Combining molecular techniques with public outreach to better understand wild and captive populations of the short-beaked echidna for applications in their conservation and management

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December 2020

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Abstract

Short-beaked echidnas are iconic Australian animals, the most widespread native mammal and both ecologically and evolutionarily important. Despite this, we have limited information on most wild populations, except for the unique population on Kangaroo Island, which is currently listed as endangered due to feral predators, environmental changes and roadkill. Echidnas are also kept in many zoos and animal parks, but captive management and breeding has been challenging due to poor gastric health and the inability to determine the sex of juveniles. It is therefore critical to obtain more information about the biology and health of wild and captive echidnas in order to aid in conservation and captive management efforts.

Echidnas are notoriously difficult to study in the wild and their wide distribution makes it difficult to gather population data. To resolve this, we developed an Australia-wide citizen science project: Echidna Conservation Science Initiative (EchidnaCSI), where thousands of participants submitted sightings of echidnas and collected echidna scats for molecular analysis. This project has provided the largest baseline distribution database for echidnas and has successfully incorporated scat collection into a national citizen science project. EchidnaCSI also provided a platform to engage and educate the public on echidna biology and conservation.

Successful scat collection by participants enabled diet and gut microbiome analysis. The gut microbiome is recognised as a good indicator of health and gastrointestinal biology. Analysis of gut microbiomes of these echidna scats from across Australia and from Perth and Taronga Zoos showed for the first time that diet is a main driver for echidna gut microbial composition. Significant differences were observed between the wild and captive echidnas and also amongst echidnas fed different diets. Furthermore, this research revealed that plants are likely a larger part of echidnas' natural diet than has previously been recognised.

During December 2019 to January 2020, Kangaroo Island had its most devastating bushfires, burning almost half of the island. This resulted in an urgent call to action for fire recovery efforts for many endemic threatened species, including the unique population of echidnas. The very active participant and researcher base on Kangaroo Island allowed us to obtain and analyse samples before and after the bushfires. This research revealed for the first time that fires dramatically affect the composition of gut microbiomes in echidnas.

Captive breeding of echidnas has been challenging, however, better diet and housing in recent years has led to several successful breeding programs. A limitation in breeding efforts has been the inability to determine the sex of individuals, juvenile animals in particular. Here, we used our genetic expertise to develop a PCR-based sexing technique on DNA from hair samples using sex chromosome genes as markers. This allowed us to determine the sex of 10 juveniles born at Perth Zoo, aiding in their captive management.

This research shows how combining areas of molecular biology with public engagement can gain new insights into wild and captive echidna populations, ultimately, to aid in echidna conservation and better captive management.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed: ___ _____ Date: 11/12/2020

Tahlia Perry

Acknowledgements

Firstly, to my supervisor Frank. Words cannot describe how lucky I feel to have had you as a mentor, role model, teacher, and my biggest supporter. You have taught me more than I could ever have imagined, not only on how to be a great researcher, but how to be kind, collaborative and optimistic in an often competitive field. Thank you for always encouraging me to follow my passions and exploring new research areas. You never faltered your belief in my ability to achieve.

Thank you to the Australian Government for the Research Training Program scholarship and thank you to the several funding bodies who provided much needed support to enable this research: The Environment Institute, Foundation for National Parks & Wildlife, Nature Conservation Society of South Australia and the Nature Foundation of South Australia. Thank you to the Australian Federation of University Women – South Australia and Adelaide University Graduate Centre who provided travel scholarships, which enabled me to visit Cambridge, UK, for a metagenomics workshop and North Carolina, USA, for an international Citizen Science conference. These were invaluable for my training and networks required for my research.

To my lab members, who became my unofficial family, thank you for making the place we spend the majority of our time the most fun and supportive environment. Special thanks to Eunice, Ellen, Tash, Bella and Ella who have become some of my closest friends (and a special shoutout to amazing women in STEM). To Tas, thank you for guiding me through my first year of experiments; I know you often say I taught you as much as you taught me but that is definitely not true, your passion for research is inspiring.

Peggy, your knowledge and passion for echidnas is unbeatable. Thank you for teaching me everything I know about the natural history of these amazing animals and being such a caring and supportive mentor. I wouldn't have been able to communicate so effectively without your knowledge and I'm always stunned by how every time we talk, you still manage to shock me with another fact I didn't know about echidnas.

Thank you to every single person who has supported and participated in EchidnaCSI. To the EchidnaCSI team, who have made this project possible (Frank, Alan, Bella, Michelle, Ella, Imma & Mollie). To the citizen science community in South Australia who have guided and

supported this project from the very beginning. To every person who downloaded the app, told a friend about the project, submitted a sighting or collected a scat sample, you have turned my PhD into something I could not have imagined it would be. This project gave me the opportunity to explore another side of research that I absolutely adore, and that is communicating science.

To Rachel, Michelle and Raphael, thank you for taking the time to share resources, materials, and knowledge in the variety of fields that have now shaped my thesis. Your ongoing support and enthusiasm for my research is forever appreciated.

To all of my friends, thank you for ensuring I still lived a somewhat normal and fun life outside of the lab. My best friend Dee, we have shared many highs and lows through our PhD journeys and I truly couldn't have done it without you, thank you for being my coffee and wine buddy and for motivating me to get work done especially when it was hard. Kate, you have been my number one cheerleader ever since I met you, thank you for celebrating every single win with me, no matter how big or small. Special thanks to my housemates, Donny, Will and Renee who made coming home so much fun, who would eat, drink and watch awful reality TV shows with me and who regularly fed me when I was too busy or stressed to cook.

To my family, who have been waiting for the day to finally call me Dr, thank you for always believing that I can do anything I put my mind to. I'm so lucky to be surrounded by loving, caring, amazing people who are always so proud of my achievements. I may be the first in the family to get a University degree, but I certainly won't be the last.

Lastly, to my Mum, who I dedicate this thesis to. You are the strongest and most caring person I know. You have always sacrificed so much in order to provide me with the opportunities to pursue what I love and I will forever be grateful.

Manuscripts in Thesis

Chapter 2: EchidnaCSI: engaging the public in research and conservation of the short-beaked echidna
Authors: Perry, T[1], Stenhouse A[1], Wilson I[1], Perfetto I[1], McKelvey MW[2], Coulson M[1], Ankeny RA[3], Rismiller PD[1,2], Grützner F[1]
Status: Submitted to Biological Conservation

Chapter 3: Characterising the gut microbiomes in wild and captive short-beaked echidnas reveals diet-associated changes
Authors: Perry T[1], West E[1], Eisenhofer R[4], Stenhouse A[1], Wilson I[1], Laming B[5], Rismiller PD[1,2], Shaw M[6], Grützner F[1]
Status: Submitted to Frontiers in Microbiology

Chapter 4: Bushfires significantly impact the gut microbiomes of short-beaked echidnas on Kangaroo Island
Authors: Perry T[1], Lu A[1], McKelvey MW[2], Rismiller PD[1,2], Grützner F[1]
Status: Submitted to Australian Mammalogy

Chapter 5: Non-invasive genetic sexing technique for analysis of short-beaked echidna (*Tachyglossus aculeatus ssp*) populations
Authors: Perry T[1], Toledo-Flores D[1], Kang WX[1], Ferguson A[5], Laming B[5], Tsend-Ayush E[1], Lim SL[1], Grützner F[1]
Status: Published in Reproduction, Fertility and Development

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Chapter One: Introduction

Short-beaked echidnas (*Tachyglossus aculeatus*) are unique, egg-laying mammals, an iconic Australia species and beloved by the public. Echidnas are the most widespread native mammal in Australia, surviving in diverse environments, from desert, to temperate and snowy alpine regions (Rismiller and Grutzner, 2019). However, due to their cryptic nature, we lack information on most wild populations, which is a concern for their conservation. Echidnas are also commonly held in captivity across the world where they have historically had poor nutritional health and low success with breeding (Ferguson and Turner, 2012; Stannard et al., 2017). It is critical we rapidly gather more information on the biology, health and distribution of echidnas, which requires a multidisciplinary approach. Incorporating molecular studies into wildlife research is becoming increasingly popular as genomic techniques can elucidate new biological information about an animal and often be used on non-invasively collected samples, such as faeces and hair (Waits and Paetkau, 2005). Microbiome studies, in particular, can shed light on both wild and captive animals' gastrointestinal biology, diet and health (Barko et al., 2018; McKenzie et al., 2017). As echidnas cover a vast geographic area, it requires a significant effort to collect data and material for their wild populations, which can be achieved through large-scale citizen science approaches (Dickinson et al., 2012). Not only can community-based research aid in rapid assessment and investigation of echidnas but provide an opportunity to educate and empower the public for the conservation of an iconic Australian mammal.

Here, I cover the remarkable biology of echidnas and the challenges associated with their conservation and captive management. Next, I explore how the rapid advances in genomic technologies provide opportunities for gathering biological information for applications in wildlife conservation. Lastly, I discuss the area of citizen science, which is a rapidly growing approach for assessing wildlife biodiversity and a great platform for public engagement in science.

History and Unique Biology of Monotremes

The short-beaked echidna is one of only three genera of egg-laying mammals (monotremes), which diverged from therian mammals (eutherians and marsupials) approximately 187 million years ago (MYA; Phillips et al., 2009; Zhou et al., 2020). Alongside the short-beaked echidna is three species of long-beaked echidna (*Zaglossus attenboroughi, Z. bartoni and Z. brujini*) and one species of platypus (*Ornithorhynchus anatinus*). Echidnas and platypuses diverged approximately 55 MYA, while there are currently no divergence date estimates between short-beaked and long-beaked echidnas (Figure 1; Zhou et al., 2020). Monotremes form an important

evolutionary group as the longest surviving mammals, with unique combinations of biological and morphological characteristics that have fascinated western scientists since the first specimen was obtained. In fact, when the platypus was first sent to British scientists in the late 18th century, they thought it was a hoax due to the unusual combination of traits (Hall, 1999). It then took scientists more than 100 years to show that monotremes did indeed lay eggs rather than give birth to live young. Surprisingly, two naturalists (Wilhelm Haacke and William Caldwell) on the same day (24th of August 1884) separately reported that echidnas and platypuses lay eggs (Caldwell and Foster, 1887; Haacke, 1885); with Caldwell's famous telegram wired to London: "Monotremes oviparous, ovum meroblastic" (Hall, 1999). The unusual biology of monotremes is not limited to them being the only mammals to lay eggs, but they are named after having a cloaca (monotreme means 'one hole'), which acts as both the gastrointestinal and reproductive opening (Rismiller and Grutzner, 2019). Monotremes also have a lower body temperature than other mammals, generally sitting at 32 °C, while echidnas can regulate their daily temperature to as low at 5 °C as they undergo torpor (Grant, 1983; Grigg et al., 1992; Nicol and Andersen, 2007). However, like therian mammals, monotremes have fur, produce milk (but lack nipples) and develop a simple placenta during their short gestation period (Niwa et al., 2008). Although monotremes share these traits, short-beaked echidnas (hereafter referred to as 'echidna') have their own unique biology in comparison to the long-beaked echidna and the platypus.

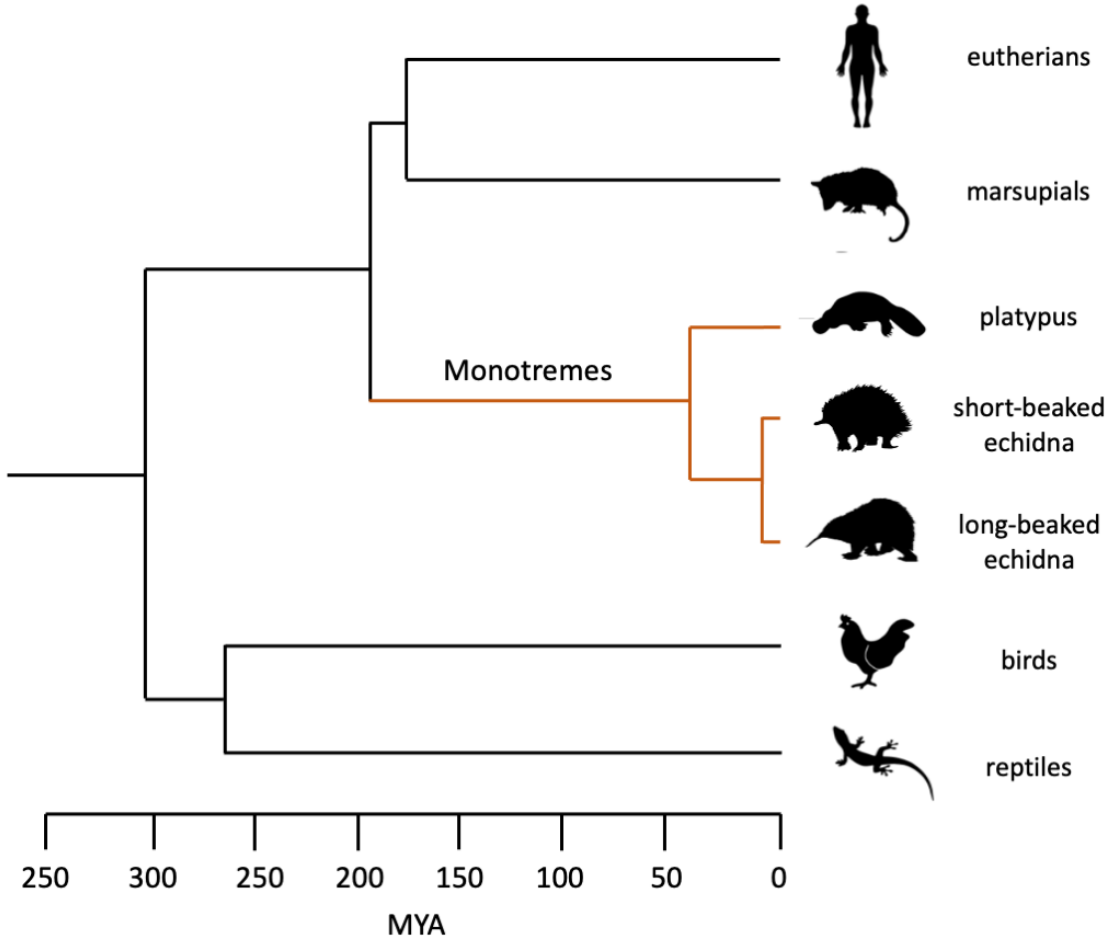

Figure 1: Phylogenetic relationships within and between monotremes. The monotreme lineage is highlighted in orange. MYA = Million Years Ago. Figure adapted from Krubitzer and Campi., 2009.

Echidnas: Extraordinary Mammals

Echidnas are well-adapted to Australia, where they can survive across a large variety of environments (i.e. desert, alpine, tropical, temperate, coastal) and are also found in Papua New Guinea (Rismiller and Grutzner, 2019). Five subspecies of echidnas have been characterised based on geographic location and some physical attributes such as fur density, spine diameter and toe length (Griffiths, 1978; Rismiller and Grutzner, 2019). While echidnas are well adapted and have survived the harsh Australian habitats there are threats to echidnas. Echidna young are vulnerable once they are dropped in a burrow and goannas and snakes are known predators (Augee et al., 2006; Overton, 1987; Rismiller and McKelvey, 2000). For adult echidnas, spines and digging into the substrate are effective defence mechanisms, however, larger birds of prey and carnivorous mammals like dingoes, foxes, cats and Tasmanian devils, are capable to overcome these defence mechanisms (Augee et al., 2006; Rismiller, 1999). Echidnas are highly

cryptic animals, will shelter in a range of locations including native vegetation, caves, other animals burrows (such as rabbits and wombats) or self-dug burrows (Rismiller, 1992), and can have home-ranges up to 192 hectares, depending on the environment (Abensperg-Traun, 1991; Augee et al., 1992; Sprent and Nicol, 2012).

Although ubiquitous across Australia, echidnas are very difficult to study in the wild as they are not attracted to baits, sound or movement (Rismiller and Grutzner, 2019). Most of what we understand about their natural history comes from observational work over the past 30 years in only two locations: Kangaroo Island and parts of Tasmania. Echidnas are most active throughout breeding season (June – September each year); during this time echidnas form mating 'trains' where one female is followed by two or more males following her for several weeks in an attempt to mate with the female (Figure 2A; Harris et al., 2014; Rismiller and McKelvey, 2000). The female will typically only mate one time during breeding season, although it has been observed in Tasmania some males will disturb a female to induce infanticide so that this new male can mate with her instead (Harris and Nicol, 2014). The female will be pregnant for approximately 23 days before she lays her egg (Figure 2B) directly into a pseudo-pouch, which forms during pregnancy, caused by the swelling of their mammary glands (Griffiths, 1968; Nicol and Morrow, 2012; Rismiller and McKelvey, 2000). The juvenile echidna (called a puggle) will hatch from the egg just 10 days later, extremely altricial with only its forearms well developed (Figure 2C; Griffiths et al., 1969). Echidnas lack nipples, so the puggle will use its snout to massage the mammary glands until milk emerges and form what are named 'milk patches' that the puggle then suckles the milk from (Figure 2D) (Figure 2D; Rismiller and Grutzner, 2019). For echidnas on Kangaroo Island, it is observed that the young stay in the mother's pouch until it is ~50 days old when it starts to produce spines, at this time, the mother will then place the puggle in a nursery burrow only returning once every five days to feed it milk until weaning at 204-210 days of age (Griffiths, 1978; Rismiller and McKelvey, 2000). After weaning, echidnas live a solitary lifestyle until reaching sexual maturity for breeding (at 5-12 years of age; Rismiller and McKelvey, 2003).

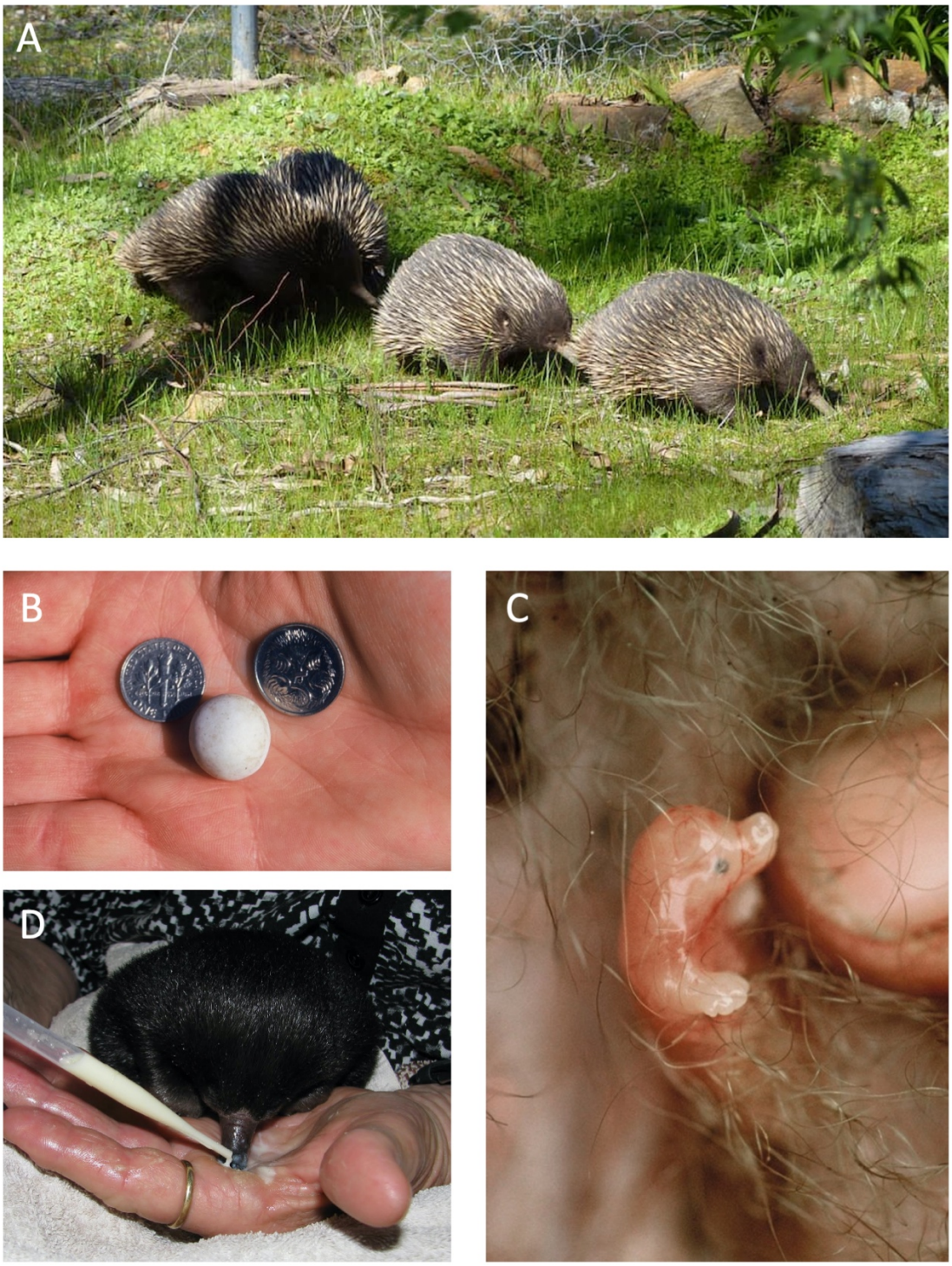

Figure 2: Life stages of the echidna. A) Mating trains form during breeding season (June – September) where one female leads, and several males follow. B) Echidna egg, showing the approximate size in comparison to an Australian 5-cent coin. C) Newley hatched echidna puggle, showing well-developed forearms and size in comparison to human thumb nail. D) Juvenile echidna being hand-raised; to feed the young, milk is pipetted into the palm of hand to resemble 'milk patches' where the juvenile then suckles the milk from. Photos A & D provided by EchidnaCSI; Photos B & C provided by Dr Peggy Rismiller.

Current Issues in Echidna Conservation and Captive Breeding

Short-beaked echidnas are considered 'least concern' across Australia according to the IUCN Redlist Database (International Union for Conservation of Nature (IUCN), http://www.iucnredlist.org, accessed 18 November 2020). However, the best studied population, which is a unique subspecies endemic to Kangaroo Island (*T. a. multiaculeatus*), is now EPBC listed as 'endangered' (EPBC Act 2015, ''Conservation Advice Tachyglossus aculeatus multiaculeatus Kangaroo Island echidna''). The listing was made due to several threats including feral cats, roadkill and habitat fragmentation. These threats (and others such as foxes and dingoes) exist on mainland Australia and so it is likely echidna populations are under a greater threat than is currently recognised. Furthermore, with climate change causing more regular and consecutive hot days, echidnas are not likely to fare well in these changing conditions due to their naturally lower body temperatures (Nicol and Andersen, 2007). The increased frequency of hotter, drier days is also leading to more intense bushfires, with Australia experiencing Black Summer from July 2019 to February 2020, which was its most devastating fire season yet (Ward et al., 2020). Echidna populations certainly overlap with these fire affected regions, including the already endangered Kangaroo Island population, which has been added to an urgent fire-recovery list for threatened species (Department of Agriculture, Water & Environment 2020, 'Rapid analysis of impacts of the 2019-20 fires on animal species, and prioritisation of species for management response'). It is important now more than ever to better understand wild echidna populations and to develop conservation approaches to ensure the survival of this unique and iconic Australian species.

Zoos have recognised the importance of echidnas, as one of the keystone species for conservation and captive breeding (Kerr, 2020). Captive breeding has been notoriously difficult for echidnas, however, with recent insights into how echidnas breed in the wild, zoos are able to prepare their breeding programs better, resulting in more successful births (Ferguson and Turner, 2012). Perth Zoo, Taronga Zoo and Currumbin Wildlife Sanctuary have been the most successful at implementing captive breeding programs in Australia so far. Since 2010, Perth Zoo has had 10 captive born echidnas (Perry et al., 2019), in 2015 Currumbin Wildlife Sanctuary reported 13 births from 2011 to 2014 (Wallage et al., 2015), and Taronga Zoo has publicly announced four births between 2016 and 2018. The success of the programs has been attributed to additions of underground boxes acting as nursery burrows and pairing individual males and females who are kept in an isolated area, as echidnas (especially females) are usually solitary in the wild (Rismiller, 1992). Housing echidnas in captivity has not gone without its difficulties though. There is no easy way to determine the sex of an adult echidna and almost impossible for juveniles, which has hindered breeding programs (Perry et al., 2019).

