disease.

A negative *C. pecorum* result from scat analysis does not necessarily mean that the individual does not carry the bacterium. Indeed, an individual could harbour *Chlamydia pecorum* without it being detectable in scats.

10.4 INDICATIVE KOALA ACTIVITY LEVELS

The indicative Koala activity levels presented in this report are indicative only and should not be viewed or interpreted as absolute. These values are only true for the time when each of the sites was sampled. Koala activity levels can vary over time, and do not represent estimates of Koala numbers. The only scientifically valid way of obtaining valid estimates of Koala numbers via scat is to have scats analysed and the individuals genetically profiled via a purposely designed mark-capture program.

The indicative Koala activity levels provided in this report are to be viewed and interpreted in conjunction with the genetics results included herein.

10.5 GENETIC RESULTS

All genetics data and results presented in this report are baseline data, based solely on the limited number of genetic profiles obtained to date from the region by OWAD. No previous genetic results and no previously collected genetic material were available from the region that could be used for this study, as this is the first study of the kind in the region. If the sample base is increased, more details about the fine-scale population structure or other processes may become apparent that could not be detected here due to restricted sample size and/or restricted number of geographic areas sampled within Port Stephens LGA.

10.6 LIMITATIONS OF DATA INTERPRETATION

The interpretations provided in this report are only valid indications for the areas and the individual Koalas sampled and profiled as part of this study.

The results included in this report are to be read and interpreted in conjunction with any future study into the genetics and health of Port Stephens LGA Koalas and/or nearby LGAs that may share Koala populations with Port Stephens LGA.

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APPENDIX 1
Laboratory test results for each individual Koala genetically profiled

Appendix 1: Koala profiling results, including sex and disease status for each individual Koala genetically profiled within Port Stephens LGA

Sampling location code(s) of unique individuals profiled	Geographic location	Sex	KoRV-A	*C. pecorum*
PS01	Balickera	Male	Detected	Detected
PS02	Balickera	Female	Detected	Detected
PS03, PS12	Balickera	Male	Detected	Not detected
PS04, PS09	Balickera	Male	Detected	Not detected
PS05	Balickera	Female	Detected	Not detected
PS06	Balickera	Female	Detected	Detected
PS07	Balickera	Female	Detected	Not detected
PS08	Balickera	Male	Detected	Not detected
PS10	Balickera	Male	Detected	Detected
PS11	Balickera	Male	Detected	Not detected
PS13, PS14, PS15, PS65	Peninsula	Female	Detected	Not detected
PS16	Peninsula	Male	Detected	Not detected
PS17	Peninsula	Female	Detected	Not detected
PS19	Peninsula	Female	Detected	Not detected
PS20	Peninsula	Female	Detected	Detected
PS21	Peninsula	Male	Detected	Not detected
PS24	Karuah	Male	Detected	Not detected
PS25	Karuah	Male	Detected	Not detected
PS26	Karuah	Male	Detected	Detected
PS27	Ferodale	Male	Detected	Detected
PS28	Ferodale	Female	Detected	Detected
PS29	Ferodale	Female	Detected	Not detected
PS30	Ferodale	Male	Detected	Not detected
PS31	Ferodale	Female	Detected	Detected
PS32	Ferodale	Male	Detected	Not detected
PS33, PS50	Peninsula	Female	Detected	Not detected
PS35	Peninsula	Female	Detected	Not detected
PS36	Peninsula	Male	Detected	Not detected
PS42	Ferodale	Female	Detected	Not detected
PS43	Ferodale	Female	Detected	Not detected
PS44	Ferodale	Female	Detected	Not detected
PS46	Ferodale	Female	Detected	Not detected
PS47	Peninsula	Male	Detected	Detected
PS52	Peninsula	Male	Detected	Detected
PS57	Peninsula	Male	Detected	Detected
PS59	Peninsula	Female	Detected	Detected
PS62	Peninsula	Male	Detected	Detected
PS63	Peninsula	Male	Detected	Not detected
PS64	Peninsula	Female	Detected	Not detected
TOTALS	**Balickera: 10 Karuah: 3 Ferodale: 10 Peninsula: 16**	**19 females 20 males**	**100% (39/39)**	**36% (14/39)**

APPENDIX 2
mtDNA control region haplotype network

Figure A2: mtDNA control region haplotype network

Each pie represents a different version of DNA sequence (haplotype) detected in the Port Stephens Koalas sampled for this study. Pies are scaled according to the number of samples for which a particular haplotype was detected. Colours within each pie show the frequency that each haplotype was detected in Koalas from each of the sampling locations. The two most common haplotypes, Pc1 and Pc4, were present within both coastal (Tomaree and Tilligerry) and inland (Karuah, Ferodale Balickera) Koalas sampled.

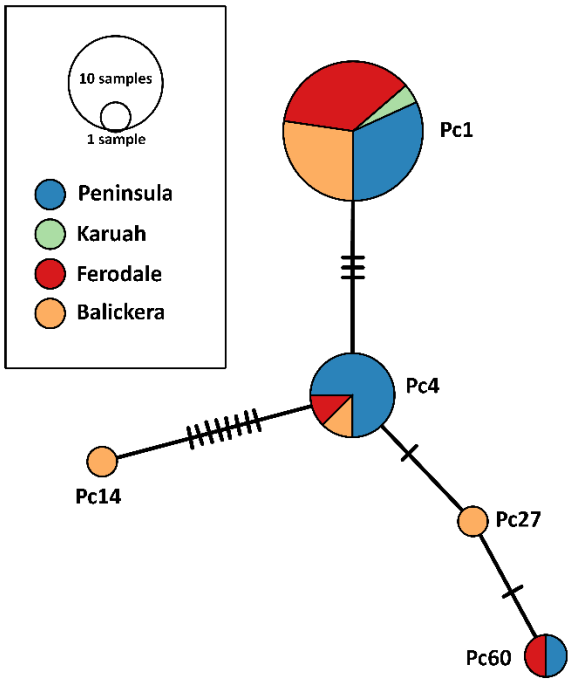

Table A2: Frequency of haplotypes among sample sites. Only samples with reliable genotype data and identified as a unique individual are included.

Haplotype	Peninsula (*n*=14)	Karuah (*n*=1)	Ferodale (*n*=10)	Balickera (*n*=9)
Pc1	7 (50%)	1 (100%)	8 (80%)	6 (67%)
Pc4	6 (43%)	–	1 (10%)	1 (11%)
Pc14	–	–	–	1 (11%)
Pc27	–	–	–	1 (11%)
Pc60	1 (7%)	–	1 (10%)	–