Furthermore, artificial diets fed to echidnas have in the past lead to many health issues, including diarrhoea, gastritis, cystitis, and obesity (Stannard et al., 2017). These diets were initially based off of carnivore (cat and dog) models as this was thought to best to represent an insectivore's nutritional needs. Recently, new diets have been created that better reflect the echidnas natural diet, by increasing protein and providing extra fibre (Stannard et al., 2017). In order for zoos to provide the best management for echidnas, we need to continue to gather information on their biology both in the wild and in captivity. As it has proven difficult to gather new information quickly using typical ecological studies, the employment of molecular approaches is another avenue to explore.

Fascinating Molecular Biology in Monotremes

Initial interest in monotremes' molecular biology began with their karyotypes, as their chromosomes seemed to be a mix of micro- and macro-chromosomes, as seen in the chicken. Later, it was discovered that the smaller chromosomes were actually unpaired sex chromosomes that form a chain during meiosis (Grützner et al., 2004; Murtagh, 1977; Rens et al., 2004). Unlike any other mammal, male echidnas have 5X and 4Y chromosomes (females 10X), while platypus males have 5X and 5Y chromosomes (females 10X). Chromosome painting showed that the chains differ between the echidna and platypus by both order and constitution, indicating that their sex chromosomes have continued to evolve after the echidna and platypus diverged (Figure 3; Rens et al. 2007),. Monotreme sex chromosomes share no homology with human sex chromosomes (Grützner et al., 2004; Watson et al., 1990) and it is still unknown what the monotreme sex determining gene is. However, due to new genomic technologies, such as Next Generation Sequencing, we are rapidly gaining new knowledge on monotreme genomics and sex chromosomes. The platypus genome was sequenced in 2008 (Warren et al., 2008), followed by transcriptomic studies, revealing gene content for Y chromosomes, which led to the candidate sex-determining gene AMHY (Anti-Müllerian hormone Y-gametalog; Cortez et al., 2014). Now, through combination of new sequencing technologies such as PacBio, Illumina and Hi-C sequencing, we have a chromosome-level genome for the platypus and a draft genome of the echidna (Zhou et al., 2020), opening more avenues for genomic research for these animals.

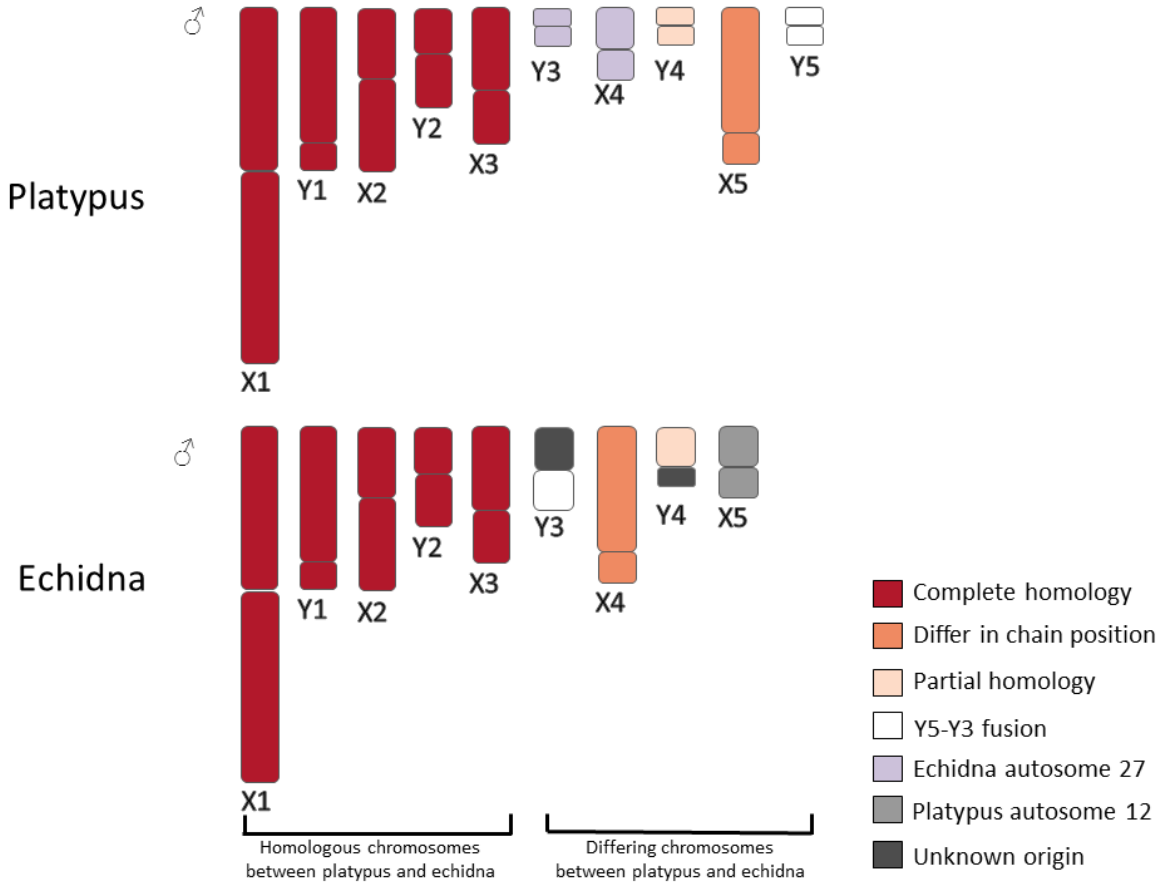

Figure 3: Schematic of male sex chromosome system for platypus and echidna. Chromosomes are coloured based on their homology; complete homology exists for chromosomes X1, Y1, X2, Y2, X3 (maroon); orange chromosomes are homologous but occupy different positions in the meiotic chain; Y4 is partially homologous between platypus and echidna (pink); Y5 in platypus has fused to Y3 in the echidna (white); mauve platypus chromosomes maps to echidna autosome 27; echidna X5 maps to platypus autosome 12 (light grey); dark grey regions of echidna sex chromosomes have unknown origins.

The Genomic Revolution in Wildlife Conservation

Over the past decade there has been a genetic and genomic revolution in wildlife conservation, due to the advances in high-throughput sequencing technologies, which can be applied to non-model organisms (Barbosa et al., 2021; Hohenlohe et al., 2020). This has democratised the field of genomics, allowing research into any organism, including wild populations of rare or difficult-to-study species (Rajora, 2019; Supple and Shapiro, 2018). Genomics can be applied to many areas of conservation, such as assessing population size and connectivity, detecting inbreeding or hybridisation, determining sex ratios in the wild or captivity, understanding traits that allow populations to survive in certain environments, and assessing the potential for populations to survive with changing environments (Hohenlohe et al., 2020; Liu et al., 2014; Romiguer et al.,2014; Postma et al., 2011). Furthermore, the use of genomics techniques can

be applied to non-invasively collected samples such as faeces or hair, creating even more opportunities to study elusive species, as this removes the requirement of the animal being tracked or captured (Piggott and Taylor, 2003; Waits and Paetkau, 2005). By assessing an animal's faeces, even more areas of an organism's biology can be investigated, such as diet and bacterial communities present in the gut (Bohmann et al., 2018; Brice et al., 2019; Iversen et al., 2013).

Microbiome Research in Conservation and Captive management

Microorganisms, consisting of bacteria, fungi and viruses, live on and within all living organisms. The composition of all genetic components of these microorganisms and the surrounding environment is characterised as the microbiome (Marchesi and Ravel, 2015). The recent growth in microbiome studies can also be attributed to high-throughput DNA sequencing technologies allowing quick and cost affective characterisation of microbial communities, through 16S metabarcoding (Bahrndorff et al., 2016). The Human Microbiome Project, which was initiated in 2007, estimated that the human microbiome contains 100x more genes than its host and highlighted the immense microbial diversity and complexity that exists across multiple bodily niches (Turnbaugh et al., 2007). The microbiome has been shown to play a significant role in human health, with microbial dysbiosis associated with diseases such as diabetes, obesity, bowel disease and asthma (Cho and Blaser, 2012; Frank et al., 2007; Turnbaugh et al., 2006; Wen et al., 2008). Due to the important relationships between microbial communities and their host, recent studies have started characterising and assessing the microbiomes in many nonhuman vertebrates, with particular attention on how these studies can be applied to conservation (Trevelline et al., 2019; West et al., 2019).

The most extensively studied region in both humans and other animals is the gut microbiome, which is commonly characterised using faecal samples. The gut microbiome is mostly influenced by an animal's diet and/or their phylogeny. For example, most mammals have been shown to cluster depending on whether they are a carnivore, herbivore or omnivore (Ley et al., 2008). However, the giant panda's gut microbiome still clusters with carnivorous bears even though their diet is mostly bamboo (Guo et al., 2018). By characterising the gut microbiomes of wild animal populations, many studies have been able to assess how the microbial communities change when animals are fed different diets or housed in different environments, such as when they are in captive settings such as zoos (Alfano et al., 2015; Haworth et al., 2019; McKenzie et al., 2017). Recently, studies have even begun to assess if gut microbiomes can recover when animals are released from captivity back into the wild (Chong et al., 2019).

Microbiome studies are becoming a powerful genetic approach to assess both wild and captive communities, often for conservation purposes.

The Power of Citizen Science

In order to perform population-level genomic studies on a species, significant sampling effort is required. Furthermore, accurate assessment of population size, distribution, biology and health are all required in order to determine conservation risk, which again requires a large amount of data. Citizen science is a rapidly growing field in order to gather population-level information, often for for biodiversity and conservation reasons (Irwin, 2018), whereby the general public aid in collecting or analysing data. Citizen science has the potential to enlist thousands of members of the general public to collect data over large geographic and time scales, which is not usually possible for researchers without significant time and costs associated (Fairclough et al., 2014). Citizen science is rapidly growing in Australia, with many successful national and local projects underway (Englefield et al., 2020; Rowley et al., 2019; Skelton et al., 2019). Advances in technology have improved the reliability of citizen science contributions to research due to increased accuracy in data validation (Kelling et al., 2015; Willett et al., 2010). While social media have been extraordinarily powerful in advancing community-based research as they facilitate engagement of new and existing audiences over large geographic scales (Graham et al., 2011; Liberatore et al., 2018). Importantly, citizen science is an effective engagement platform that can be used to increase the public's knowledge of science and raise awareness for environmental issues, which is also an important part for species conservation (Dickinson et al., 2012; McKinley et al., 2017). The potential for using citizen science in echidna research and conservation is therefore exciting.

Aims

The aims throughout my PhD were to develop and apply small-scale and large-scale genetic tools to advance our understanding of echidna populations and health using a combination of molecular biology and public outreach, ultimately to aid in echidna conservation and management. Specifically, I aimed to:

1) Develop, manage and analyse an Australia-wide citizen science project in order to gather baseline distribution data and collect echidna scats for molecular analysis (Chapter 2).

2) Develop and apply methods for DNA isolation from echidna scats for diet and microbiome analysis in wild and captive populations (Chapters 3 & 4).

3) Develop and apply a non-invasive genetic sexing technique to aid in echidna captive management and breeding (Chapter 5).

This research provides the basis for using molecular techniques in echidna conservation and captive management by successfully combining genetics and microbiology with citizen science.

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Chapter Two:

EchidnaCSI: engaging the public in research and conservation of the short-beaked echidna

Statement of Authorship

Title of Paper	EchidnaCSI: engaging the public in research and conservation of the short-beaked echidna
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Submitted to Biological Conservation

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Name of Principal Author (Candidate)	Tahlia Perry		
Contribution to the Paper	Project design, established methods, sample processing, data analysis, created figures and wrote manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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EchidnaCSI: engaging the public in research and conservation of the short-beaked echidna

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Abstract

The short-beaked echidna is an iconic Australian animal and the most widespread native mammal, inhabiting diverse environments including deserts, rainforests and alpine regions. The cryptic nature of echidnas has limited research into their ecology and conservation needs. In this paper, we present data obtained over three years running the Echidna Conservation Science Initiative (EchidnaCSI) citizen project that we established to obtain sighting data and scat samples from echidna populations Australia-wide. EchidnaCSI encourages members of the public to submit photographs of wild echidnas and to learn to identify and collect echidna scats for molecular analysis. In order to facilitate participation, we developed an app and implemented ongoing social media and traditional media activities. In three years, more than 9000 members of the public have downloaded the EchidnaCSI app, collecting 400 scats and submitting >8000 sightings of echidnas from across Australia. Scats were confirmed as echidna both visually and by polymerase chain reaction of an echidna-specific gene following DNA extraction, validating the approach of using citizen science for scat collection and viability for molecular analysis. To assess the impact of the project through public participation, we surveyed our participants (n = 944) to understand the users' demographics and motivations for engagement. Survey results also revealed that EchidnaCSI served as a gateway into citizen science more generally for many participants. EchidnaCSI demonstrates the potential for using citizen science approaches to collect data and material from a cryptic species over a very large geographic area and the potential educational value of community-based research.

Key words: citizen science, public engagement, mammal, Australia, scat, DNA

1. Introduction

The short-beaked echidna (*Tachyglossus aculeatus ssp*) is one of Australia's most iconic mammals and is of both evolutionary and ecological importance. Echidnas and platypuses form the unique group of egg-laying mammals (monotremes), which is the most ancient surviving mammalian lineage, diverging from all other mammals approximately 190 million years ago (Phillips et al., 2009;). The short-beaked echidna (hereby referred to as 'echidna') is characterised into five subspecies found in Australia and parts of New Guinea and is the most widespread native Australian mammal, inhabiting environments from desert to temperate and snowy alpine regions (Griffiths, 1978; Rismiller and Grutzner, 2019). Despite this, we lack basic population information as they are a highly cryptic species, making echidnas difficult to study in the wild (Nicol and Andersen, 2007; Rismiller and McKelvey, 2003). Due to lack of information, the short-beaked echidna is considered 'least concern' according to the IUCN Red List database (http://www.iucnredlist.org, accessed 13 November 2020). However, the subspecies *T. a. multiaculeatus*, which inhabits Kangaroo Island (a large island off the coast of South Australia) has recently been listed as 'endangered' under the EPBC Act 2015 ('Conservation Advice *Tachyglossus aculeatus multiaculeatus* Kangaroo Island echidna'). The greatest threats to echidnas, like many Australian mammals, are feral cats, roadkill and habitat loss (Rismiller and McKelvey, 2000). These threats exist on mainland Australia (along with additional predators such as foxes and dingoes) and have been further exacerbated by the recent devastating Australian bushfires in 2019 and 2020 (Ward et al., 2020). It is therefore a matter of urgency to obtain more information to determine the conservation status of echidnas across Australia. As far as we are aware there are no concerted efforts in place to ascertain and monitor echidna populations. As echidnas are difficult to study in the wild, gaining Australia-wide information on their populations is a very challenging task. Echidna sightings have been reported in the past via paper-based reports to citizen science 'Echidna Watch' projects hosted by Wildlife Queensland (https://wildlife.org.au/echidnawatch/, accessed 13 November 2020), and by leading echidna ecologist Dr Peggy Rismiller, which shows promise for using these types of approaches for nationwide and more coordinated data collection long term.

The citizen science approach is increasingly recognised as producing valuable and large datasets in environmental biology as well as other fields (Irwin, 2018). Citizen science is an excellent platform for research, as it has the potential to enlist thousands of members of the general public to collect data over large geographic and time scales, which is not usually possible for researchers without significant time and costs associated (Fairclough et al., 2014). Secondly, advances in technology have improved the reliability of citizen science contributions to research due to increased accuracy in data validation (Kelling et al., 2009; Willett et al.,

2010). The level of engagement of the community can be different, ranging from co-designing to the most common "Contributory" projects, where participants collect or analyse data for projects designed by researchers (Shirk et al., 2012). Many contributory projects focus on data collection of plants and animals for biodiversity and conservation purposes (Battersby and Greenwood, 2004; Gorta et al., 2019; Matteson et al., 2012; Pecl et al., 2017; Sardà-Palomera et al., 2012). The ubiquitous smartphone has revolutionised this approach and many projects use apps that allow photos to be taken on a smartphone, providing validation and additional information (date, time, location), as well as limiting user error in data submission (Ellul et al., 2013; Luna et al., 2018; Singh et al., 2018). Apps can also be linked directly with larger databases (in Australia the Atlas of Living Australia: http://www.ala.org.au, accessed 13 November 2020) allowing accessibility to the wider community. Social media have also been extraordinarily powerful in advancing community-based research. Effective use of social media (i.e. Facebook) and email lists facilitate engagement of new and existing audiences over large geographic scales (such as entire countries) (Graham et al., 2011; Liberatore et al., 2018). Importantly, citizen science is an effective engagement platform that can be used to increase the public's knowledge of science and raise awareness for environmental issues and conservation (Dickinson et al., 2012; McKinley et al., 2017).

A critical part of designing a successful citizen science project is how to recruit participation and sustain engagement over time. In order to ensure good communication and outreach for targeted audiences, many citizen science projects have evaluated the demographics and motivations of their participants through surveys in order to gain a deeper understanding of why they volunteer their time for certain scientific tasks (Domroese and Johnson, 2017; Land-Zandstra et al., 2016; Tinati et al., 2017). By evaluating how project participants rank these motivations, project leaders can better implement targeted strategies to increase engagement and more diversity in participation (Domroese and Johnson, 2017). It is important to engage diverse audiences in citizen science, especially for biodiversity and conservation type projects, as direct involvement can empower individuals to make significant changes in their attitudes and behaviours around environmental and sustainability issues (Cooper et al., 2007; Novacek, 2008).

A high proportion of citizen science projects collect sightings of one or multiple species of interest. These studies have led to important ecological milestones such as gaining baseline population information (Sullivan et al., 2010), finding new pockets of habitat (Ashcroft et al., 2012), showing distribution changes (Wilson et al., 2013), or declines in species numbers (Gorta et al., 2019), ultimately leading to tangible conservation outcomes (Devictor et al.,

2010). An exciting new avenue of citizen science is incorporating material collection for analyses (such as genomic or microbiome studies), which would greatly increase the information that can be obtained from the species of interest (Biggs et al., 2015; Chauhan et al., 2020; Hulcr et al., 2012; McDonald et al., 2018). Animal's scat (faeces) contains DNA and hormones that can provide information about the animal's sex, population genetics, diet, stress level, microbiome gut health and reproductive activity (Barba et al., 2014; Dallas et al., 2003; Rolland et al., 2005; Sheriff et al., 2011; Yildirim et al., 2010). Analysing animal scats has become increasingly used in field studies over the past 20 years due to new technologies and more robust techniques, which allow valuable information about an animal to be gained in a non-invasive way (Browett et al., 2020). However, faecal material collection from animals is rarely used in citizen science projects and thus could provide a new and powerful avenue for wildlife research.

With the Echidna Conservation Science Initiative (EchidnaCSI), we created a citizen science project that incorporates both echidna sighting submission and scat collection in order to begin Australia-wide research on echidnas for conservation purposes. After two years, we surveyed participants of the project in order to determine the demographics and motivations behind their participation. Here, we provide an overview of EchidnaCSI, where our aims were to (1) generate the largest database for echidna sightings in order to develop a baseline distribution map to track population changes in the future; (2) collect echidna scats from across Australia and validate their use for future molecular work in order to show the feasibility of incorporating scat collection in large-scale citizen science efforts; (3) engage the public in scientific research which stressed the importance of echidna conservation; and (4) determine current participants' demographics and motivations in order to evaluate the project and develop strategies to increase future public engagement and participation.

2. Materials and Methods

2.1 Data and sample collection

EchidnaCSI collects data via a smartphone app using both iOS (Apple, Cupertino, California, USA) and Android (Google, Mountain View, California, USA) operating systems. Three main functions exist within the app: First, users can submit a live photo of an echidna, where the app collects the date, time and GPS location of the photo; Second, users can submit photos of echidnas that they have previously taken on their smartphones, so long as the photo has the date, time and location data embedded within the photo. Following the taking or selection of the photo, the app then guides the user through questions about the echidna itself, such as whether it was alive or dead, what activity it was doing, approximately how large it was, and what environment it was in. Finally, users can submit any scats that they collect, which requires them to take a photo of the scat at the time and location of collection, again to capture the related metadata associated with the collection. Participants are encouraged to collect scats if they are long, cylindrical in shape, dry in texture, and mostly composed of soil and insect exoskeletons. Once the photo is taken, the app then guides the user on how to collect the scat, such as placing the scat in a plastic bag without touching it with bare hands (to avoid contamination of the scat), and then placing the scat in a freezer until ready to send to the University of Adelaide. Users can submit data immediately in areas with mobile data or internet access; if out of range, the data can be submitted later by selecting a specific 'upload' button within the app, or if the next data submission occurs within phone data range then all previous submissions will be uploaded along with the current submission. For participants who could not or did not wish to use the smartphone app, an online submission form was created through the Atlas of Living Australia's BioCollect platform (http://www.ala.org.au/biocollect, accessed 13 November 2020). The online form allows users to submit both sightings and scat collections by uploading a photo and self-selecting the GPS location, along with answering the same sets of questions embedded within the EchidnaCSI app.

2.2 Communication and Engagement

EchidnaCSI was launched with a media release from the University of Adelaide and nationally televised interviews of lead researchers in September 2017. Following this, regular media engagements have further advertised the project. Over three years, EchidnaCSI has been the topic of >40 radio interviews, two television appearances, and >50 newspaper articles or online blog posts. Leaders of EchidnaCSI have also participated in 20 in-person talks within South Australia. A dedicated webpage was created for hosting information about EchidnaCSI,

including how to use the app, what the research was aiming to achieve and FAQs. Facebook, Twitter and Instagram accounts were created and updated at least weekly with information about the project and to share photos and videos that participants had submitted. When a participant downloads the EchidnaCSI app, there is the option to submit user contact details such as name, email address and postcode. The users' email addresses were used to send a welcome email with links to the EchidnaCSI webpage and social media pages, and to send updates about the project via an e-newsletter. Scat identification information and images are embedded in the app itself, as well as on the EchidnaCSI webpage and social media channels.

2.3 Survey

The survey was designed and run through Qualtrics software (Provo, UT). A link to the survey was sent to 5720 registered users via email and posted on all EchidnaCSI social media accounts (Facebook, Twitter and Instagram) on the 22nd August 2019. The survey was active for approximately 3 weeks, closing on the 8th of September 2019 and participants were incentivised to complete the survey with the chance of winning one of five $50 gift cards. Human ethics clearance was obtained through the University of Adelaide (HREC-2019-156). Survey participants were required to be 18 years or older to participate and submission of the survey acted as user consent. The survey contained questions regarding their demographics and motivations; most questions were multiple choice, Likert-scale or open answer (Supplementary File 1).

2.4 Scat DNA extraction and PCR

Total genomic DNA was extracted from scat samples using the Qiagen QIAamp Mini Stool Kit (Qiagen, Hilden, Germany) as per the manufacture's protocol. The extractions took place in a Flow Cabinet Biological Safe Level 2 that was cleaned with 10% bleach (sodium hypochlorite) to reduce contamination. Approximately a third of the sample was crushed up in the presence of liquid nitrogen using a mortar and pestle, prior to adding the sample to InhibitX Buffer and then processed according to the manufacturer's instructions.

Samples were PCR amplified using primers designed to specifically target a unique region of the echidna mitochondrial dloop (Summerell et al., 2019). DNA was amplified with the primer pair Forward 5'-TGCATTCATCTTTTATCCCCATAC-3' and Reverse 5'-TAATCTGTCAGAACCTCAATTATG -3'. Single reactions of 18.9 µL dH2O, 2.5 µL 10X buffer, 1 µL 50mM $MgCl_2$, 0.1 µL IMMOLASE Polymerase (Bioline), 0.5 µL 10 mM dNTP, mix, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer and 1 µL DNA. DNA

was amplified using an initial denaturation at 96 °C for 10 min, followed by 35 cycles of denaturation at 96 °C for 30 sec, annealing at 50 °C for 1 min, elongation at 72 °C for 2 min, with final adenylation for 7 min at 72 °C. To validate that the primers amplify on DNA extracted from scats, each round of PCR also contained a positive control, where genomic DNA from echidna liver was used, as well as a negative control. PCR product size (200bp) was check by gel electrophoresis (2.5% agarose).

3. Results

3.1 EchidnaCSI Participation and Engagement

From the launch of EchidnaCSI on 4th September 2017 until 4th September 2020, EchidnaCSI had 9079 downloads of the dedicated app, resulting in the submission of 8090 echidna sightings and 406 echidna scats. A total of 2816 users had submitted data (either photo sighting or scat collection); most participants (56.8%) had submitted data once, 33.2% had submitted data between 2-5 times, and 10% had submitted data more than five times. Although there was consistent increase of app downloads, Facebook Likes and data submissions, large increases in app downloads were mostly associated with nationwide media broadcasts, in particular news articles (Table S1; Figure 1A), while increases in Facebook Likes were associated with viral social media posts. Data submissions varied from 0-30 submissions per day, with a cyclic trend (Figure 1B). Although echidnas are most active during breeding season (June-September), these were not the months with the highest data submissions. Instead, September to February were the most active months, which is the Australian Spring and Summer/holiday season, where we suspect participants are outdoors more often to submit sightings of echidnas.

Main forms of communication with participants were through email newsletters and a Facebook page (Twitter and Instagram accounts were also launched on 3rd April 2019). Since the launch of EchidnaCSI til the 4th September 2020, the Facebook page has grown to 2734 Likes (Figure 1), consistently engaging new audiences. Responses from the survey indicated that Facebook was the most common mode for participants to be introduced to EchidnaCSI, followed by 'word of mouth'. Facebook posts have a high engagement rate (max: 68%, min: 3%) with the base rate considered as 'good' engagement by marketing standards being 1% (Buhalis and Mamalakis, 2015); one EchidnaCSI post 'reached' over 100,000 people, according to Facebook's metrics.

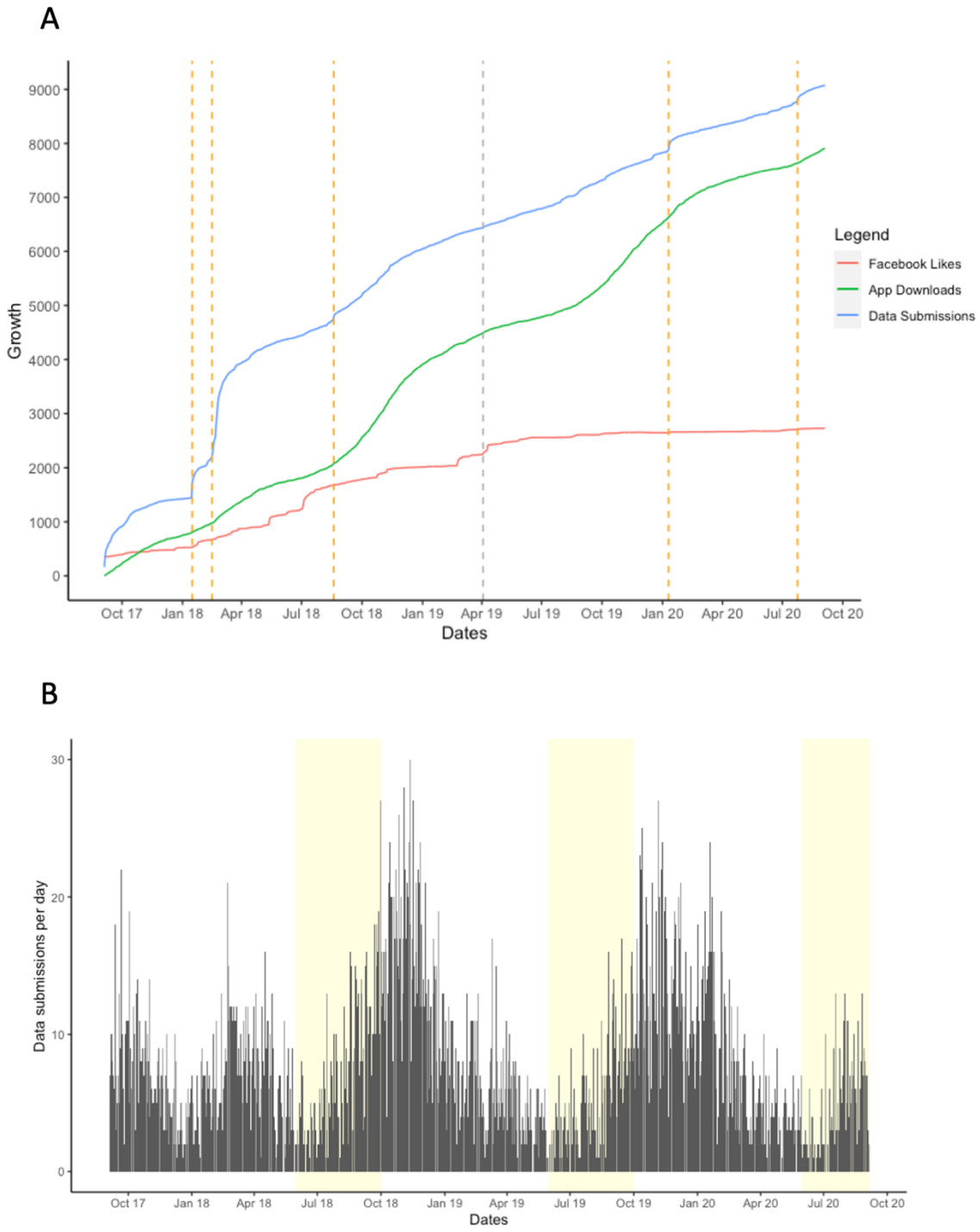

Figure 1: Change over time for app downloads, social media reach and data submissions. A) Accumulative growth of number of EchidnaCSI app downloads, submissions of data (either echidna sightings or scats;) and Likes on the EchidnaCSI Facebook page. Orange dotted lines indicate dates of large increase in app downloads associated with media and events (Table S1). Grey dotted line indicates launch of EchidnaCSI Twitter and Instagram accounts. B) Number of data submissions per day; submissions can fluctuate between 0 and 30 submissions per day, in a cyclic trend. Echidna breeding season is indicated in yellow shading. Data is visualised from 4th September 2017 until 4th September 2020.

3.2 Echidna Sightings

8090 echidna sightings were received from across Australia (Figure 2A), with data submitted from every state and territory. Many submitted sightings are from densely populated areas, city fringes, and even within major cities (Figure 2A). Users are asked to submit sightings of both alive and deceased echidnas; 314 sightings (4% of total sightings) were recorded as deceased and of those 82% were due to road collision (Figure 2B). Users were asked to self-report the kind of environment in which the echidnas were sighted: 35% were in native vegetation, 26% roadside, 23% agriculture or farmland, 11% urban and 3% coastal (Figure 2C). Although size of echidnas does not generally correlate with age or maturity, if echidnas were described as able to 'fit in one hand', then these sightings were attributed to juvenile echidnas; only 2% of total sightings were considered as juvenile. During breeding season (June – September), echidnas form mating 'trains', where one female is followed by multiple males attempting to mate with her, these trains were seen frequently during breeding season; however, the action of mating was rarely observed (1% of total sightings).

3.3 Echidna Scats

Collection of echidna scats has never been attempted before and only few community-based projects include material collection. 406 echidna scats were collected from across Australia, providing the largest collection of echidna material to date, including invaluable samples from remote locations such as Kimberley in WA, APY-lands in central Australia, Arid Recovery in South Australia, far north and central Queensland, as well as along the east coast of Australia and throughout many regions in South Australia (Figure 3A). Scats were identified as belonging to echidna first by visual identification, which is possible by their unique physical characteristics (Figure 3B). Secondly, echidna scats were identified by polymerase chain reaction (PCR) by amplifying a 200bp genomic region specific to echidnas (Summerell et al., 2019; Figure 3C). PCR amplification not only aided in confirming the origin of the scat from echidna, but also showed that the scat material collected through citizen science can reliably be used for molecular work.

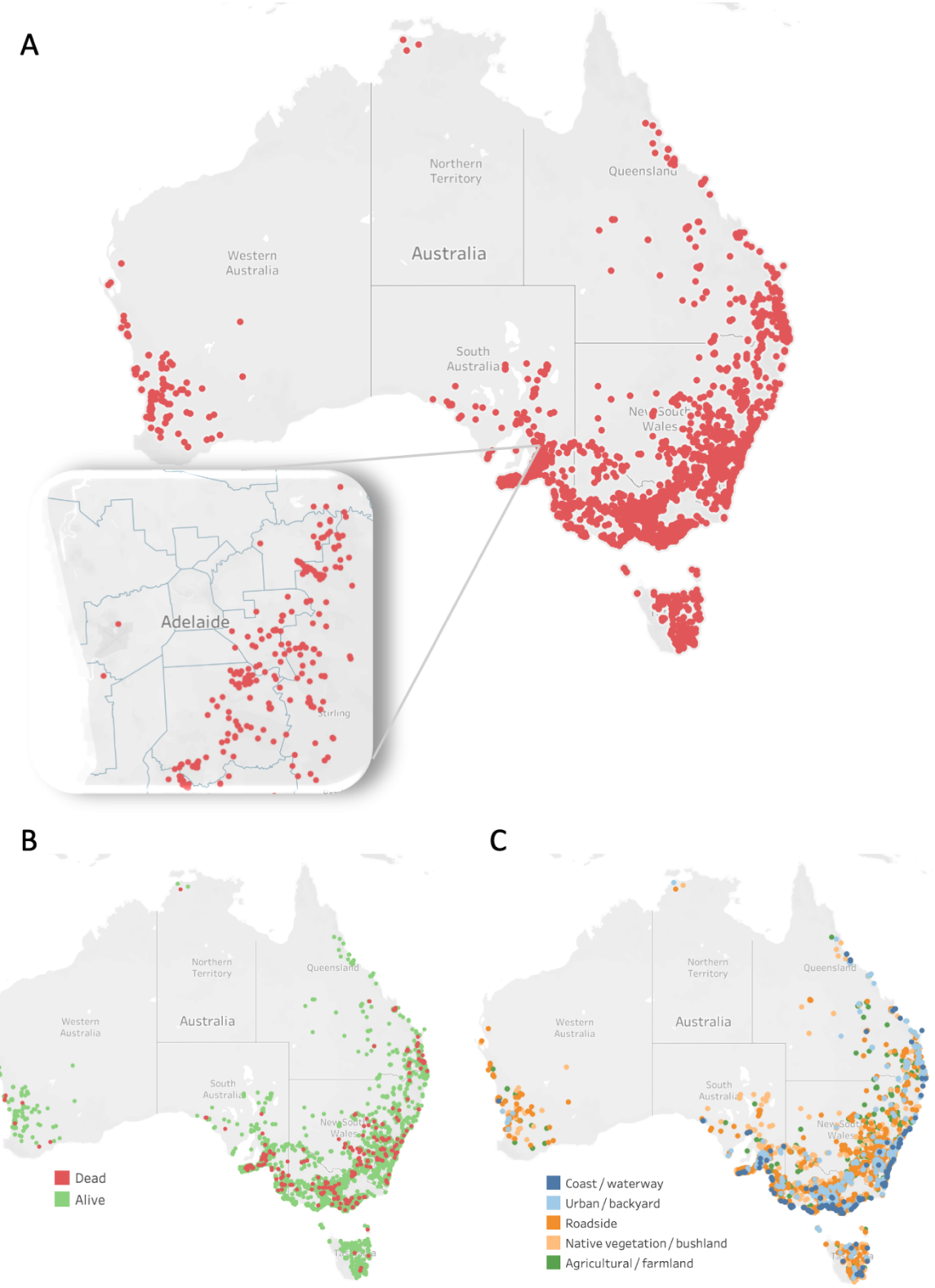

Figure 2: Echidna sightings submitted to EchidnaCSI. A) All sightings submitted between 4th September 2017 to 4th September 2020 are shown in red across Australia, with Adelaide highlighted as one of the major cities where a high density of echidna sightings were submitted immediately surrounding the city. B) Sightings are coloured according to whether the echidna was alive (green) or dead (red). C) Sightings are coloured according to the type of environment in which the echidna was were seen in.

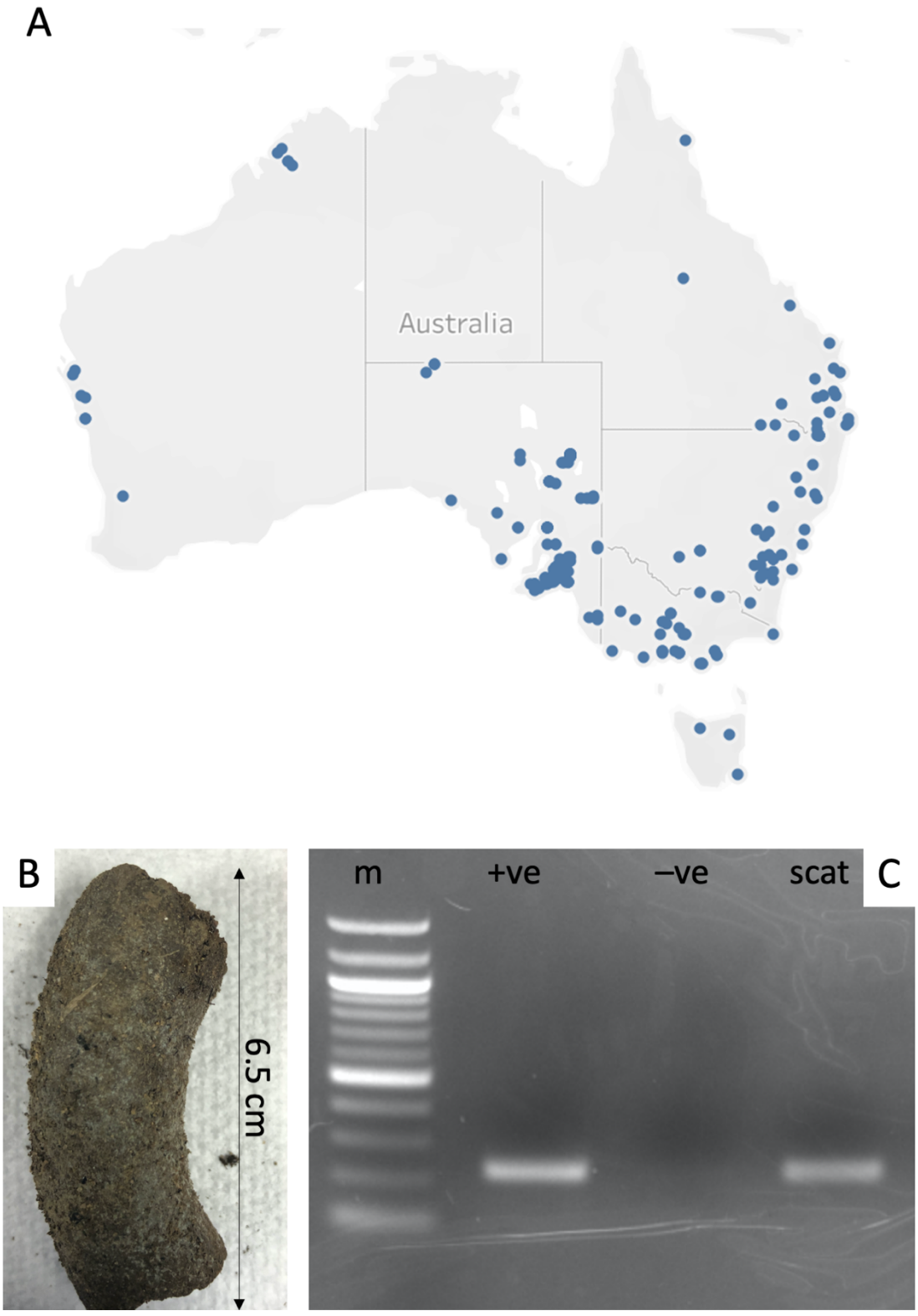

Figure 3: Echidna scats collected by the public and validating their use in molecular biology. A) Locations across Australia where echidna scats were collected by the public between September 4th 2017 to September 4th 2020. B) Photograph of an echidna scat, showing the distinct long, cylindrical shape with blunt ends, and dry soil texture; colour depends on the soil the echidna was feeding in. C) PCR of the mitochondrial dloop region specific to echidnas (200bp); m = 100 bp marker; +ve = positive control; -ve = negative control; scat = DNA from echidna scat.

3.4 Participant demographics

A survey was emailed on 22nd August 2019 to 5720 participants. Survey responses were received within 3 weeks from 944 participants who were engaging with EchidnaCSI; responses were received from across Australia, clustering in major cities, and also internationally (Figure 4A). 64% of survey respondents had submitted data (either echidna sightings or scats), while 36% had not but were still engaging with the project; these latter participants expressed they had not submitted data due to not seeing echidnas or scats since downloading the app (or not being able to capture a photo of an echidna) but not because they were no longer with the project. More females (62.5%) than males (36.6%) participate in EchidnaCSI, with less than 1% preferring not to state their gender (Figure 4B). EchidnaCSI participants were spread across all age groups from 18 years and older, ~ 50 % between 18-54 and ~ 50% 55 and older (Figure 4C). In terms of education, 55% had a Bachelors' degree or above (Figure 4D), while 60% of participants had a maximum of year 11 or 12 high school education specifically in science (Figure 4E). The largest proportion of participants were fully employed (29%), followed by retirees (25%) and part-time employment (15%); students (3.5%) and unemployed persons (2.5%) were the smallest categories. When asked who they submit data with, 41% participants contribute data on their own, 29% with a partner, 12% with children, 8% with a friend, 3% with a colleague, 2% with grandchildren and 2% with a parent.

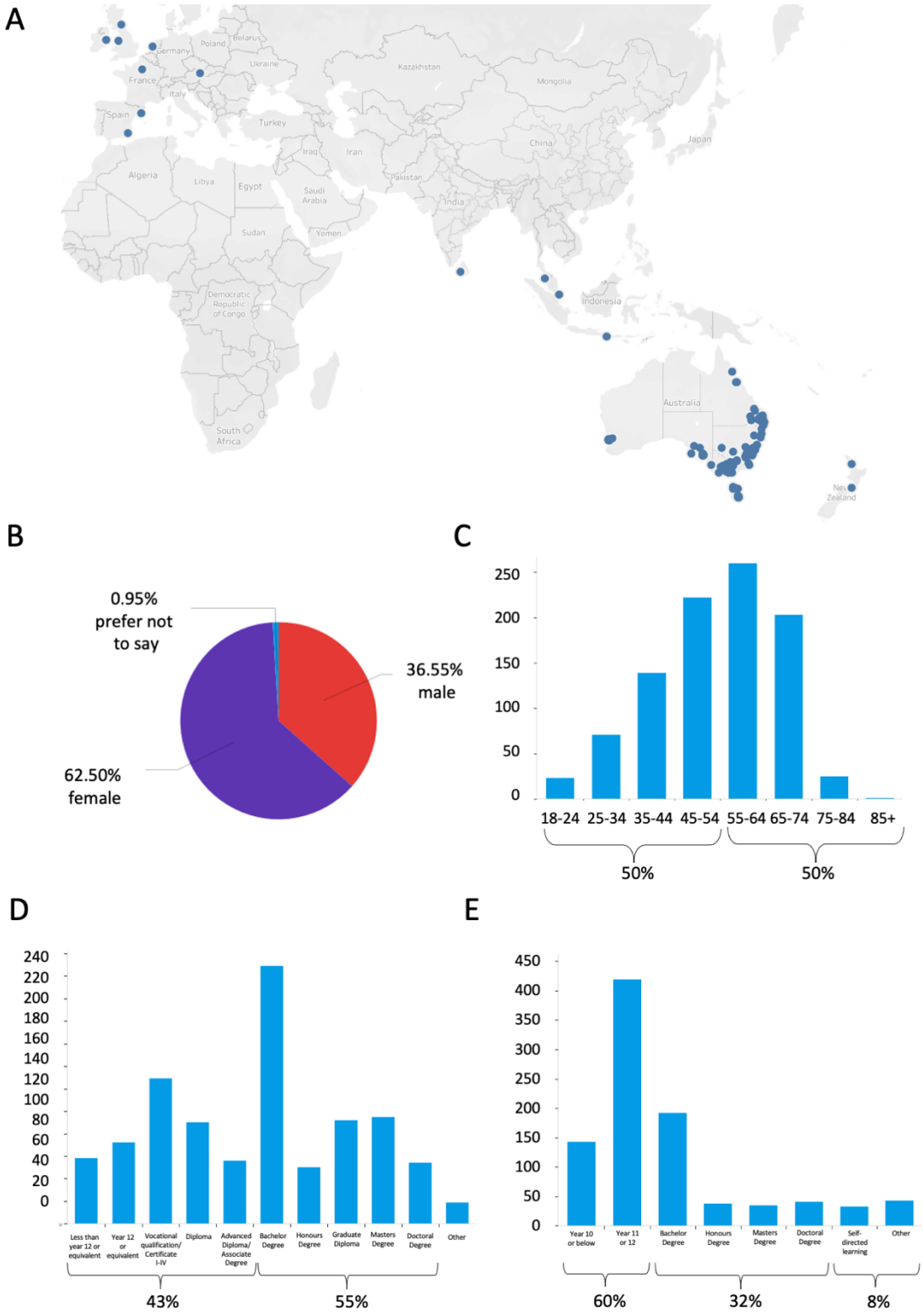

Figure 4: Survey demographic information of EchidnaCSI participants. A) Locations participants took the survey from shown in red; B) Pie chart of gender; C) Histogram of ages, showing the percentages of participants that were under the age of 55 and those that were 55 years and older; D) Histogram of level of education, showing percentages of those that had an education below a Bachelor's degree and those that had at least a Bachelor's degree or higher; E) Histogram of level of science education, showing percentages of those that had up to a high school level (max year 12) science education and those that had a Bachelor's degree or above in Science.

3.5 Introducing participants to citizen science

Survey results show that EchidnaCSI is the first citizen science project in which the majority (63%) of survey respondents had participated, thus introducing a large cohort of the public to citizen science. When comparing those who had submitted data to EchidnaCSI (submitters) to those who had not submitted any data but still engaged with the project (non-submitters), there was no difference between submitters and non-submitters in terms of how many were actively involved in other citizen science projects (37%). However, there was a larger proportion of submitters who had joined other citizen science projects after participating in EchidnaCSI (22%) and they indicated that they were more likely to be involved in citizen science in the future due to their participation in EchidnaCSI (66%) in comparison to non-submitters (13% and 53% respectively). This finding suggests that submitting data increases the likelihood of joining other citizen science projects, likely due to having had a positive experience. This interpretation is further reinforced by the survey indicating that 92% of submitters agree with the statement that 'citizen science is worth my time', with 50% saying their views increased towards that statement since their involvement in EchidnaCSI. As for non-submitters, 91% agreed with the statement; however, only 32% had increased the strength of their view that citizen science was worth their time (Tables S2 and S3).

3.6 Participants' changes in attitudes and their motivations for involvement

Next, we wanted to determine what participants' attitudes are to certain statements followed by the question of if their views had changed since engaging with EchidnaCSI. Survey results indicate that EchidnaCSI attracts participants who are passionate about echidna conservation and environmental health in general, as more than 90% agreed to the importance of these statements (Table S2). However, since participating in EchidnaCSI, a large proportion (42% of submitters and 36% of non-submitters) indicated that echidna conservation had become more important to them, and their views and actions towards the health of the environment had also increased (Table S2). Interestingly, 36% agreed with or were neutral to the statement that 'I do what I can, but the environment is not my biggest concern'.

The survey identified the motivations that most greatly influenced participants as a combination of 'wanting to contribute to wildlife conservation', 'liking echidnas', 'contributing to scientific research' and 'learning about echidnas'. When comparing submitters to non-submitters, these four motivations were in their top five responses at varying levels (Tables S3 and S4). However, for the non-submitters, 'I intend to submit data in the future' was a high motivation to continue

to engage with the project, while for submitters 'the project is easy to participate in' was ranked highly. The motivations that were ranked consistently the lowest included 'interest in molecular biology', 'seeing recognition of my or other participants' contributions', and 'enjoy the time spent with family and/or friends' (Tables S3 and S4).

4. Discussion

4.1 New Data and Information for Wild Echidna Populations

EchidnaCSI was able to achieve the main goal to produce a large number of echidna sightings across Australia. In three years, EchidnaCSI has produced the equivalent to 25% of all echidna sightings in the Atlas of Living Australia, which covers more than the past 100 years of data (Atlas of Living Australia (ALA) website species page: https://bie.ala.org.au/species/urn:lsid:biodiversity.org.au:afd.taxon:0d4c9c0c-51d3-44e0-a365-fe0f8b791c66, accessed on 13 November 2020). As we require a photo of the echidna upon submission as evidence of sighting, the data are high-quality additions to the Atlas of Living Australia's biodiversity database. Thus, this study has made a significant contribution that enables better assessment and understanding of echidna populations in Australia. Without a citizen science approach, which engaged thousands of members of the public, this coverage would never have been achieved. These sightings provide a considerable increase in available baseline information about echidna presence, which can now be used to monitor changes in wild echidna populations. For example, this data will be powerful in assessing the effects of the devastating 2019/2020 bushfire season in Australia (Ward et al., 2020), where echidna distributions overlap with regions that were significantly affected, including Kangaroo Island, where echidnas are already recognised as endangered. Due to the nature of the project, we receive far more echidna sightings in areas where there is higher human population density (mostly around the coastal areas of Australia), which is a typical finding in citizen science studies (Geldmann et al., 2016; Matteson et al., 2012). This finding does not indicate that echidnas do not exist or are in lower numbers in regional and remote areas of Australia, but that more targeted engagement strategies are required to receive sightings from these locations. Although we expected most sightings to occur close to human populated areas, we did not anticipate as many echidna sightings to occur within or immediately surrounding all major cities in Australia. This evidence raises a number of concerns as there is very little appropriate habitat or food sources available for echidnas in these environments and proximity to densely populated areas increases their risk of being hit by vehicles. Unlike common ring-tailed possums or koalas, echidnas have not previously been considered to be an 'urban' native

species, but our findings indicate that it may be important to consider echidnas when establishing policies surrounding biodiversity in cities (Łopucki et al., 2020; Stanford and Bush, 2020).

EchidnaCSI has also made a significant methodological contribution by successfully incorporating wildlife scat collection into a nationwide citizen science project, a strategy that has not previously been utilized to our knowledge. This approach has resulted in the largest material collection for echidnas to date and from geographically unique locations that would have been impossible to obtain in any other way than through community participation. Unlike other material collection projects (Chauhan et al., 2020; McDonald et al., 2018), the scats were collected by participants without specific training or kits; instead, a combination of resources were provided (e.g. in app instructions, scat identification guide in app and on website) which, together with the distinct appearance of echidna scats, were sufficient for the general public to successfully identify, collect and ship the scat samples. The scats were validated for their use in molecular work, as DNA was successfully isolated and amplified, and by targeting an 'echidna specific' gene we were able to further confirm the identity of the scat belonging to echidna. These findings not only further validate the approach of scat collection in citizen science, but also open exciting avenues for understanding more about wild echidna biology such as diet, gut health, reproductive success and potential stressors as well as providing a model for how to incorporate scat collection and analysis for other animals into citizen science projects.

4.2 EchidnaCSI Recruitment and Engagement

Gaining knowledge of wild animal populations using citizen science approaches is not a new phenomenon. However, EchidnaCSI has successfully scaled this approach up to continent size, using new technology to submit data and online social media platforms for communication. Using traditional media (e.g. radio, television and news articles) early in recruitment was effective in reaching large audiences, which has been observed in other Australia-wide projects, for example, on wombats (Skelton et al., 2019). Social media became an important form of recruitment later in the project when a cohort of participants were already registered, as the majority of users first heard about the project via Facebook. In-person events were also an effective form of recruitment as seen by an increase in app downloads during National Science Week in 2018 where we held or spoke at six events over seven days. It is very well documented in citizen science that feedback and project updates are important for the retention of participants (Battersby and Greenwood, 2004; Bell et al., 2008; Crall et al., 2017; Wald et al.,

2016). As can be seen from our findings, social media is both a good platform for recruiting new participants and an effective form of engagement to sustain participation. Our approach of using social media as the main platform of communication also meant that we were able to engage a significant number of people who had not directly contributed data or material to EchidnaCSI (36%). Therefore, we have engaged a large group of people about echidna biology and conservation even if they have not yet formally contributed sightings or scats to the project.

4.3 Participants' Demographics

Concerns have been raised that a lack of demographic diversity exists in many citizen science projects, as most volunteers tend to be highly educated males who are 50 years or older and are often retired (Hobbs and White, 2012; Pandya, 2012). Our survey revealed that EchidnaCSI has more female participants than male, which has only been documented in one other citizen science project that also had a conservation focus (Domroese and Johnson, 2017). Although the age range in our participants is diverse, 50% were still over the age of 55. Due to the survey being limited to those over 18 years of age, we could not accurately gauge our engagement with younger audiences; however, as we have presented at events that were specifically aimed at primary and high school children and 12% of the survey respondents reported submitting data with their children, we expect the actual age demographics of our EchidnaCSI community to be younger than what we have been able to capture. The survey also highlighted that EchidnaCSI caters to those who are both 'time poor' and 'time rich', as the largest cohort of participants are fully employed and the second largest are retirees. This is likely a key factor in our ability to have more variety in the diversity of participants, along with the many strategies of recruitment and engagement (e.g. traditional media, social media, in-person events). However, we would like to further improve the diversity of EchidnaCSI participants, especially for varying ethnicities (particularly indigenous and remote communities) and those without university qualifications, as well as continue to reach younger audiences, which will require more targeted engagement strategies to be developed.

4.4 Participants' Motivations

Details of the core motivations of current participants can be used as a powerful tool to increase recruitment and engagement. Our survey revealed, similar to other citizen science studies, that the main motivations for the participants of EchidnaCSI were: 1) wildlife conservation; 2) interest in echidnas; 3) contributing to science; and 4) learning. Interestingly, unlike findings in many other conservation and biodiversity type citizen science projects (Bell et al., 2008;

Berg et al., 2009; Rotman et al., 2012), spending time with family or friends was ranked amongst the lowest motivators. Perhaps this is unsurprising, given that echidna and scat sightings mostly occur from opportunistic circumstances rather than planned activities, due to the cryptic nature of echidnas. This interpretation is further underscored by the fact that the majority of individuals said they submitted data when they were alone, and that the main reason that participants were unable to submit data was because they had not yet seen an echidna (or their scat), not because they lost interest in the project. The citizen science literature has indicated that some participants are motivated by wanting to be recognised for their contributions (Lawrence and Turnhout, 2010; Rotman et al., 2012); however, in this project, recognition was a low ranked motivator. Therefore, although providing regular feedback or recognition is likely an important factor for maintaining engagement with the project (Bell et al., 2008), it seems not a major motivator for participation in this project. Although our marketing had already focussed on echidnas and their conservation (the highest motivators), incorporating more about the contributions that this research is making and opportunities to learn from participation will be important for future communications and engagement strategies, based on evidence from our survey. In addition, given that our research in part aims to have a positive environmental impact, the fact that 36% agreed with or were neutral to the statement that 'I do what I can, but the environment is not my biggest concern', highlights the need to make more explicit connections between echidna populations and environmental health.

4.5 Conclusion and Future Directions

EchidnaCSI has proven to be a successful citizen science project, which produces high quality and quantity data, engaging a large, geographically varied and diverse audience, and instilling passion and care for echidna conservation. Because of EchidnaCSI, we now have an unprecedented and continuously growing baseline dataset for wild echidna populations, which is essential for long term conservation of echidnas in our changing environment. This project is the first to incorporate nation-wide scat collection into citizen science with limited technical guidance and its validity for use in molecular biology. It is clear from our experience that a combination of both traditional and social media is key for reaching and engaging a large audience over a continental scale, although there still needs to be significant effort placed on engaging those in more rural, regional and remote areas across Australia, which should simultaneously increase the diversity of participants according to some demographics. We see EchidnaCSI as a powerful educational platform and a gateway for more of the public to become engaged in citizen science, as well as a way to obtain high quality data and material for this iconic yet cryptic species.

Acknowledgments

The authors would like to thank and acknowledge every one of the more than 8000 participants of EchidnaCSI over these past 3 years. Thank you to all that have submitted echidna sightings and scats samples for the project. Without our shared passion and excitement for echidnas this research could not have been done. Authors would also like to thank Peter Brenton from Atlas of Living Australia (ALA) for his support integrating EchidnaCSI with the ALA and the Australian Citizen Science Association (ACSA) for their guidance and ongoing support in the citizen science field. Tahlia Perry and Alan Stenhouse are funded by the Research Training Program. Frank Grützner is an Australian Research Council Future Fellow. Acknowledgements to the Pelican Lagoon Research Centre for research support and resources. Funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

CRediT author statement

Tahlia Perry: Conceptualisation, Investigation, Writing (Original Draft, Review & Editing), Visualisation, **Alan Stenhouse:** Conceptualisation, Software, Data Curation, **Isabella Wilson:** Data Curation, Methodology, **Imma Perfetto:** Methodology, Resources, Investigation, **Michael W. McKelvey:** Supervision, Resources, **Michelle Coulson:** Supervision, Methodology, **Rachel A. Ankeny:** Supervision, Methodology, Resources, Writing – Review & Editing, **Peggy D. Rismiller:** Conceptualisation, Supervision, Writing – Review & Editing, **Frank Grützner:** Conceptualisation, Supervision, Project Administration, Funding Acquisition, Writing – Review & Editing.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter Three:

Characterising the gut microbiomes in wild and captive short-beaked echidnas reveals diet-associated changes

Statement of Authorship

Title of Paper	Characterising the Gut Microbiomes in Wild and Captive Short-beaked Echidnas Reveals Diet-Associated Changes
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Submitted to Frontiers in Microbiology

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Overall percentage (%)	75%		
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Characterising the gut microbiomes in wild and captive short-beaked echidnas reveals diet-associated changes

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Abstract

The gut microbiome plays a vital role in health and wellbeing of animals and an increasing number of studies investigate microbiome changes in wild and managed populations to improve conservation and welfare. The short-beaked echidna (*Tachyglossus aculeatus*) is the most widespread native mammal in Australia and commonly held in zoos. Here, we used 16S metabarcoding of scat samples to characterise and compare the gut microbiomes of echidnas in wild (n=159) and managed (n=44) populations, which were fed four different diets. Overall, the analysis reveals a high level of variability in the gut microbiome between samples; however, they are mostly dominated by taxa belonging to the phyla Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Fusobacteriota. Diet plays a significant role in shaping the gut microbiomes in echidnas; this work demonstrates significant differences between zoo held and wild echidnas, as well as managed animals on different diets. Wild echidnas exhibit gut microbial diversity and compositional changes associated with season, climate, and land-use changes, which is likely due to food availability as they are opportunistic foragers. Although echidnas are often mistakenly categorised as myrmecophagous mammals (diet consisting mostly of ants and termites), their gut microbiome consists of many putative plant-fermenting bacteria, suggesting plant matter may play a significant role in their diet. This first analysis of echidna gut microbiome highlights extensive microbial diversity in wild echidnas and changes in microbiome composition in managed populations as well as changes as a result of different diets. This is a first step towards using microbiome analysis to better understand gastrointestinal biology and improve management in these iconic animals.

Keywords: citizen science, scat, faecal, 16S metabarcoding, bacteria, conservation, Tachyglossus

1. Introduction

The influence of the gut microbiome on host fitness has been well-established in humans, with many diseases and health problems associated with microbial dysbiosis, including obesity, diabetes and bowel disease (Cho and Blaser, 2012; Frank et al., 2007; Turnbaugh et al., 2006; Wen et al., 2008). How microbiomes affect the fitness and health in non-human animals has only recently been investigated, but is recognised as vital for wildlife conservation and captive management (Bahrndorff et al., 2016; Chong et al., 2020; McKenzie et al., 2017; Trevelline et al., 2019). The short-beaked echidna (*Tachyglossus aculeatus*) is the most widespread native mammal in Australia, found across all types of habitats from desert, temperate regions, to snowy alpine (Archer, 1983; Rismiller and Grutzner, 2019). Although echidnas are an iconic Australian species, we have relatively little information about most wild populations due to their cryptic and solitary lifestyles and large home-ranges (Abensperg-Traun, 1991; Rismiller and Mckelvey, 1994). The only long term studies of echidnas are on Kangaroo Island, South Australia, and specific areas in Tasmania (Nicol and Andersen, 2002, 2007; Rismiller, 1992; Rismiller and McKelvey, 2003). On Kangaroo Island, work over more than 25 years revealed fundamental aspects of echidna biology and recorded the impacts of feral animals and environmental changes, which led to the subspecies (*T. a. multiaculeatus*) being recognised as endangered (EPBC Act, 2015 'Conservation Advice *Tachyglossus aculeatus multiaculeatus* Kangaroo Island echidna'). Characterising the gut microbiomes for wild echidnas across their range of habitats can inform us more about the biology of these remarkable egg-laying mammals and may be a good indicator of health.

Diet is a major determinant of the bacterial communities in the gut microbiome, with many phylogenetically distant mammals clustering together as carnivores, omnivores and herbivores, with herbivores even forming distinct groups of foregut and hindgut fermenters, based on the location and the composition of these microbes living in their gut (Ley et al., 2008). Echidnas eat a wide variety of invertebrates including ants, termites, beetles, worms, and a range of insect larvae (Griffiths, 1968; Smith et al., 1989; Sprent and Nicol, 2016), and have even been associated with the distribution of mycorrhizal fungi (Feuerherdt et al., 2005). Echidnas are opportunistic foragers, and their diets will change depending on the food availability, season and temperature (Smith et al., 1989; Sprent and Nicol, 2016). In some parts of Australia and times of the year when echidnas' diet consists of mostly ants and termites, their gut microbiomes may be similar to myrmecophagous species. A comparative study suggested that eutherian myrmecophagous species show converging gut microbiomes due to their specialised diet (Delsuc et al., 2014). Echidnas have often been mistakenly characterised as

myrmecophagous but have a much more diverse diet than simply ants and termites, and physiologically echidnas also differ from eutherian myrmecophagous species. Echidnas lack teeth and instead will masticate their food in between the horny plates at the back of their tongue and palate to aid in digestion (Augee et al., 2006). They have a non-acidic stomach (pH >6), with a loss of pepsin genes that encode some digestive enzymes, which are also features shared with their closest relative, the platypus (Ordoñez et al., 2008; Zhou et al., 2020). It is important to understand how the combination of diet and unique digestive physiology relate to the gut microbiome in wild echidnas.

Characterising the gut microbiomes of wild animals is also important for comparison to animals kept in captivity, as it is well-documented that mammals in captivity often have altered gut microbiomes compared to their wild conspecifics due to differences in diet and habitat (Haworth et al., 2019; McKenzie et al., 2017; Prabhu et al., 2020; West et al., 2019). In managed populations, diets for echidnas have been based on carnivore (i.e. cat and dog) models, as this was believed to be comparatively the most similar digestive system and therefore have similar nutrient requirements (Augee et al., 2006; Stannard et al., 2017). However, in the past, echidnas fed these diets were reported to have nutrition-related problems such as diarrhoea, gastritis, cystitis, and obesity (Stannard et al., 2017). Therefore, new diets have been, and continue to be, developed to address these problems. Key changes in diet include an increase in protein and fat to better reflect the natural insectivorous diet and higher fibre content to account for the high soil and organic matter echidnas usually ingest when foraging (Griffiths and Greenslade, 1990; Stannard et al., 2017), with recent diets balancing macro and micronutrients to meet expected requirements. However, how these different diets affect the gut microbiome of echidnas is yet to be investigated.

Faeces (or scats) are commonly used materials for studying animals' gut microbiomes as they can be non-invasively collected and do not require the animal itself to be present in order to collect the sample. In zoos, scat samples can be easily collected by animal care staff, however, collecting an adequate number of samples across multiple locations for wild populations can be difficult. Some studies have successfully collected scat or other material (such as swabs or ticks) to analyse microbiomes from large and geographically dispersed datasets through citizen science initiatives (Chauhan et al., 2020; Hulcr et al., 2012; Klimenko et al., 2018; McDonald et al., 2018). As echidnas cover a vast geographic range, citizen scientists have been enlisted to collect echidna scats through the project EchidnaCSI (Echidna Conservation Science Initiative) (Perry et al., 2020, submitted). Echidna scats can be easily identified as they are a smooth cylindrical shape, approximately 2 cm in diameter, and mostly consist of soil and undigested exoskeletons of prey items (Augee et al., 2006; Rismiller and Grutzner, 2019).

Here, we present the first comparative gut microbiome study for wild and managed populations of echidnas. We aimed to 1) characterise the gut microbiome of wild echidnas across their diverse habitats in Australia; 2) investigate how captivity influences the echidna gut microbiome; and 3) determine if different diets alter the gut microbiome in zoo-held echidnas.

2. Materials and Methods

2.1 Wild echidna faecal sample collection and metadata

Faecal samples from wild echidnas were collected through a collaborative effort with volunteers throughout Australia as a part of the citizen science project: Echidna Conservation Science Initiative (EchidnaCSI; www.grutznerlab.weebly.com/echidna-csi.html). Participants were instructed to download the EchidnaCSI app, which housed photographs and detailed instructions on how to identify an echidna scat. Participants were then instructed to take a photo of the echidna scat through the EchidnaCSI app when the sample was found so that the date, time and GPS location could be matched to the physical samples. Once a photo was taken, the app directed the participant to place the faecal sample in a clean zip-lock bag without touching the faecal samples, or instead using gloves, to avoid contamination. Samples were shipped immediately to The University of Adelaide and then stored in the freezer. A total of 159 wild samples from across Australia were used in this study from a large variety of locations and environments (Figure 1; Table S1). Although most scats were collected opportunistically and could have been in the environment for an unknown time prior to collection, we worked closely with a subset of citizen scientists in South Australia, who collected fresh scats from their properties where echidnas frequented often. As samples were collected opportunistically, diet information for wild samples were unknown, for simplicity we have labelled the wild samples as having an 'insect' diet (Figure 1). Based on GPS coordinates, each sample was given metadata associated with its location (e.g. climate, land use, anthropogenic biomes, land cover; Table S1) by using the Atlas of Living Australia's Spatial Portal (https://spatial.ala.org.au/layers).

2.2 Captive echidna faecal sample collection

Faecal samples were collected from managed echidnas in two locations: Perth Zoo and Taronga Zoo (Figure 1; Table S2). Faecal material was collected from nine echidnas at Perth Zoo, Western Australia (31.9755° S, 115.8523° E), where biological triplicate faecal samples were collected from each individual (collected consecutively across three days; n=27). Faecal material was collected from ten echidnas from Taronga Zoo, New South Wales (33.8435° S, 151.2413° E; sample number varied per individual; n=18). Samples were collected by zoo

personnel, where fresh faecal samples were handled with gloves and placed in a clean plastic zip-lock bag or screw-capped tube and then immediately frozen. Samples were shipped to The University of Adelaide (from Perth Zoo on dry ice and from Taronga Zoo on ice) and again stored immediately in the freezer.

Echidnas at Perth Zoo were only fed the Meat diet, which consisted of lean beef mince, microcrystalline cellulose, hardboiled egg, banana, multivitamin supplement with iron (Pentavite), calcium carbonate, mealworms and water. Echidnas at Taronga Zoo were fed three different diets (see Table S3 for comprehensive diet information): The Updated Meat Diet (UMD), which is similar to the Meat diet fed in Perth Zoo; Vetafarm diet, manufactured by Vetafarm (Wagga Wagga, NSW), where the main sources of protein are meat meal, corn and soy; and Wombaroo diet, manufactured by Wombaroo Food Products (Mount Barker, SA), which contains meat meal, soy and whey protein isolate as the protein sources.

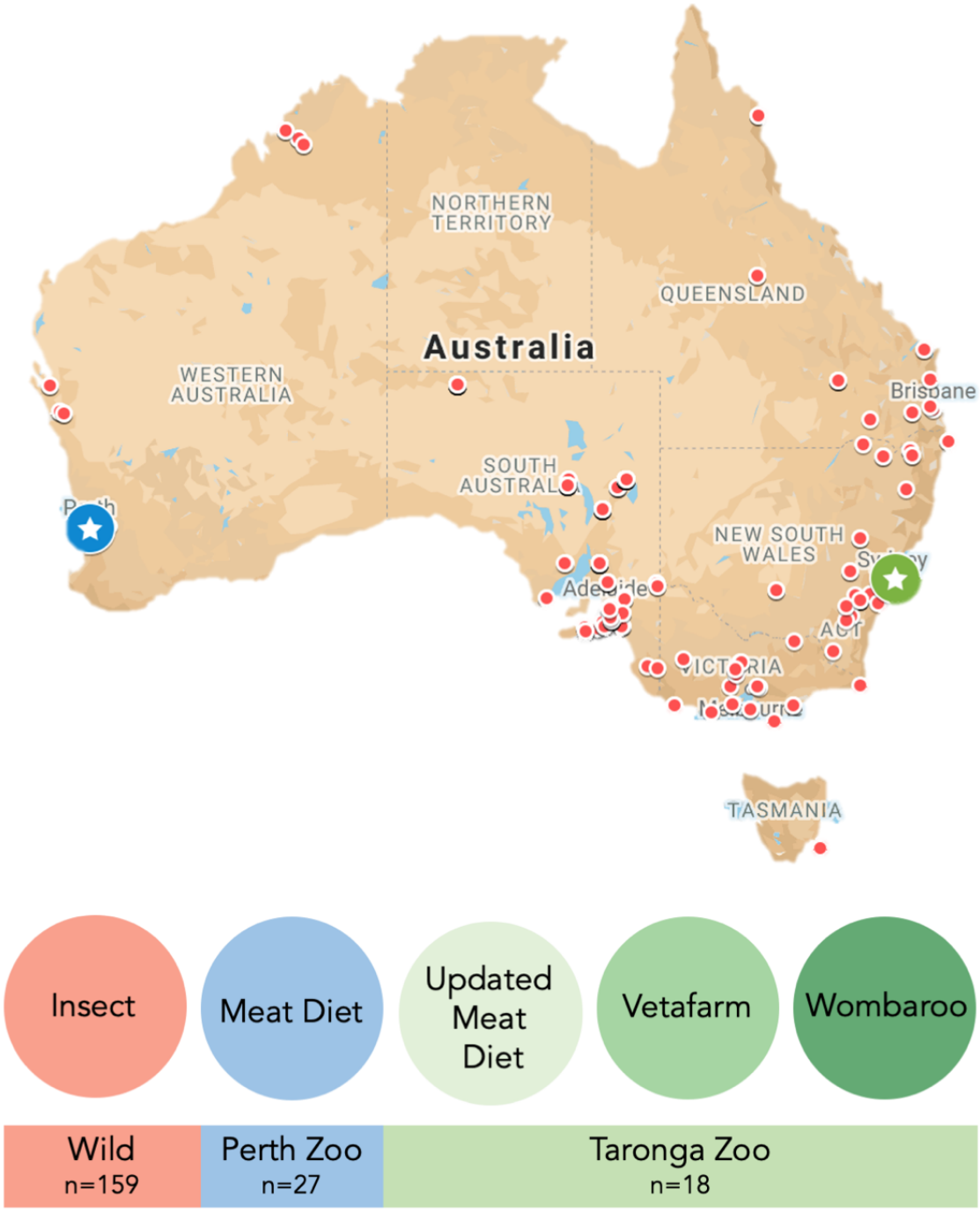

Figure 1: Location and diet information for faecal samples collected from wild and captive echidnas in this study. Red dots on map indicate locations of faecal samples collected from wild echidnas, diet labelled as 'insect' for simplicity; blue circle with star is the location of Perth Zoo, where faecal samples were collected from echidnas that were fed exclusively the Meat diet; green circle with star is the location of Taronga Zoo, where faecal samples were collected from echidnas fed three different diets: Updated Meat Diet (UMD), Vetafarm diet and Wombaroo diet.

2.3 DNA extraction

Total genomic DNA was extracted from 204 faecal samples using the Qiagen QIAamp Mini Stool Kit (Qiagen, Hilden, Germany) as per the manufacture's protocol, apart from some details outlined below. The extractions took place in a Flow Cabinet Biological Safe Level 2 that was cleaned with 10% bleach (sodium hypochlorite) to reduce contamination. A third of the scat sample was crushed up in the presence of liquid nitrogen using a mortar and pestle, prior to adding the sample to InhibitX buffer. Next, samples were centrifuged at 20,000 g for 3 min and ~1 mL eluate transferred to a new 1.5 mL tube. Samples were again centrifuged at 20,000 g for 1 min and ~700 µL of eluate transferred to a new 1.5 mL tube, carefully avoiding any transfer of physical material. Samples were centrifuged one last time at 20,000 g for 1 min and 600 µL added to 25 µL Proteneise K such as in the protocol; from here the rest of manufacturer's protocol was followed.

2.4 PCR amplification

All samples were PCR amplified and uniquely barcoded, using primers targeting the V4 region of the bacteria 16S ribosomal RNA (rRNA) gene (Caporaso et al., 2011). DNA was amplified with the primer pair 515F (5'-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3') and uniquely barcoded 806R (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnnnnnnnAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3'). Single reactions of 18.7 µL dH_2O, 2.5 µL 10X HiFi buffer, 1 µL 50mM MgSO4, 0.1 µL Platinum Taq DNA Polymerase (ThermoFisher), 0.2 µL 100 mM dNTP, mix, 0.5 µL of 10 µM forward primer, 1 µL of 5 µM reverse primer and 1 µL DNA. DNA was amplified using an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 50 °C for 1 min, elongation at 68 °C for 90 sec, with final adenylation for 10 min at 68 °C, in line with the Earth Microbiome Protocol (Thompson et al., 2017).

Gel electrophoresis was carried out for each PCR reaction on a 2.5% agarose gel to ensure the samples contained library constructs of the desired length (~390bp). Each sample was then quantified using Qubit 2.0 Fluorometer and pooled to equimolar concentration. Pooled samples were cleaned following the Agencourt AMPure XP PCR purification protocol (Beckman Coulter), quantified and quality checked by the LabChip® GX Touch™ nucleic acid analyser. A final concentration of 4 nM was run on the Illumina Miseq (v2, 2 x 250bp) at ACRF (Australian Cancer Research Foundation) Cancer Genomics Facility.

2.5 Data processing and statistical analyses

DNA sequencing data were processed and analysed using QIIME2 v2020.2 (Bolyen et al., 2019). Demultiplexed paired-end sequence reads were merged, quality filtered and denoised into Amplicon Sequence Variants (ASVs) using the *deblur* plugin (Amir et al., 2017) and trim length of 247 bp. The feature table was rarefied to a depth of 1300, using the minimum number of sequences per sample for diversity analysis. Representative sequences were assigned taxonomy using the *feature-classifier* plugin (Naïve Bayesian approach) on the pre-trained SILVA (Quast et al., 2013) 138 V4 region classifier (Bokulich et al., 2018). Alpha diversity was assessed by diversity metrics including observed ASVs, Faith's phylogenetic diversity, Shannon's entropy and Pileou's evenness and statistical significance was assessed using the Kruskal-Wallis tests. Beta diversity was assessed by weighted and unweighted UniFrac metrics and visualized by Principal Coordinates Analysis (PCoA), with statistical significance assessed with Permutational Multivariate Analysis of Variance (PERMANOVA) tests, with 999 permutations.

3. Results

3.1 Characterising the wild echidna gut microbiome

DNA sequencing of the 210 samples resulted in 14,323,679 reads with a mean of 60,951, which underwent read-pair joining and were denoised into 10,646 Amplicon Sequence Variants (ASVs). First, the gut microbiomes of wild echidna samples were analysed, revealing extraordinary variability of the individual samples. We endeavoured to correlate this variation with climate, vegetation, land-use, and seasonal aspects based on the locations each scat was collected (Table S1). Analysis of alpha diversity revealed significant differences in samples collected in areas with differing climate; typically, samples collected from subtropical and tropical regions had greater number of ASVs (observed ASVs), greater phylogenetic diversity (Faith's phylogenetic diversity) and greater richness (Shannon's diversity) than samples collected in desert, grassland and temperate locations (Figure 2). Due to limited number of samples from tropical regions (n=4), these results should be tested in the future with greater sample size.

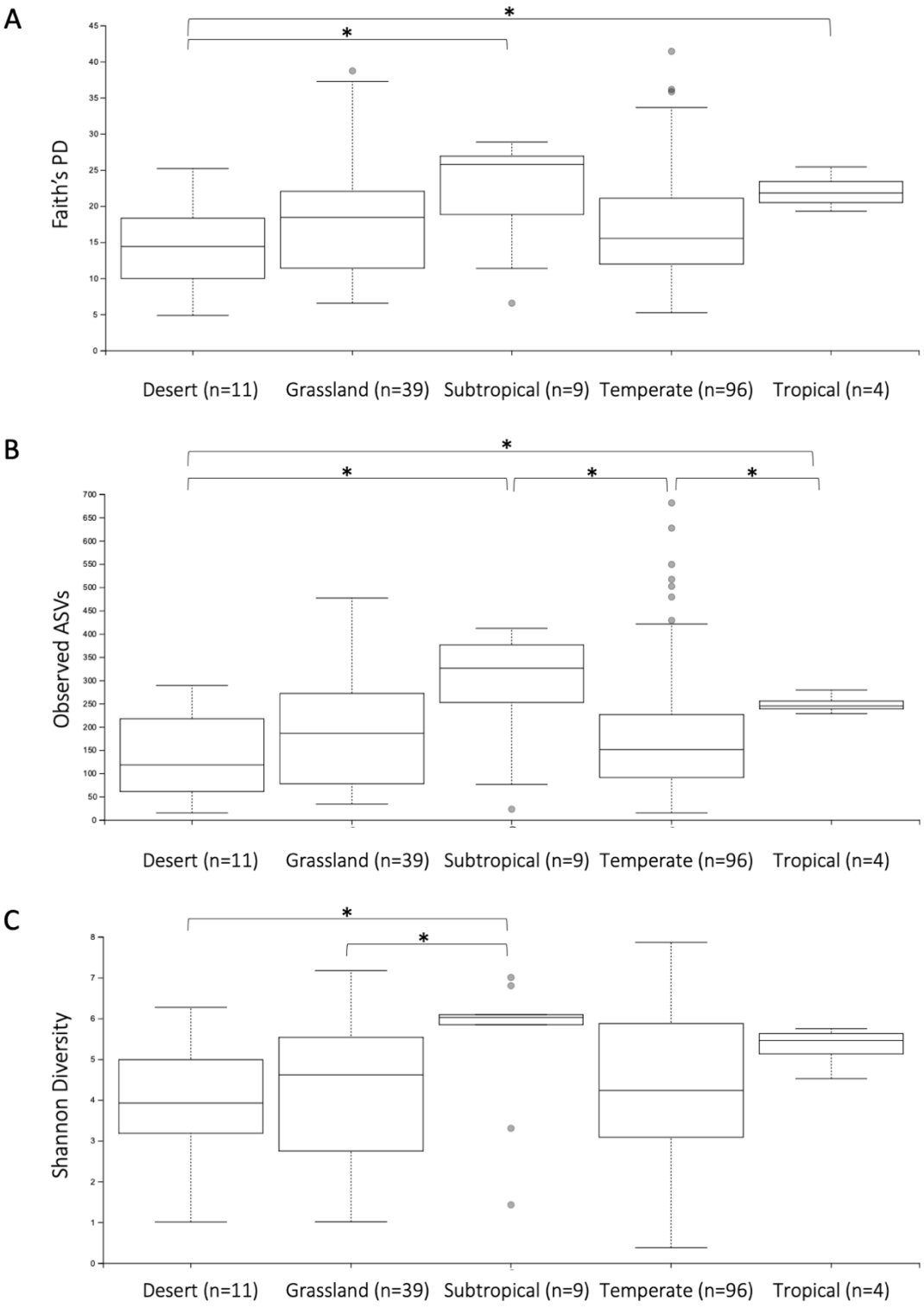

Figure 2: Alpha diversity analyses of gut microbiomes from wild samples collected in different climate regions across Australia. Whisker-box plots depict the following metrics: A) Faith's phylogenetic diversity (Faith's PD); B) Observed ASVs; C) Shannon's Diversity index. Horizontal lines indicate median values, upper and lower bounds represent the 25th and 75th percentiles, and top and bottom whiskers indicate maximum and minimum values. Outliers are shown as grey circles. * = significance ($p<0.05$).

Next, we investigated microbial composition and saw significant differences in beta diversity (unweighted UniFrac) influenced mostly by seasonal changes ($p = 0.002$; PERMANOVA) and anthropogenic biomes ($p = 0.005$; PERMANOVA), which describes how the land is used by people (i.e. croplands, rangelands, urbanised; Table S1). Land-cover was also considered statistically different between some groups, for example, samples from native grasslands (n=27) had significantly different microbial communities to samples collected from land used for annual crops (n=34; $p = 0.039$; PERMANOVA). The microbiomes of wild echidna scat samples were dominated by bacteria from phyla Proteobacteria, Firmicutes, Bacteroidota, Actinobacteriota, and Fusobacteriota, with some samples also containing low abundances of Verrucomicrobiota, Myxococcota, Cyanobacteria, Acidobacteriota and even lower abundances of several other phyla (Figure 3). Despite an overall high variability of the bacteria seen between wild samples (n=159), the most prevalent bacteria were from the following genera (numbers in brackets represent number of wild samples with the bacteria present): *Arthrobacter* (143), *Enterococcus* (119), *Enterobacteriaceae* (114), *Escherichia-Shigella* (104), *Fusobacterium* (98), *Lactococcus* (98), *Bacillus* (96), *Romboutsi*a (92), *Pseudomonas* (86), *Pediococcus* (80), *Paeniclostridium* (63), *Acinetobacter* (63), and *Sanguibacter* (61) (Figure S1); no taxon was found in all samples.

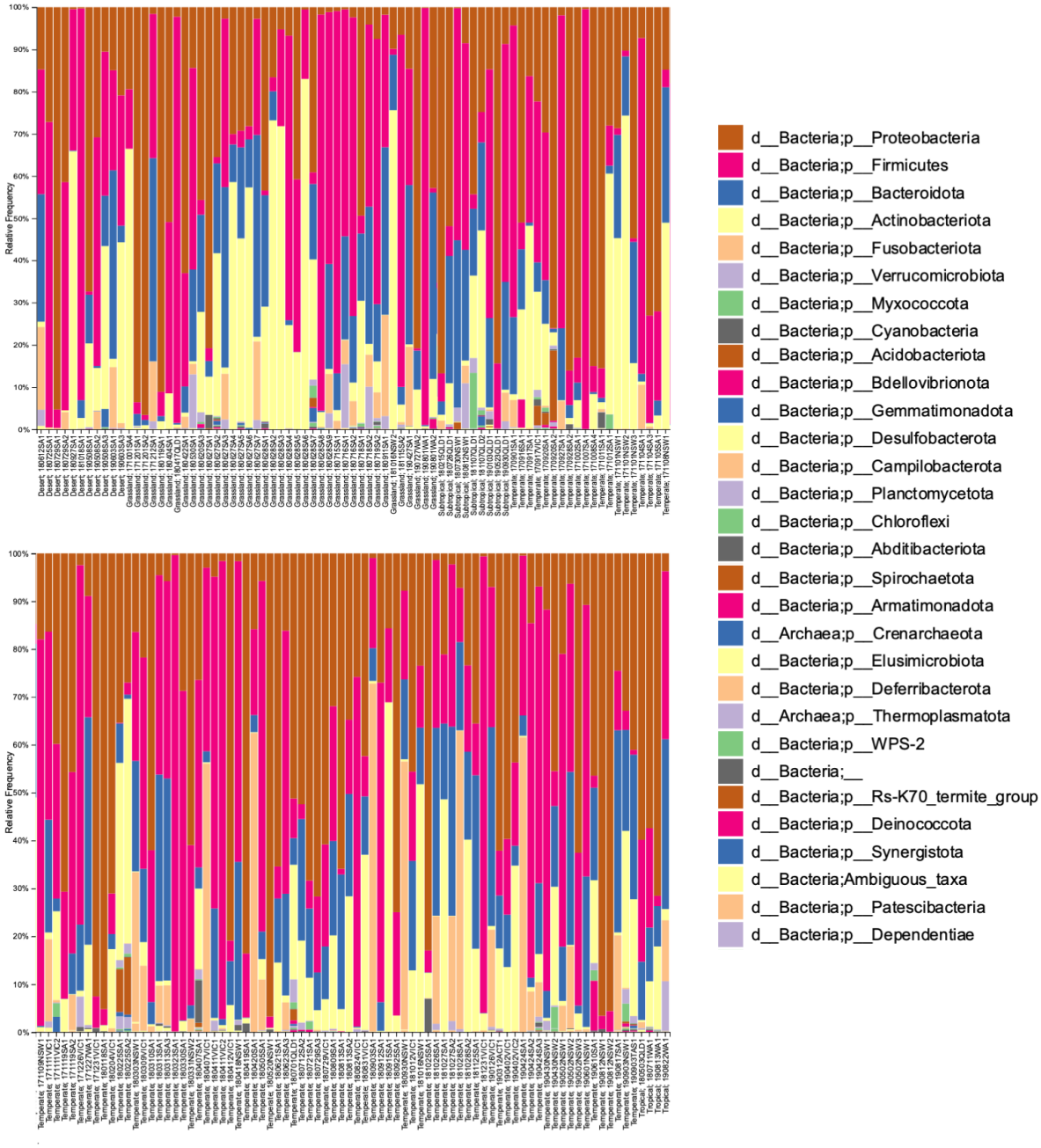

Figure 3: Taxonomy bar plots of relative frequency of bacteria present in all wild echidna scats at the phylum level. Samples are labelled and organised by their sample ID and climate class (Table S1). All phyla present are included in the legend, however only the most abundant are easily visualised; d = domain (Bacteria or Archaea); p = phyla.

3.2. Gut microbiomes of echidnas held in captivity are vastly different to wild echidnas

Captive animals often feature different microbiomes when compared to wild conspecifics. We assessed this in echidnas and in addition investigated if different diets had an effect on their microbiomes. There were no differences observed for alpha diversity metrics ($p > 0.05$; Faith's PD, Observed ASVs, Shannon Diversity) between samples collected in the wild and in zoos (Figure S2). However, the two groups (wild vs captive) were significantly different in regard to microbial composition (unweighted UniFrac distances $p = 0.001$; PERMANOVA; Figure 4A). Both wild and zoo-held echidnas shared most of the same abundant bacteria phyla (Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota and Fusobacteriota), however there was very little overlap in the most abundant genera observed between the two groups. For example, echidnas fed captive diets had abundances of *Bacteroides*, *Proteus*, *Lactobacillus*, *Peptostreptococcus*, *Lactococcus*, uncharacterised *Lachnospiraceae*, and *Peptoniphilus*. Very small abundances of *Acinetobacter* were observed, which was one of the most prevalent bacteria in the wild samples. *Fusobacterium* was the only prominent bacteria in the zoo samples that was seen frequently in wild samples (Figure 4B).

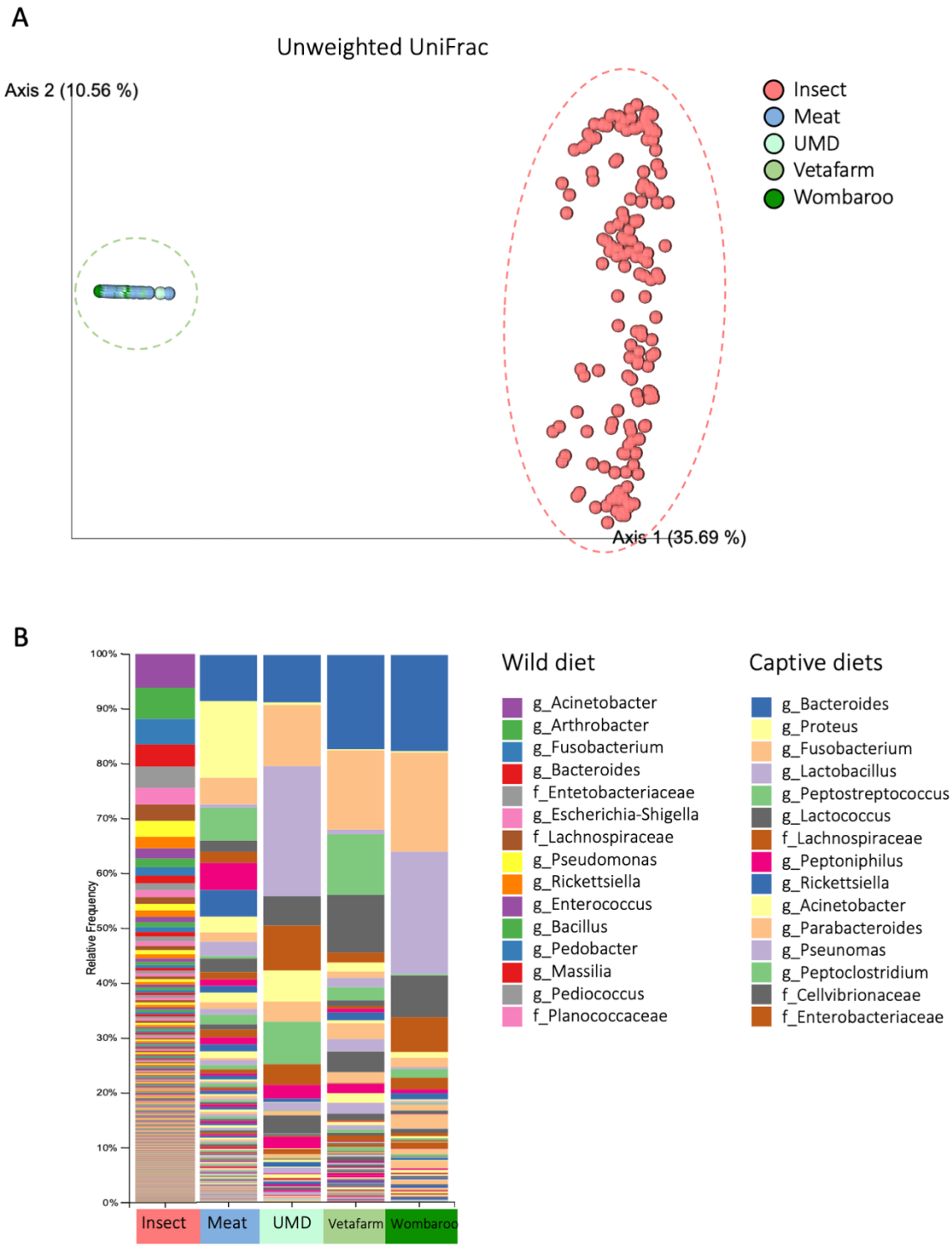

Figure 4: Differences in microbial composition are observed between samples collected in wild compared to samples collected in captivity fed four different diets. A) PCoA plot of unweighted UniFrac distances showing complete separation between wild samples (insect diet) and zoo samples (Meat, UMD, Vetafarm and Wombaroo diets). B) Taxonomy bar plots showing relative frequencies of bacteria present in echidna scats shown at the genus or family level; all samples have been aggregated according to their diet and an average relative frequency is shown. Samples are labelled by their diet where insect refers to wild collected samples. As there is very little cross over of bacterial genera between wild and captive samples, the top 15 genera or families have been provided for these two groups separately. UMD = Updated Meat Diet; g = genus; f = family.

3.3 Different diets fed in captivity affect the echidnas' gut microbiomes

Lastly, we assessed how different diets in captivity may affect the microbiomes of echidnas. Of the four diets tested, we found that the gut microbiome from echidnas fed the Meat diet was more phylogenetically diverse than all other diets, had greater number of ASVs than UMD and Vetafarm, and had a greater Shannon's diversity when compared to the Vetafarm diet ($p<0.05$; Figure S3). There were no statistically significant alpha diversity differences observed between UMD, Vetafarm and Wombaroo diets ($p>0.05$). Unweighted UniFrac distances also showed significant differences in microbial composition between Meat and all other diets ($p = 0.001$; PERMANOVA; Figure 5A).

Similar to the samples collected from wild echidnas, the major phyla present in samples collected from captive echidnas include Firmicutes, Proteobacteria, Bacteroidota, Fusobacteriota, and Actinobacteriota, with lower abundances of Verrucomicrobiota, Desulfobacterota, Campilobacterota, Bdellovibrionota, Cyanobacteria, and Myxococcota. Several genera were observed in most or all zoo samples including *Bacteroides*, *Fusobacterium*, *Lactococcus*, *Acinetobacter*, *Parabacteroides*, *Enterococcus*, *Erysipelatoclostridium*, *Escherichia-Shigella*, and uncharacterised genera of *Enterobacteriaceae* family. *Rickettsiella* and *Peptoniphilus* were only present in samples from echidnas fed the Meat diet. *Pseudomonas* mostly appeared in Meat diet and *Proteus* was more abundant in Meat diet. *Peptostreptococcus* was found commonly in echidnas fed the Meat or Updated Meat Diet, while *Lactobacillus* and *Lachnospiraceae* were found exclusively in samples from echidnas fed the Vetafarm and Wombaroo diets (Figure S4).

In order to assess if these results are affected by independent sampling and experimental variation, we included technical replicates (DNA extracted in triplicate from individual scats) and investigated longitudinal variation (scats sampled from echidnas across 3 days). This did reveal daily variation in the gut microbiome of some echidnas, even being fed the same diet and housed in the same environment (Figures 5B and S4). Technical triplicates from two samples confirm that this is not a result of technical variation (S5).

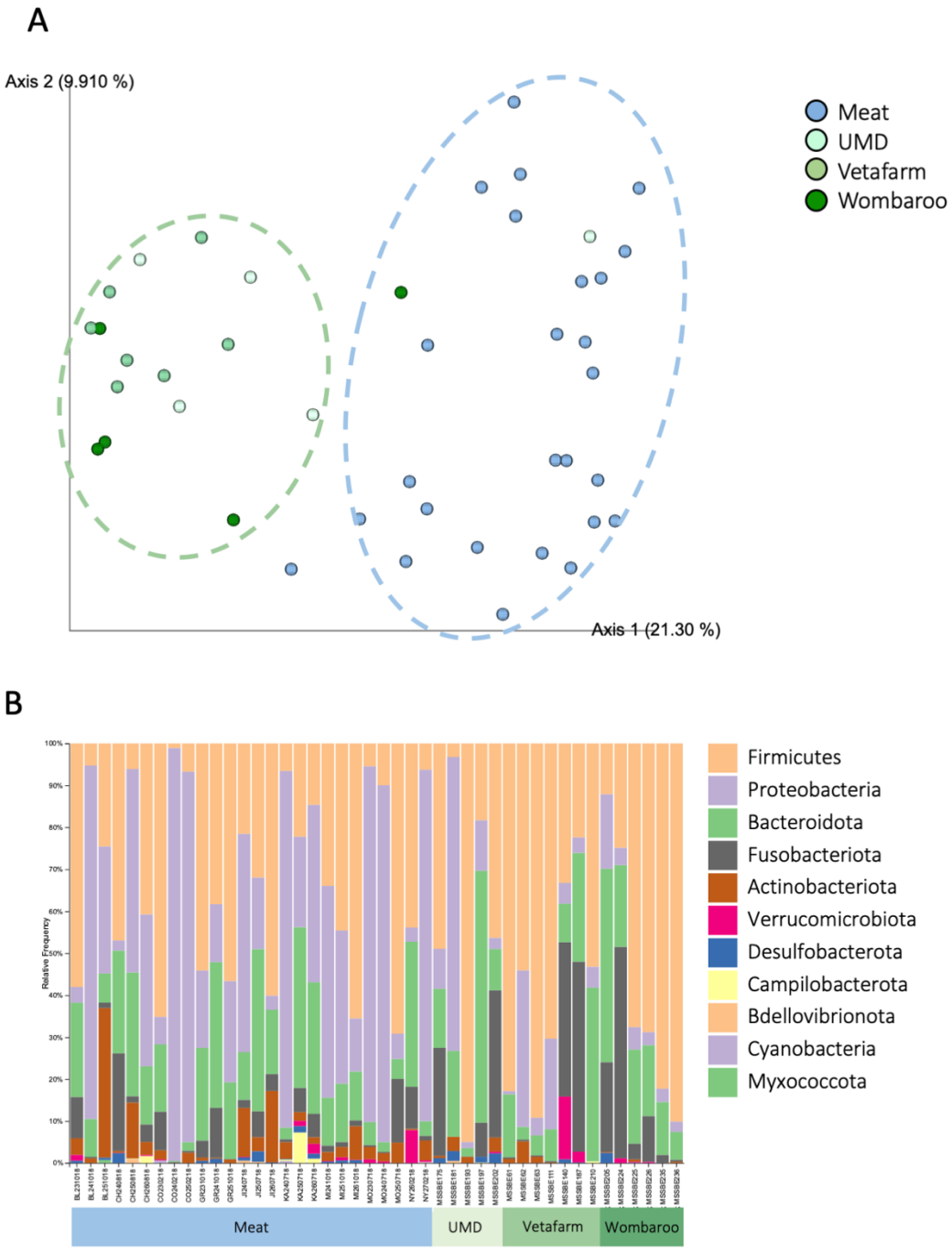

Figure 5: Differences in microbial composition are observed between Meat diet and all other diets fed in captivity. A) PCoA plot of unweighted UniFrac distances showing separation between Meat diet clustering further to the right of Axis 1 and other diets clustering to the left of Axis 1. B) Taxonomy bar plots showing relative frequencies of bacteria present in echidna scats shown at the phylum level. Samples are labelled by their sample ID and diet (Table S2). All phyla present are included in the legend, however only the most abundant are easily visualised; as bar colours repeat, the legend is labelled with most abundant taxa on top to least abundant taxa on bottom of legend. UMD = Updated Meat Diet.

4. Discussion

This study is the first characterisation of the short-beaked echidna gut microbiome. We show here that the major phyla forming the gut microbiome consist of Proteobacteria, Firmicutes, Bacteroidota, Actinobacteriota, and Fusobacteriota. These are consistent with gut microbiota in most mammals, where Firmicutes and Bacteroidota are usually the dominant groups (Ley et al., 2008). Wild echidnas have a much greater abundance of Proteobacteria, which has often been associated with gut dysbiosis (Shin et al., 2015). However, in echidnas, this is due to the dominating genus *Acinetobacter* (which belongs to Proteobacteria phylum); *Acinetobacter* is a common soil bacterium (Acer et al., 2020), which is consistent with echidnas consuming large amounts of soil when foraging. This is further supported by the presence of *Arthrobacter*, another prolific soil bacterium and the second most abundant bacteria genus in wild samples (Radkov et al., 2016). Interestingly, along with soil and environmental bacteria, the next most abundant groups were plant-fermenting and lactic acid bacteria, including *Lachnospiraceae, Pedicococcus, Enterococcus*, *Lactococcus*, and *Oscillospiraceae* (Biddle et al., 2013; George et al., 2018; Porto et al., 2017; Teuber, 1995). This suggests that plant material may be a much more prominent part of the echidna diet than has been previously recognised. It also raises the question whether the echidna gut system can be considered fermentative with the combination of an abundance of these putatively fibre-fermenting bacteria and a monogastric digestive tract, which is how hindgut-fermenting mammals, such as odd-toed ungulates (horses and rhinoceroses), rodents, rabbits and koalas, digest cellulose (Prins and Kreulen, 1990).

A large proportion of *Fusobacterium* is not commonly seen in mammal gut microbiomes (Ley et al., 2008), however, some wild echidna samples had up to 70% relative abundance of this bacterium. In humans, some species of *Fusobacterium* can be attributed to colorectal cancer (Castellarin et al., 2012), and other diseases (Han, 2015), but it is rare to find it in faecal samples. It has, however, been observed in the proximal and distal intestines of Atlantic cod (Zhou et al., 2013), large intestine of vultures (Roggenbuck et al., 2014) and more recently in faecal material collected from the rectum of jackals (Menke et al., 2017), with no evidence of being pathogenic. Furthermore, as *Fusobacterium* was also present in the zoo echidna samples, it is likely a gut commensal in echidnas as these animals were considered healthy at time of sampling.

This work also revealed the presence of *Rickettsiella* in wild samples collected in South Australia, New South Wales and Victoria. This bacterium is associated with hard ticks and can be pathogenic to both the tick hosts and to mammals if transmitted (Leclerque and Kleespies, 2012). Echidnas can often be infested with hard ticks, there is even a species of tick that is

recognised to live almost exclusively on echidnas (*Bothriocroton concolor*; Roberts, 1970), although this species has also been observed on Kangaroo Island kangaroos (Oorebeek and Rismiller, 2007). In three samples (located in Kangaroo Island and Waitpinga, SA, and Wamboin, NSW), *Rickettsiella* was 80-90% of total bacterial abundance indicating echidnas may be frequently ingesting ticks, which has been observed in the wild (P. Rismiller, Pers. Comms.).

Our finding of significant changes in gut microbiome of captive echidnas has also been observed in many different mammalian taxa (McKenzie et al., 2017) and is likely due to different diets and environments. These changes are less frequently observed in omnivores and herbivores as their diets provided in captivity may more closely resemble their natural diet (Delsuc et al., 2014; McKenzie et al., 2017). A similar dramatic shift was observed in the aardvark and giant ant eater, which are myrmecophagous species that have also had nutrition-related health problems due to the difficulty in creating appropriate diets (Clark et al., 2016; McKenzie et al., 2017). Rather than soil and environmental bacteria forming the majority of the microbiome like is seen in wild echidnas, zoo-held echidnas had a greater proportion of Bacteroidota, especially *Bacteroides* and *Parabacetroides*, which are common gut commensals (Hiippala et al., 2020). Interestingly, although captive echidnas are fed a carnivorous-modelled diet with meat as the main ingredient, there were still high proportions of putative plant-fermenting and lactic acid bacteria present in their gut, suggesting that echidnas may naturally be herbivorous hindgut fermenters. In 2017, Shaw suggested that echidnas be reclassified as insectivorous herbivores, which is supported by our findings (M. Shaw, Pers. Comms.).

Diet appears to play a significant role in the formation of echidna gut microbiomes. Even subtle differences in diets fed in zoos resulted in microbial community and diversity changes, particularly when comparing the Meat diet to the three other diets at Taronga Zoo. Location effects, such as water source or soil in enclosure, may also enhance these differences, as the Meat diet was exclusively fed at Perth Zoo and showed the greatest dissimilarity. The Meat diet shows greater microbial diversity but also potentially pathogenic bacteria such as *Proteus* (a pathogen found in beef mince; Doulgeraki et al., 2011) and *Rickettsiella*. Whereas in the diets fed to echidnas in Taronga Zoo, there were greater abundances of gut commensals and even *Lactobacillus*, however this may be coming directly from the food source as the Vetafarm and Wombaroo diets include a dry yeast probiotic that may contain *Lactobacillus*. Both zoo populations were healthy at the time of sampling and have had recent reproductive success (Ferguson and Turner, 2013; Perry et al., 2019), so future research is needed to understand the pathogenic and probiotic properties of gut bacteria in relation to echidna health.

Another feature of the echidna gut microbiome is how variable it can be. Even in captivity, where echidnas are housed in the same environment and provided the same food, there were (sometimes major) differences observed within individuals from samples collected across three consecutive days. This daily variation, in combination with diet heavily influencing the gut microbiome, may explain the large variability observed in the wild scats and how multiple samples collected from the same location contained different bacterial profiles. As echidnas will opportunistically forage throughout the day, often travelling large distances, their daily gut microbiomes will likely depend on what food is available and in what environments they forage in. It would be ideal to examine the diet contents in these scats (either physically or genetically) to determine if these correlations exist.

This study investigates, for the first time, the gut microbial diversity and composition in echidnas. We find that diet plays a major role in defining the echidna gut microbiome, with striking differences observed between the wild samples and those from echidnas kept in captivity. An enormous amount of variation was seen in wild echidnas, which was only able to be characterised due to the great collaborative effort of sampling by citizen scientists across Australia. Some common gut commensals were present in both wild and zoo samples, however, most of the gut bacteria observed were soil or plant associated and large abundances of Actinobacteria and Fusobacteria which appear unique to echidnas, as they are not common in other mammals' guts. It will be important in the future to determine the pathology and probiotic relationships between the bacteria and echidnas in order to make meaningful connections to echidnas' health and determine if we should be concerned with the differences observed in managed echidnas. Furthermore, this research has provided new insights into the biology and gastric functions of an iconic and unique Australian mammal.

Acknowledgements

Authors would like to acknowledge the citizen scientists of the EchidnaCSI project who collected echidna scat samples used in this study. Thank you to Perth and Taronga Zoos' zookeepers for collecting echidna scat samples used in this study. This research was funded by the Environment Institute at The University of Adelaide. Tahlia Perry and Alan Stenhouse are funded by the Research Training Program. Frank Grützner is an Australian Research Council Future Fellow. Peggy Rismiller and Michael McKelvey acknowledge the Pelican Lagoon Research Centre for research support and resources. Funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Author Contributions

TP performed sample processing, laboratory work, data analysis, created figures and wrote the manuscript; EW performed laboratory work, data analysis and cowrote sections of the manuscript; RE provided guidance and resources for the microbiome sequencing methods and aided in interpretation of data; AS created the EchidnaCSI app which enabled sample collection; IS performed sample processing and data management; BL collected samples and provided guidance on echidna biology; PDR collected samples, guided the design of the project, aided in interpretation of results and edited manuscript; MS collected samples, guided the design of the project, aided in interpretation of results and edited manuscript; FG contributed to design and supervision of the project as well as manuscript preparation and evaluation.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter Four:

Changes observed in gut microbiomes of Kangaroo Island echidnas (*Tachyglossus aculeatus multiaculeatus*) following bushfires

Statement of Authorship

Title of Paper	Changes observed in gut microbiomes of Kangaroo Island echidnas (*Tachyglossus aculeatus multiaculeatus*) following bushfires
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Submitted to Australian Mammalogy

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Name of Principal Author (Candidate)	Tahlia Perry
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Overall percentage (%)	80%
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Changes observed in gut microbiomes of Kangaroo Island echidnas (*Tachyglossus aculeatus multiaculeatus*) following bushfires

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Abstract

Kangaroo Island experienced extensive bushfires in December 2019 and January 2020, affecting almost half of the island. This has impacted several of the threatened species including the Kangaroo Island echidnas (*Tachyglossus aculeatus multiaculeatus*). Echidnas were amongst the first animals observed foraging in the burnt areas once the fires had subsided. Changes in soil chemistry and food availability in the burnt areas raises questions about the impact on the gut health and foraging behaviours of echidnas. Here, we assessed the gut microbiome of Kangaroo Island echidnas before and after the fires. Metabarcoding of scat microbiota revealed substantial changes in diversity and composition in echidnas post-bushfire when compared to samples collected prior to the bushfires. Before the fires, echidna gut microbiomes were more variable and contained mostly soil-associated bacteria, whereas post-fire samples shifted to more uniform bacterial communities consisting of lactic acid and gut commensal bacteria. Interestingly, changes were observed in scats collected in both burnt and unburnt areas on the island, suggesting echidnas are foraging between these areas, depending on their home ranges. This is the first study to document changes in gut microbiomes in echidnas following bushfires. More work is needed to investigate if the gut bacterial communities continue to change as the areas recover from the fires and to understand the effects on animal health.

Keywords: bacteria, bushfire recovery, endangered, Tachyglossus, 16S metabarcording

1. Introduction

Fire plays an important role within the Australian ecosystem, however, extreme weather events such as wildfires are becoming more frequent and intense as climate change extends the number of hot and dry days throughout the year (Bowman et al., 2009; Burrows, 2008; Lindenmayer et al., 2020; Nicholls and Lucas, 2007). Australia experienced its most historically devastating bushfire season from July 2019 to February 2020 (named the Black Summer), which was estimated to burn 97,000 km^2 of land, affecting 832 vertebrate species (Ward et al., 2020). These fires have had a huge effect on plant and animal biodiversity, food availability and habitat (Ward et al., 2020). Kangaroo Island is the third largest island in Australia and from December 2019 to January 2020 almost 50% of the 4,405 km^2 island was affected by fire (Department for Environment and Water, 2020). This resulted in a call to action to assess the effects of bushfires on priority flora and fauna species (31 plant and 23 animal) (Department of Agriculture, Water & Environment 2020, 'Kangaroo Island regional bushfire recovery workshop report').

According to current nomenclature, the Kangaroo Island echidna (*Tachyglossus aculeatus multiaculeatus*) is one of five subspecies of short-beaked echidna (Rismiller and Grutzner., 2019). The Kangaroo Island echidna was recently recognised as endangered due to threats from habitat destruction/fragmentation, roads increasing roadkill, and introduced predators such as cats (EPBC Act 2015, 'Conservation Advice *Tachyglossus aculeatus multiaculeatus* Kangaroo Island echidna'). The bushfires have further intensified the threats that these echidnas face, resulting in this subspecies being placed on an urgent fire-recovery list for threatened species (Department of Agriculture, Water & Environment 2020, 'Rapid analysis of impacts of the 2019-20 fires on animal species, and prioritisation of species for management response').

Egg-laying mammals, including platypuses and echidnas, are the oldest surviving mammals and well-adapted to fire. Depending on substrate, echidnas are able to dig underground as the fire front passes. In addition they go into torpor (hibernation-like state) until it is safe to re-emerge (Nowack et al., 2016). Echidnas foraging habits change depending on the intensity of the fire; after low intensity and patchy fires where most habitat remains intact within their home range, echidnas are able to still find shelter to protect themselves against predators, whilst also foraging more easily. Whereas after an intense burn where most (if not all) remnant shelter has been destroyed, echidnas will forage across a smaller area, likely to avoid predation (McKemey et al., 2019). Echidnas where amongst the first animals observed foraging in the burnt areas once the fires had subsided on Kangaroo Island (P. Rismiller, Pers. Comms.). The changes in soil chemistry and food availability in the burnt areas raises questions about the impact on the gut health and foraging behaviours of echidnas.

The gut microbiome plays a vital role in health and wellbeing of animals and an increasing number of studies investigate microbiome changes in wild populations to improve conservation efforts (Bird et al., 2019; Chong et al., 2019; McKenzie et al., 2017). Although there is strong evidence of how changes in an animal's environment (especially diet and habitat) significantly affect their gut microbiomes (Bird et al., 2019; Brice et al., 2019; McKenzie et al., 2017), research into the effects of bushfires on animal microbiomes is currently limited to few human oral and gut studies (Perera and Perera, 2018, Gillings et al., 2015). However, there is a better understanding of how soil microbiomes are affected by fire (Certini, 2005). Although microbial communities are significantly altered immediately following fire, recovery is observed after one year post fire, where even increases in the diversity is seen (Shen et al., 2016; Xiang et al., 2014). As fires are common in the Australian ecosystem, it will be important to assess how the microbial communities respond in regards to native wildlife.

The Kangaroo Island echidna is the best studied echidna population in Australia and echidnas can be found anywhere on the island (Rismiller, 1999; Rismiller and Grutzner, 2019; Rismiller and McKelvey, 2003); The Echidna Conservation Science Initiative (EchidnaCSI) is a citizen science project in which researchers collaborate with the community to collect echidna sightings and scat samples. Strong participation, in particular on Kangaroo Island, has provided us with sufficient samples before and after the 2019 fires to assess the effects of bushfires on the echidna gut microbiome. Here, we provide the first analysis and comparison of gut microbiomes of echidnas before and after bushfires. Our finding of major changes in scats collected in burnt and unburnt areas, before and after the fires, raises important questions about the health effects of fires on native Australian species.

2. Materials and Methods

2.1 Wild echidna faecal sample collection and metadata

Faecal samples from wild echidnas prior to the 2019/2020 bushfires were collected through a collaborative effort with volunteers as a part of the citizen science project: Echidna Conservation Science Initiative (EchidnaCSI; www.grutznerlab.weebly.com/echidna-csi.html). Two participants had collected echidna scats (n=6) from their properties on Kangaroo Island from October 2017 – October 2019 (Figure 1; Table 1). Faecal samples from wild echidnas after the bushfires were collected by Dr Peggy Rismiller, either found defecated in their habitat or harvested from the lower intestine of a deceased (due to roadkill) echidna (n=7).

Two scat samples were collected within the burnt region of Kangaroo Island, while five samples were collected outside the burnt region, on the eastern side of the island (Figure 1; Table 1). After collection, samples were shipped immediately to The University of Adelaide and then stored in a freezer.

Table 1: Information relating to all scat samples used in analysis. Fire = whether the scat was collected before or after the 2019 bushfires; fire area = whether or not the sample was collected in an area affected by fire (Figure 1); sex was known if the scat was taken from a deceased animal and could be correctly identified; echidna breeding season falls between June – September each year.

Sample ID	Collection Date	Latitude	Longitude	Fire	Fire Area	Material Type	Sex	Season	Breeding Season
171002SA1	2/10/17	-35.60	137.58	before	no	scat	unknown	spring	no
171002SA2	2/10/17	-35.60	137.58	before	no	scat	unknown	spring	no
171008SA4	8/10/17	-35.74	137.64	before	no	scat	unknown	spring	no
171205SA1	5/12/17	-35.74	137.64	before	no	scat	unknown	summer	no
180914SA1	14/9/18	-35.74	137.64	before	no	scat	unknown	spring	no
191015SA1	15/10/19	-35.60	137.58	before	no	scat	unknown	spring	no
200125SA1	25/1/20	-35.79	137.04	after	yes	scat	unknown	summer	no
200423SA1	23/4/20	-35.82	137.56	after	no	scat from intestine	male	autumn	no
200508SA1	8/5/20	-35.81	137.79	after	no	scat	unknown	autumn	no
200707SA1	7/7/20	-35.78	137.54	after	no	scat from intestine	male	winter	yes
200707SA3	7/7/20	-35.78	137.54	after	no	scat from intestine	male	winter	yes
200805SA1	5/8/20	-35.77	137.49	after	no	scat from intestine	male	winter	no
200814SA1	14/8/20	-35.99	137.04	after	yes	scat from intestine	male	winter	no

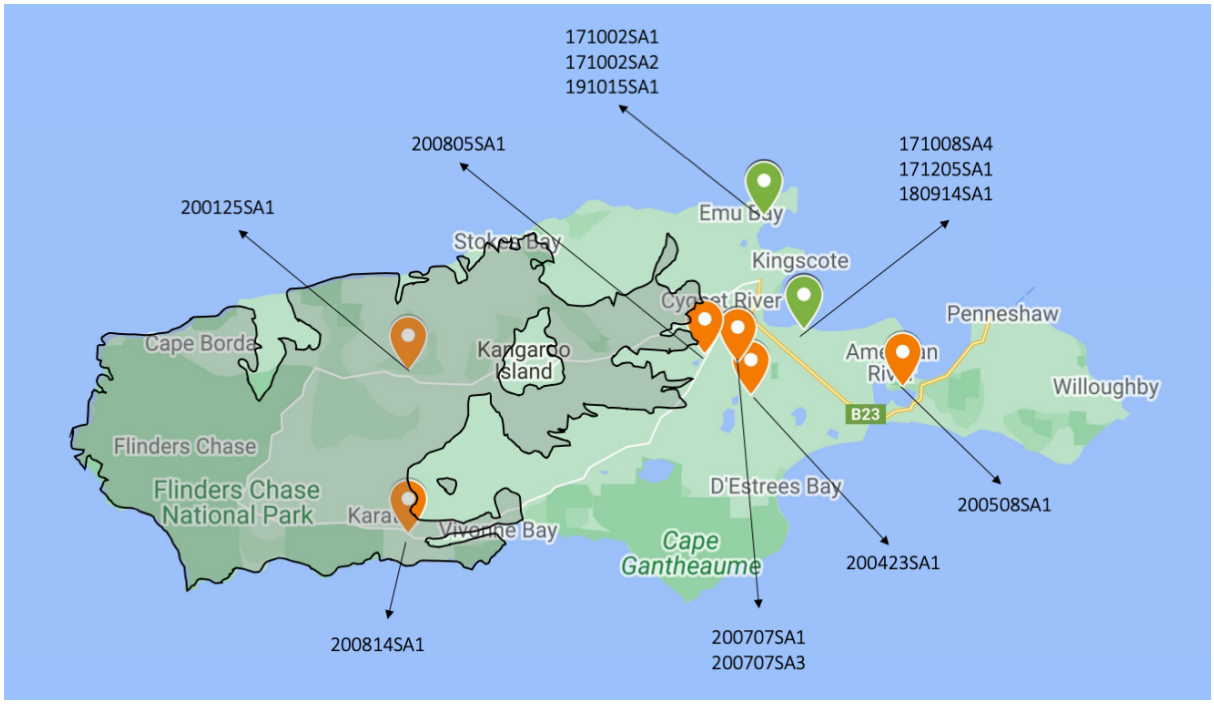

Figure 1: Locations of scat samples collected from Kangaroo Island, South Australia. Samples are labelled with ID number (Table 1); green = samples collected prior to bushfire, orange = samples collected after bushfire. Bushfire impacted regions are shaded grey with black outline.

2.2 DNA extraction

Total genomic DNA was extracted from 13 faecal samples using the Qiagen QIAamp Mini Stool Kit (Qiagen, Hilden, Germany) as per the manufacture's protocol with some modifications. The extractions were performed in a Flow Cabinet Biological Safe Level 2 that was cleaned with 10% bleach (sodium hypochlorite). Approximately a third of the sample was crushed up in liquid nitrogen using a mortar and pestle, prior to adding the sample to InhibitX Buffer. Samples were centrifuged at 20,000 g for 3 min and ~1 mL eluate transferred to a new 1.5 mL tube. Samples were again centrifuged at 20,000 g for 1 min and ~700 uL of eluate transferred to a new 1.5 mL tube. Samples were centrifuged one last time at 20,000 g for 1 min and 600 uL added to 25 uL Proteinase K and processed according to the manufacturer's protocol.

2.3 PCR amplification

All samples were PCR amplified and uniquely barcoded, using primers targeting the V4 region of the bacteria 16S ribosomal RNA (rRNA) gene. DNA was amplified with the primer pair 515F (5'-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3') and uniquely barcoded 806R (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnnnnnnnAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3'). Single reactions of 18.7 µL dH_2O, 2.5 µL 10X HiFi buffer, 1 µL 50mM MgSO4, 0.1 µL Platinum Taq DNA Polymerase (ThermoFisher), 0.2 µL 100 mM dNTP, mix, 0.5 µL of 10 µM forward primer, 1 µL of 5 µM reverse primer and 1 µL DNA. DNA was amplified using an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 50 °C for 1 min, elongation at 68 °C for 90 sec, with final adenylation for 10 min at 68 °C, as per accepted Earth Microbiome Protocol (Thompson et al., 2017).

PCR products where checked (2.5% agarose gel) for length (~390 bp), quantified (Qubit 2.0 Fluorometer) and pooled to equimolar concentration. Pooled samples were cleaned following the Agencourt AMPure XP PCR purification protocol (Beckman Coulter), quantified and quality checked by the LabChip® GX Touch™ nucleic acid analyser. A final concentration of 4 nM was run on the Illumina Miseq (v2, 2 x 250bp) at ACRF (Australian Cancer Research Foundation) Cancer Genomics Facility.

2.4 Data processing and statistical analyses

Sequence data were processed and analysed using QIIME2 v2020.2 (Bolyen et al., 2019). Demultiplexed paired-end sequence reads were merged, quality filtered and denoised into Amplicon Sequence Variants (ASVs) using the *deblur* plugin (Amir et al., 2017) with a trim length of 247 bp. The feature table was rarified to a depth of 18,150, using the minimum number of sequences per sample for diversity analysis. Representative sequences were assigned taxonomy using the *feature-classifier* plugin (naive bayesian approach) on the pre-trained SILVA (Quast et al., 2013) 138 V4 region classifier (Bokulich et al., 2018). Alpha diversity was assessed by diversity metrics including observed ASVs, Faith's phylogenetic diversity, Shannon's entropy and Pileou's evenness and statistical significance was assessed using the Kruskal-Wallis tests. Beta diversity was assessed by weighted and unweighted UniFrac metrics and visualized by Principal Coordinates Analysis (PCoA), with statistical significance assessed with Permutational Multivariate Analysis of Variance (PERMANOVA) tests, with 999 permutations.

3. Results

3.1 Changes in microbial community post-bushfire

DNA sequencing of the 13 scat samples providing an output of 3,263,715 reads, with a mean of 171,774 per sample, resulting in a total of 1,111 Amplicon Sequence Variants (ASVs).

First, we analysed the alpha diversity between samples collected before and after the bushfires, as well as those collected within or outside the burned regions. This revealed that samples collected after the bushfires appeared to have greater (yet not significant) alpha diversity in comparison to samples collected before the bushfires. Samples collected after the fires had greater phylogenetic diversity (Faith's phylogenetic diversity), greater number of ASVs (observed ASVs), greater richness (Shannon's entropy) and greater evenness (Pielou's evenness) than samples collected before the fires ($p > 0.05$; Figure 2). Next, we assessed the microbial compositions of the samples, where a complete change in microbial community was observed for all samples (except one) collected after the bushfires. This was independent of whether they were collected within or outside bushfire affected areas ($p = 0.005$, unweighted and weighted UniFrac; Figure 3). No effect was observed when comparing seasonal differences, material type, or whether the echidna was in breeding season or not ($p > 0.05$, unweighted and weighted UniFrac).

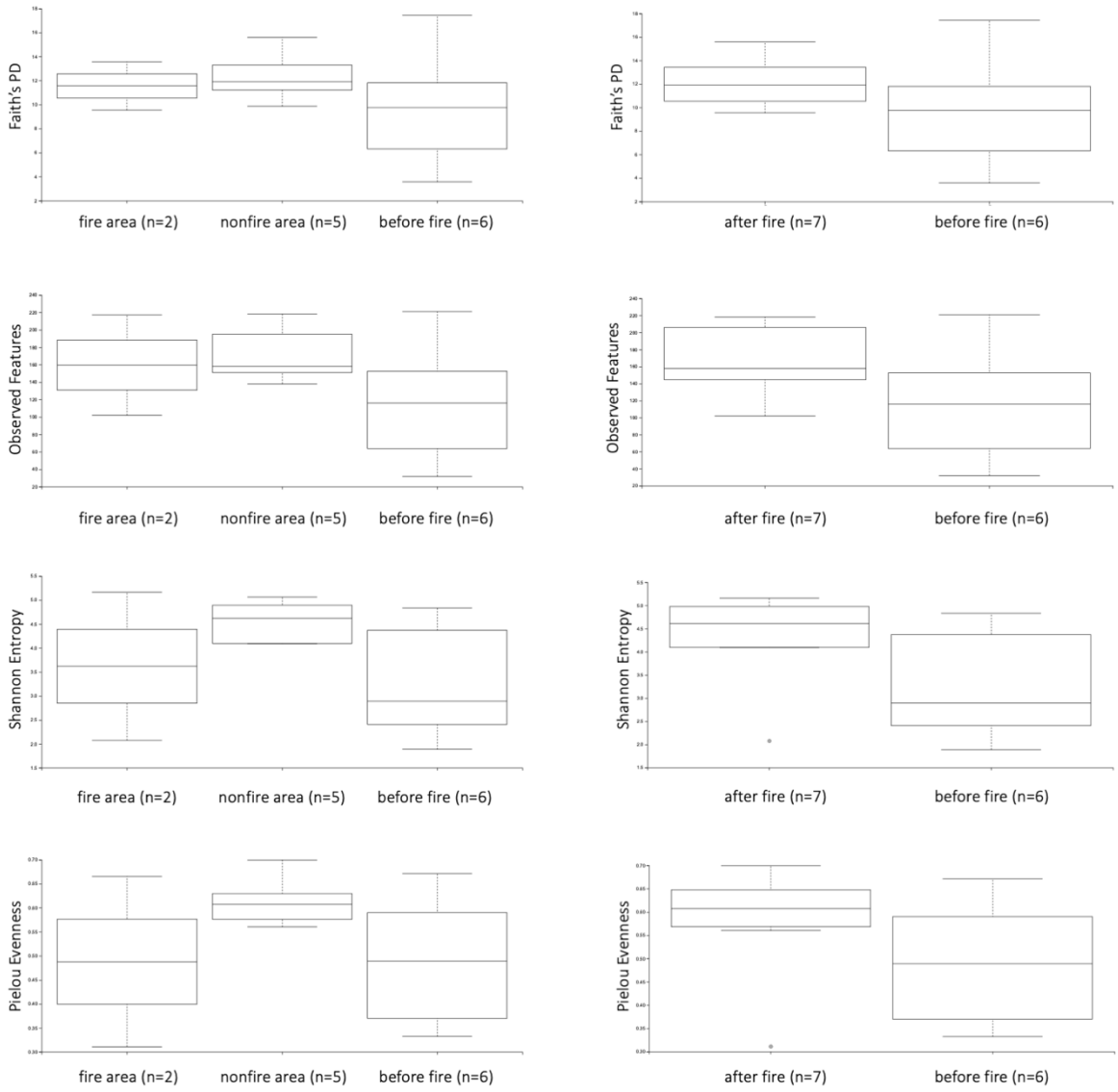

Figure 2: Alpha diversity analyses of scat samples, showing greater microbial diversity in samples post-bushfire. Whisker-box plots depict the following diversity tests: Faith's phylogenetic diversity (Faith's PD), Observed features, Shannon's entropy, and Pielou's evenness. Left side figures compare samples collected before the 2019 bushfires to samples collected after the fires either within fire-affected regions (fire area) or outside fire-affected regions (nonfire area; Figure 1), while the right side figures compare if samples were collected before or after the fires, irrespective of region. Whisker-box plot horizontal lines indicate median values, upper and lower bounds represent the 25th and 75th percentiles, and top and bottom whiskers indicate maximum and minimum values.

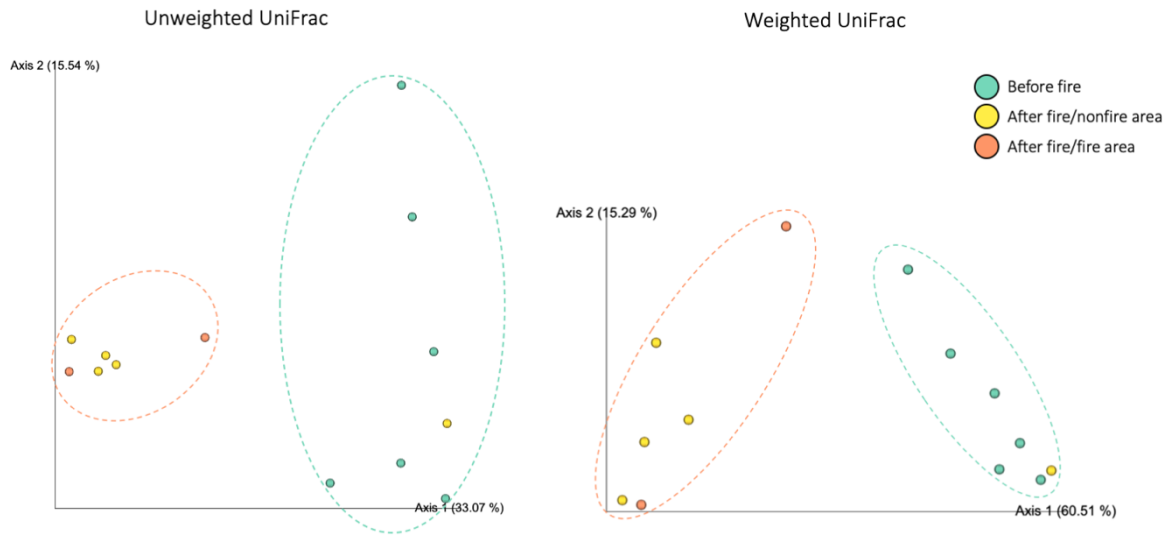

Figure 3: Effect of bushfire on echidna scat microbial composition. Principal Coordinates Analysis plots show unweighted and weighted UniFrac distances for echidna faecal samples. Before fire = sample collected prior to the 2019 bushfires; After fire/nonfire area = after the bushfire but not in a fire-affected region; After fire/fire area = after the bushfire and collected within a fire-affected region. The PCoA plots clearly separate almost all samples collected after the bushfire (dotted orange circle) from samples collected before the bushfire (dotted green circle).

3.2 Bacterial taxa changes in samples pre- and post-fire

Visualisation of the bacterial communities within samples, shows that samples collected prior to the bushfires were dominated by bacteria from phyla Proteobacteria, followed by Actinobacteriota and Firmicutes; while samples collected after the bushfires were dominated by Firmicutes, followed by Bacteroidota and Proteobacteria (Figure 4A). At a genus level, there were few genera that were consistently seen in the samples collected prior to the fires (Figure 4B); *Arthrobacter* and *Acinetobacter* were the most common, found in 6/6 and 5/6 samples respectively, with 171002SA2 and 171008SA4 dominated by *Acinetobacter*. 171002SA1 was dominated by *Rickettsiella*; 191015SA1 had large proportions of *Arthrobacter* and *Solibacillus*; 171205SA1 consisted of relatively even frequency of *Acinetobacter*, *Arthrobacter*, *Bacillus* and was the only sample with a large proportion of *Massilia*. 180914SA1 had the most unique microbial composition with dominating genera consisting of *Rhodococcus*, *Ochrobactrum*, *Stenotrophomonas*, *Brevundimonas*, *Pseudomonas*, *Achromobacter*, and *Sanguibacter*. Samples collected after the bushfires had a much more consistent microbial composition (Figure 4B); The following genera were present in either all or 6/7 samples: *Pediococcus*, *Bacteroides*, uncharacterised genera of *Oscillospiraceae*, *Enterobacteriaceae*, and *Parabacteroides*. There was also a large proportion of an uncharacterised genus and several characterised genera belonging to the family *Lachnospiraceae*, including *Lachnoclostridium*,

Roseburia, *Marvinbryantia*, *Tyzzerella*, and *Frisingicoccus*. *Rickettsiella* was seen more consistently in samples collected after bushfires, however, in low frequencies. 200508SA1 had a similar microbial profile to samples collected before the bushfire; it was mostly dominated by *Acinetobacter* and the only sample collected after fires to consist of *Arthrobacter*. As seen in Figure 1, this sample was collected from much further east than the other samples.

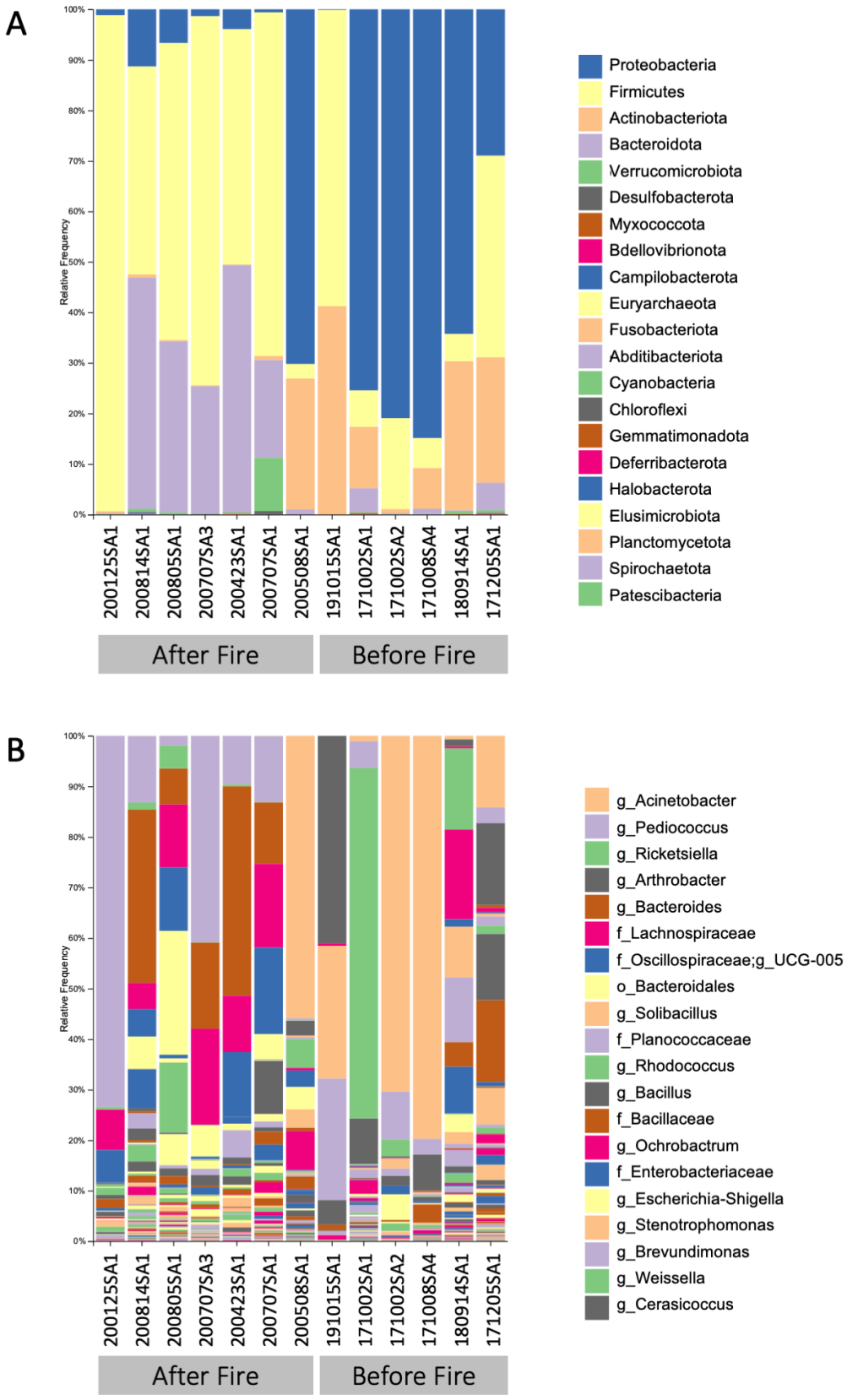

Figure 4: Taxonomy bar plots visualising the effect of bushfire on presence and relative frequency of bacteria in echidna scat samples. A) shows all bacteria phyla present in samples; B) shows all bacteria genera present in samples (only top 20 most abundant appear in legend). Samples are labelled by their sample ID (Table 1). After fire = samples collected after the 2019 bushfire; before fire = samples collected prior to the bushfire; o = order; f = family; g = genus.

4. Discussion

It is well established that the gut microbiome can be affected by environmental changes (Spor et al., 2011). In the context of bushfires, factors impacting on gut microbiota can be related to changes in diet, compositional changes in soil, as well as addition of chemicals used to put out fires. Echidnas are known to survive fires and forage in the affected areas immediately following a fire (McKemey et al., 2019; Nowack et al., 2016). As echidnas ingest large quantities of soil whilst foraging, this exposes them to changes in soil composition and chemistry.

Here, we investigated how bushfires affect the gut microbiomes of the endangered Kangaroo Island short-beaked echidna, where results show bushfires significantly influence the gut microbiome. Microbial communities in samples collected from echidnas after the 2019/2020 bushfires were completely changed compared to those collected prior to the bushfires. Interestingly, this included samples collected outside the burnt areas on Kangaroo Island, as they shared the same microbiome signature changes as those collected inside the burnt regions. Echidnas on Kangaroo Island have a home range of up to 88 hectares (Rismiller and Mckelvey, 1994), therefore, it is likely that the scat samples collected outside the burnt regions belonged to echidnas that had foraged within the burnt regions prior to defecating, which is possible as echidnas only defecate once every two days (Snipes et al., 2002). The only post-bushfire sample that had a microbial composition similar to samples collected prior to the bushfires was collected much farther east on the island, where that echidna had most likely not foraged in the burnt areas or was otherwise less exposed to the effects of the fires.

Soil-bacteria were abundant in samples collected before the fires, including *Acinetobacter* (Proteobacteria) and *Arthrobacter* (Actinobacteriota) (Acer et al., 2020; Radkov et al., 2016). This has been documented in echidnas across many different regions in Australia (Perry et al., 2020, submitted). Interestingly the post-fire samples shifted to having more gut commensal (Bacteroidota) and plant-fermenting lactic acid (Firmicutes) bacteria. Soil makes up a significant part of echidna scats, as they ingest soil while foraging. However, intense bushfires will remove a large proportion of topsoil (0-10 cm) as well as change the properties of soil including pH, nutrient content, and organic matter content, as well as the bacterial communities (Ngole-Jeme, 2019; Shen et al., 2016). Echidnas foraging in fire-affected regions are likely not consuming the same bacteria from topsoil as they usually do. Instead, the soil they are ingesting may have a different and more diverse bacterial community, which would explain the greater bacterial diversity seen in scat samples post-fire.

Echidnas are known to eat a variety of insects, worms, beetles and fungi (Feuerherdt et al., 2005; Griffiths, 1968; Rismiller and Grutzner, 2019; Smith et al., 1989; Sprent and Nicol, 2016), with previous microbiome research suggesting that plants form a significant portion of echidna diet (Perry et al., 2020, submitted). Bushfires alter the habitat and food sources available, however, ants and termites are very good at surviving fires (Avitabile et al., 2015; York, 2000). The high abundances of Firmicutes and Bacteroidota in post-fire samples is much more similar to what was observed in the gut microbiomes of eutherian obligate ant and termite eating mammals (Delsuc et al., 2014). Potentially, echidnas foraging in burnt habitat are consuming more ants and termites, and not the varied diet they usually do, which may explain why their microbiomes are more uniform in comparison to echidnas feeding prior to the bushfires.

There is surprisingly little research in the effects of fires on gut microbiomes, where limited studies focus on humans (Gillings et al., 2015; Perera and Perera, 2018). The unprecedented fires in 2019/20 have led to research efforts to understand the effects predominately in humans. As far as we know this is the first study to investigate how bushfires impact gut microbiomes in a non-human mammal. Through EchidnaCSI and local expertise we had the opportunity to investigate this on a small set of samples, which revealed significant changes in echidna microbiomes between samples collected after bushfires on Kangaroo Island compared to those collected prior to the fires. The changes are possibly due to the availability of differing soil composition and food sources. These results encourage more comprehensive future monitoring in order to assess whether microbial communities continue to change as habitat and food sources recover. Ideally, monitoring will include tracking individuals in order to determine if echidnas are foraging across burnt and unburnt areas and how quickly the gut bacterial communities change in response to their feeding behaviour. This research highlights that fires can dramatically change gut microbiomes, which may have major effects on animal health.

Acknowledgements

Authors would like to acknowledge the citizen scientists of the EchidnaCSI project, Peter Hastwell and Bill Jenner, in particular, who collected echidna scat samples used in this study. This research was funded by the Foundation for National Parks & Wildlife. Tahlia Perry is funded by the Research Training Program. Frank Grützner is an Australian Research Council Future Fellow. Peggy Rismiller and Michael McKelvey acknowledge the Pelican Lagoon Research Centre for research support and resources. Funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Author Contributions

TP performed sample processing, laboratory work, data analysis, created figures and wrote the manuscript; AL aided in laboratory work, data analysis and figure creation; MWM collected samples and guided the research; PDR collected samples, guided the design of the project, aided in interpretation of results and edited manuscript; FG contributed to design and supervision of the project as well as manuscript preparation and evaluation.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter Five:

Non-invasive genetic sexing technique for the analysis of short-beaked echidna (*Tachyglossus aculeatus ssp*) populations

Statement of Authorship

Title of Paper	Non-invasive genetic sexing technique for analysis of short-beaked echidna (*Tachyglossus aculeatus ssp*) populations
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Perry, T., Toledo-Flores, D., Kang, W. X., Ferguson, A., Laming, B., Tsend-Ayush, E., Lim, S. L., Grützner, F. 2019, Non-Invasive Genetic Sexing Technique for Analysis of Short-Beaked Echidna (Tachyglossus aculeatus ssp) Populations, Reproduction, Fertility and Development, 31(7), 1289 – 1295.

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Contribution to the Paper	Discovery of second genetic marker for sexing, sample processing, data analysis, created figures, wrote manuscript.
Overall percentage (%)	60%
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Non-invasive genetic sexing technique for analysis of short-beaked echidna (*Tachyglossus aculeatus ssp*) populations

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Abstract

Identifying male and female echidnas is challenging due to the lack of external genitalia or any other differing morphological features. This limits studies of wild populations and is a major problem for echidna captive management and breeding. Non-invasive genetic approaches to determine sex minimise the need for handling animals and are used extensively in other mammals. However, currently available approaches cannot be applied in monotremes as their sex chromosomes share no homology to sex chromosomes in other mammals. Here, we used recently identified X and Y chromosome specific sequences to establish a non-invasive polymerase chain reaction-based technique to determine the sex of echidnas. Genomic DNA was extracted from echidna hair follicles followed by amplification of two Y chromosome (male-specific) genes and one X chromosome gene: C*RSPY*, *AMHY* and *AMHX*, respectively. Using this technique, we identified the sex of 10 juvenile echidnas born at Perth Zoo, revealing that eight out of 10 echidnas are female. Future use of the genetic sexing technique in echidnas will inform captive management, continue breeding success and can be applied to investigate sex ratios and population dynamics in wild populations.

Keywords: Echidna sexing, hair sample, blood sample, sex-specific PCR, FISH, captive breeding.

1. Introduction

Echidnas and platypuses are the only living monotremes and are amongst Australia's most iconic animals. The short-beaked echidna (*Tachyglossus aculeatus ssp*) is the most wide-spread mammal in Australia and also found in parts of New Guinea. Five geographically distinct subspecies of short-beaked echidnas have been proposed based on their distinct distribution (Griffiths 1978). In contrast, the three species of the critically endangered long-beaked echidna (*Zaglossus bruijnii, Z. bartoni and Z. attenboroughi*) are found exclusively in New Guinea (International Union for Conservation of Nature (IUCN), http://www.iucnredlist.org, accessed 15 May 2019) and the single species of platypus (*Ornithorhynchus anatinus*) is found along the east-coast of Australia, including Tasmania (Grant and Temple–Smith 1998).

All monotremes feature a single cloaca (Figure 1) and internal testes (Griffiths 1978). In platypus, the well-developed spur in males can be used to identify their sex (Grant 2004), but echidnas lack any externally obvious sexual dimorphism in morphology including body mass, dimensions, or colour (Rismiller and McKelvey 2000). In addition, some of the known sex-specific traits such as the female pouch or the male spur (without the sheath) can be temporary (pouch) or unreliable (spur) and are only established once the echidna reaches sexual maturity, meaning the young cannot be sexed using these traits (Rismiller and McKelvey 2003; Augee et al. 2006). Even in adults, these characteristics cannot be unequivocally identified. The female pouch, for example, is not well developed outside their breeding season and it can be confused with the contraction of the longitudinal muscles of the abdomen, which also occurs in males (Rismiller 1993; Rismiller and McKelvey 2000). In the case of the male spur, at least 25% of the adult male population lose one of their spurs while 25% of mature females have one well developed spur; this stems from both sexes having spurs when juveniles, which normally regress in females during adulthood (Griffiths 1989; Rismiller 1993). Given the lack of obvious sexual dimorphism the only current reliable way to determine the sex of an echidna is by ultrasound or by palpation – the act of physically pressing on the abdominal area to feel for reproductive organs such as testes (Rismiller 1992; Rismiller 1993; Rismiller and McKelvey 2000). Palpation is quite invasive, requires a well-trained practitioner and is impossible in juvenile echidnas (puggles) or out-of-breeding-season adult animals (Rismiller and McKelvey 2003), whereas ultrasounds require sedating the echidnas, inducing unnecessary stress, and are impractical for field studies.

Figure 1: Juvenile echidna (puggle) born in Perth Zoo. Photo of puggle ID 5 (Table 1) at 5 months of age. The underside is showing, revealing the cloaca (labelled) and lack of outward reproductive features, which is common between males and females.

Determining sex in both wild and captive echidna populations is a vital part of understanding their ecology and reproductive biology. To date, few ecological studies describe wild echidna populations across Australia because they are cryptic animals. The most well-studied populations are on Kangaroo Island, South Australia, which was recently listed as endangered (Rismiller 1992; Rismiller and McKelvey 2000, 2003, EPBC Act 2015, ‘‘Conservation Advice Tachyglossus aculeatus multiaculeatus Kangaroo Island echidna’’) and Tasmania (Nicol and Andersen 2007; Morrow et al. 2009; Morrow 2013; Morrow and Nicol 2013). However, due to the lack of sex specific markers we lack systematic analysis of sex ratios in these and other echidna populations across Australia, which is an important aspect of understanding wildlife ecology and breeding in any species (Woods et al. 1999; Lucchini et al. 2002).

The inability to confidently determine sex of echidnas not only affects wild population studies but hinders appropriate husbandry of captive echidnas and limits captive breeding efforts. Echidnas are kept in many zoos but have proven to be difficult to breed in captivity (Temple-Smith and Grant 2001; Jackson 2003; Johnston et al. 2007), with fewer than 30 echidnas born in zoos across the world prior to 2007, and over half of those not surviving (Perry 2007). Therefore, a considerable amount of planning and effort is required to successfully breed

echidnas (Ferguson and Turner 2012). In 2012, Perth Zoo reported successful breeding of echidnas in captivity for three consecutive years, producing a total of five animals (Ferguson and Turner 2012). Their breeding continued, bringing the current total of captive bred echidnas to 10 animals.

Currumbin Wildlife Sanctuary and Taronga Zoo also recently initiated successful captive breeding programs: Wallage et al., (2015) reported 13 echidnas born between 2011 and 2014, while Taronga Zoo announced four births from 2016 to 2018. Much of these captive breeding successes can be attributed to long term observational studies of wild echidnas, which revealed key components of their mating and reproductive habits (Rismiller 1992; Rismiller and McKelvey 2000, 2003, Nicol and Andersen 2006, 2007; Morrow et al. 2009; Nicol and Morrow 2012) and improved housing and diet (Stannard et al. 2017). For example, echidnas are solitary animals in the wild and so are kept in separate enclosures in the zoo until their breeding season, when one male and female are paired together. The keepers then monitor both the male and female's behaviour and are able to recognise when the female has become pregnant and laid her egg (Ferguson and Turner 2012). Once the embryos successfully hatch from their eggs and develop, the question as to their sex arises, which is relevant for appropriate management and succession planning. Having access to a quick, reliable and non-invasive sexing technique would be invaluable for captive echidna management and breeding programs.

Over the past 20 years, genetic tests to determine the sex of mammals have been popular techniques to use in similarly difficult animals such as otters (*Lutra lutra*), pine martens (*Martes martes*), giant pandas (*Ailuropoda melanoleuca*), white-tailed deer (*Odocoileus virginianus*) and brown bears (*Ursus arctos*) (Taberlet et al. 1993; Dallas et al. 2000; Lynch and Brown 2006; Durnin et al. 2007; Lindsay and Belant 2008). Typically, these techniques involve amplifying a Y chromosome-specific gene, such as sex determining region Y (*SRY*) the sex-determining gene in therian mammals, to discriminate between males and females. For this, genomic DNA is reliably extracted from non-invasively collected samples such as hair and faeces, followed by gene specific PCR (Vigilant 1999; Dallas et al. 2000; Li et al. 2013). Such non-invasive approaches are ideal for captive animals where handling and the need for anaesthesia is kept to a minimum, and for monitoring wild populations. To date, no genetic sexing technique has been established for monotremes, as male or female specific sequences were unknown.

Monotremes have a remarkably complex sex chromosome system, with female echidnas having 10 X chromosomes while male echidnas have 5 X and 4 Y chromosomes. The male platypus,

however, has 5 X and 5 Y chromosomes, whereby it appears Y_5 has fused to Y_3 in the echidna (or these chromosomes have undergone fission in the platypus) (Rens et al. 2004; Grutzner et al. 2004; Rens et al. 2007). These sex chromosomes share no homology to sex chromosomes in therian mammals and arose independently from the therian sex chromosomes, instead sharing homology to the chicken Z chromosome (Grutzner et al. 2004; Veyrunes et al. 2008). The *SRY* gene, which is the sex determination gene on the therian Y chromosome, does therefore not exist in monotremes and SRY-box 3 (*SOX3*; from which *SRY* evolved) is autosomal (Wallis et al. 2007).

Since sequencing of a female platypus genome (Warren et al. 2008), significant effort has gone into identifying genes on these 10 (or nine) sex chromosomes and potential genes associated with the sex-determining pathway, which is still unknown in monotremes (Warren et al. 2008; Cortez et al. 2014). Mediator complex subunit Y- gametolog (*CRSPY*) was the first Y-specific gene identified, which was mapped to Y_5 in platypus (Tsend-Ayush et al. 2012), therefore presumed to be on Y_3 in echidna. This gene has an X-linked gametolog (*CRSPX*), which has significantly diverged from the Y-linked gametolog and maps to X_1 in platypus (Tsend-Ayush et al. 2012). A recent transcriptomic study identified a number of novel genes on X and Y chromosomes including a Y-gametolog and X-gametolog of the Anti-Müllerian hormone gene (*AMHY* and *AMHX*, respectfully), which plays an important role in sex differentiation in therian mammals and is the current candidate gene for sex determination in monotremes (Cortez et al. 2014).

Using the new genetic information from X and Y chromosomes in platypus and echidna, we sought to establish the first non-invasive PCR-based method to genetically determine the sex of echidnas using gDNA from hair samples and amplifying sex-chromosome genes *CRSPY, AMHY* and *AMHX*. To independently verify the results, we performed Fluorescence *in situ* Hybridisation (FISH) using Y chromosome-specific probes as well as PCR with gDNA extracted from blood. In addition to successfully establishing a simple, non-invasive genetic technique to sex echidnas, the application of this technique in one of the largest captive bred echidna populations revealed that eight out of 10 echidnas born were female.

2. Materials and Methods

2.1 Sample preparation

Hair samples were obtained from 10 echidnas born in captivity at Perth Zoo during a period of 7 years from 2009 to 2016. Each of the samples consisted of 10 – 50 hairs from which only the follicles were used for the DNA extraction protocol to minimize contaminants. Hair samples with follicle attached were collected from the abdominal area of the echidna under manual restraint using sterile tweezers and placed immediately in a sterile 15 mL Falcon® tube (Fisher Scientific). Echidna hair samples were stored at room temperature post-collection and shipped in 70% ethanol. Blood samples were collected from two echidnas (ID 7 and ID 8; Table 1) from the venous beak sinus under general anaesthesia, into green-capped sodium heparin tubes. Blood samples were stored at room temperature and shipped within 1 day of collection, arriving at the University of Adelaide the following day.

2.2 gDNA extraction from hair and blood samples

Hair follicles were cut under light microscope and each sample was placed in a 1.5 mL tube containing 0.5-1 mL of 70% ethanol. The tubes were briefly spun to collect follicles in the bottom and ethanol was then removed. Follicles were allowed to dry at room temperature. For Echidnas numbered 1-8 (Table 1), hair follicles were incubated at 55 ºC overnight while shaking at 700 rpm in 300 µL of lysis buffer (50 mM TrisChloride, pH 8; 100 mM EDTA, pH 8; 100 mM NaCl; 1% SDS), 0.17 µg μL^{-1} of proteinase K and 0.1 M DTT. Once lysis was finalized, 0.6 µL of RNaseA (10 mg mL^{-1}) were added and the cell lysate was incubated for further 30 min at 37 ºC. Samples were allowed to cool down at room temperature, after this 100 µL of 7.5 M ammonium acetate were added to the samples and they were vigorously vortexed, followed by a 5 min incubation on ice. Samples were centrifuged for 5 min at 11,270 g; supernatants were then transferred to a new 1.5 mL tube. DNA was then precipitated at -20 ºC overnight with 300 µL of 100% isopropanol in the presence of 0.5 µL of Glycogen solution (20 mg mL^{-1}). Centrifugation was carried at 12,000 rpm for 20 min, supernatants removed, and the DNA pellet was resuspended in 10 µL of pre-warmed (55 ºC) Milli-Q water.

For Echidnas 9 and 10 (Table 1), DNA was extracted from hair follicles using the Tissue and Hair Extraction Kit (Promega) and DNA IQ System (Promega), according to the manufacturer's instructions. DNA was resuspended in 30 µL of Milli-Q water.

Genomic DNA from blood samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.3 PCR and gel electrophoresis

Each PCR had a final volume of 25 µL, which consisted of 0.5-10 µL of the extracted gDNA (depending on the concentration obtained), 5 µL of 5x PCR buffer with $MgCl_2$ (Promega), 5 U μL^{-1} Taq DNA polymerase, 0.4 µM of each forward and reverse primer and 0.1 mM dNTPs. In the case of PCRs using hair-extracted gDNA, 2.5 µL of BSA was used per reaction. Initial denaturation was carried at 96 ºC for 3 min followed by 40 cycles of denaturation at 96 ºC for 30 s, annealing at 50-58 ºC for 1 min and extension at 72 ºC for 2 min. Final extension was performed at 72 ºC for 7 min. The primers used were *CRSPY* 5'-ACCAGTAAATGCTGTGAAACCTC-3' (forward) and *CRSPY* 5'-TTCTTTTTATTGGCTGGTTCTGA-3' (reverse) at 50 ºC; *AMH* 5'-ACAGGGTCCACGGGTCAGTT-3' (forward), *AMH* 5'-CCAAAAGCAGCAACAGGTCC-3' (reverse) at 58 ºC; and β–actin (*ACTB*) 5'-GCCCATCTACGAAGGTTACGC-3' (forward) and 5'-AAGGTCGTTTCGTGGATACCAC-3' (reverse) at 55 ºC. A 1.5% agarose gel was used to visualize the products.

2.4 Culture of peripheral blood cells for chromosome analysis

A 500 µL of echidna blood was cultured with 10 mL of PB-MAX medium (Life Technologies) for 72 hrs at 32 ºC (because of the lower body temperature of monotremes), according to the manufacturer's suggested protocol. The 72 hr incubation period was followed by a further 30 min incubation with 0.5 µg mL^{-1} of colcemid at 32 ºC. Samples were then centrifuged at 112 *g* for 5 min at room temperature, the supernatant was discarded and the cell pellet was slowly resuspended in 10 mL of hypotonic (0.075 M) KCl and incubated at 37 ºC for 10 min. Samples were then centrifuged again at 112 *g* for 5 min at room temperature, the cell pellet collected and the cells were slowly resuspended in 10 mL of fresh ice-cold fixative made of 1 part acetic acid and 3 parts ethanol. Cells were then incubated for 10 min at 4 ºC, washed five times with fixative and resuspended in a final volume of 0.5 mL of fixative. Cells were fixed to slides by dropping onto methanol-washed glass slides in a humid environment; slides were stored at 4 ºC.

2.5 Fluorescence in situ hybridization of BAC clones

Y_3-specific bacterial artificial chromosome (BAC) EAmhy2 and Y_3X_4-PAR BAC 48g5 (Dohm et al. 2007) were used to perform FISH on echidna metaphase spreads under standard conditions, as previously described in (Tsend-Ayush et al. 2009). Images were taken with a Zeiss AxioImager Z.1 epifluorescence microscope equipped with a charge-couple device camera and Zeiss Axiovision software.

3. Results

3.1 Echidna sexing using PCR on hair gDNA

Echidna hair follicles have a characteristic conical shape compared to the typical bulb shape found in humans (Figure S1). We observed some variation in the efficiency of the DNA extraction protocol used on individuals 1-8, however, we generally obtained 190 ng μL^{-1} from an extraction of only 10 hair follicles. The Promega Tissue and Hair Extraction Kit used for individuals 9 and 10 yielded concentrations of approximately 50-100ng μL^{-1} using the same number of hair follicles.

The sex of all individuals was then determined through PCR using primers that amplify a platypus male-specific gene, *CRSPY*. Primers were tested using gDNA from deceased echidnas whose sex was known due to dissection of testes or ovaries. This approach confirmed that the platypus *CRSPY* primers amplify a male specific product and no products in females (Figures 2 and 3). *ACTB* was used as a positive control and to rule out technical reasons for the absence of a product in females. A second *ACTB* band was observed in some samples (Echidnas 1-5, 9 and 10) and may be due to polymorphism of this gene in the tested individuals. Due to lack of sufficient DNA this could not be investigated further. Running the PCR with *CRSPY* on 10 echidna puggles from Perth Zoo identified that two out of the 10 captive-born echidnas were males (Echidnas 8 and 9) and eight were females (Figure 3; Table 1; Figure S2).

CRSPY primers amplify no products in females due to divergence between the X and Y gametologs, therefore we tested a second gene (*AMH)* to confirm the sex of two captive-bred echidnas. Primers were designed based on the platypus *AMHY* sequence. PCR on echidna gDNA amplify the X and Y copy of the gene (*AMHX* and *AMHY*) in males, and only the X copy (*AMHX*) in females (Figure 2). This shows that both a male and female (Echidnas 9 and 10) can be positively identified through amplification of this gene, confirming the results found using *CRSPY*.

Table 1: Results of sexing for all echidnas born at Perth Zoo. ID numbers are given for each individual, along with information such as date of birth (hatching), age when hair samples were collected, sex determined, sexing method used and fertility status. NA = not available; FISH = fluorescence in situ hybridisation; PCR = polymerase chain reaction.

Echidna	ID number	Date of birth (hatching)	Age at sampling (months)	Sex	Sexing method	Fertile
1	A80281	06/8/2008	6	♀	PCR	Yes (Mother of B20282)
2	A80284	20/8/2008	6	♀	PCR	Yes (Mother of B20300)
3	A70246	25/7/2007	18	♀	PCR	Yes (Laid egg but lost young)
4	A90273	27/8/2009	5	♀	PCR	NA
5	A90272	24/8/2009	5	♀	PCR	NA
6	B10297	12/9/2011	10	♀	PCR	NA
7	B20282	17/8/2012	19	♀	PCR & FISH	NA
8	B20300	27/8/2012	19	♂	PCR & FISH	NA
9	B50173	5/8/2015	19	♂	PCR	NA
10	B60165	15/9/2016	6	♀	PCR	NA

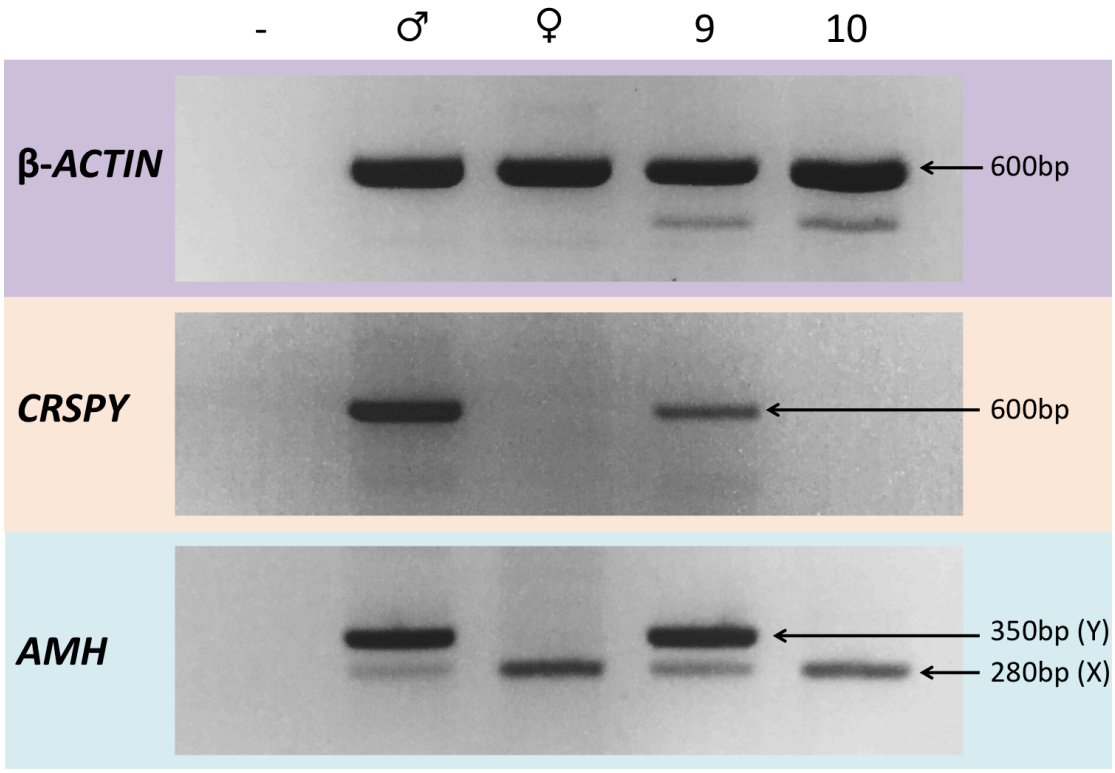

Figure 2: PCR using primers to amplify sex specific genes clearly identifies male and female echidnas. Amplification of *CRSPY* gives a single band in males only. Amplification of *AMH* gives two bands in males and one band in females. Upper band is *AMHY* while the lower band is the *AMHX*. Banding pattern for Echidna 9 shows it is male; banding pattern for Echidna 10 shows it is female. ♂ = known male, ♀ = known female, - = negative control. β-actin (*ACTB*) is positive control indicating that genomic extraction was successful for all individuals and that lack of amplification with *CRSPY* is not due to insufficient or poor quality DNA.

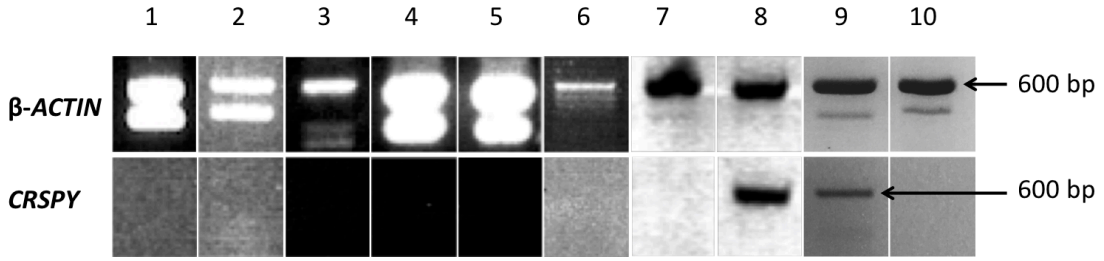

Figure 3: Sex of all 10 echidnas determined by PCR of *CRSPY* reveals eight females and two males. Amplification of *CRSPY* gives a single band in males only. *CRSPY* is only amplified in echidna ID 8 and 9, indicating they are the only two males and the remaining echidnas are female. β-*ACTIN* is positive control indicating that genomic extraction was successful for all individuals. Echidnas are labelled 1-10 as per Table 1. (For original gel images, see Figure S2).

3.2 Echidna sexing validation through FISH and PCR on blood genomic DNA

In order to validate our method with independent techniques, we confirmed our PCR results with gDNA extracted from blood samples of two echidnas (Echidnas 7 and 8). PCRs with *ACTB* and *CRSPY* on blood gDNA confirmed that Echidna 7 is female and Echidna 8 is male (Figure S3). In addition, we performed chromosome analysis and DNA FISH using short-term lymphocyte culture from blood of Echidnas 7 and 8. For Echidna 7, we observed two signals for the red-labelled Y_3X_4-PAR BAC and no signals for the green-labelled Y_3 BAC (Figure 4A), showing that this echidna is female. For Echidna 8 we observed two signals for the now green-labelled Y_3X_4-PAR BAC and one signal for the red-labelled Y_3 BAC, which colocalized with one of the Y_3X_4-positive chromosomes (Figure 4B), showing that this echidna is male. These results confirm the results obtained by PCR from hair follicle DNA. Further validation of sex was confirmed for three females as they later produced offspring or laid eggs (Table 1).

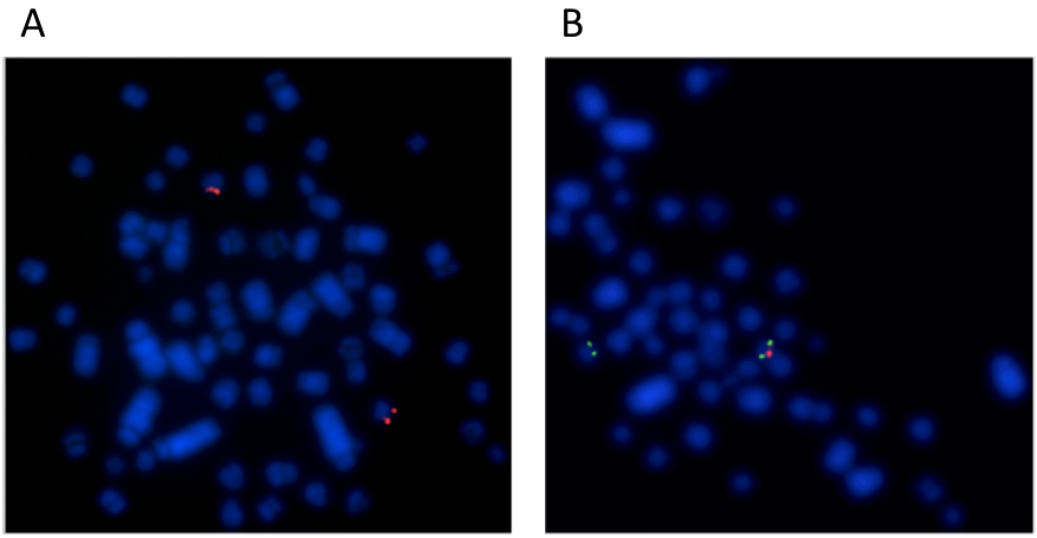

Figure 4: FISH on metaphase spreads confirms sex of two echidnas determined by sexing PCR. Metaphase spreads were prepared from short-term culture of peripheral blood lymphocytes. (A) FISH on metaphase spread from echidna ID 7 with a red-labelled Y_3X_4 BAC which yielded 2 signals and a green-labelled Y_3 specific BAC that yielded 0 signals, confirming that this animal is female. (B) FISH on metaphase spread from echidna ID 8 with a green labelled Y_3X_4 BAC that produced 2 signals and a red-labelled Y_3 specific BAC which produced 1 signal which colocalized with one of the Y_3X_4 BAC positive chromosomes, confirming that this animal is male.

4. Discussion

Determining the sex of an echidna is challenging due to the lack of external genitalia and other reliable morphological differences (Rismiller 1993; Figure 1). Current approaches, such as palpation or ultrasound, require handling (and potentially anaesthesia), adding undesirable stress to echidnas and are impractical for both field studies and juvenile animals. With the recent success of echidna captive breeding in Australia, there is a need for a reliable sexing technique to ensure proper husbandry and future breeding success. Furthermore, sex ratios are still unknown in most wild populations. A better understanding of echidna ecology, including breeding biology, is becoming more urgent with the decline in populations, which is well documented in the extensively studied (and now endangered) echidna population on Kangaroo Island. A genetic test using samples that can be collected in a non-invasive or minimal invasive way is invaluable in determining sex in captive and wild populations and addressing important questions and challenges in echidna management and conservation.

Here, we successfully developed the first PCR-based genetic sexing technique for echidnas. We show that this technique can reliably be used with gDNA, extracted from non-invasively collected hair samples, which is important for removing the intensive handling strategies currently used for sexing adult echidnas (Rismiller and McKelvey 2000). The process is quick and low cost: using commercially available extraction kits (Promega Tissue and Hair Extraction Kit and DNA IQ System), DNA extraction can be performed in approximately 2 hours followed by a PCR and gel to visualise the amplified products. With this method, identifying the sex of an echidna can be achieved in one day with a basic laboratory setup.

The combination of amplifying both an X chromosome gene (*AMHX*) and two Y chromosome genes (*AMHY* and *CRSPY*) allows confident genetic identification of both males and females. Males can be clearly identified with *CRSPY* by gel visualisation of a single PCR product and with *AMH* by visualising a double band showing both the X- and Y-linked gametologs. Females result in no product when amplifying *CRSPY* (due to lack of Y chromosomes), but a distinct product of *AMHX*. We validated our PCR results through chromosomal sexing via FISH and obtained biological confirmations for the sex of three female echidnas because they later had puggles or laid eggs. The application of our genetic sexing technique will allow captive echidnas to be housed in their male- and female-specific enclosures as early as possible and provide an easier approach for mate pairing during breeding season.

From our successful sexing of 10 echidnas, we found a first indication that sex ratio in the echidnas born at Perth Zoo may be skewed, with eight out of 10 being female. Sex ratio biases

have been observed to occur commonly in zoos, with a bias favoured in one direction (e.g. more females) for many years in a row (Glatston 1997; Faust and Thompson 2000). However, the sex bias observed in the Perth Zoo population cannot be statistically evaluated yet due to small numbers. In addition, studies in wild populations based on morphological methods yield different results. A regional Tasmanian population has been estimated at an approximate 1:1 sex ratio (Nicol and Morrow 2012), whereas the Kangaroo Island population is closer to 2:1 males to females (Rismiller 1992). Mainland Australia populations are yet to be investigated. As genetic sexing works well with gDNA extracted from hair samples, it has the potential to also be used with scat material, which is an even less invasive approach to gain a better understanding of the dynamics and behaviour of echidnas in the wild. It would also allow comparison of sex ratios between more wild and captive populations.

In conclusion, we have developed the first genetic sexing technique for echidnas that confidently identifies both males and females by the unique banding pattern achieved through PCR of sex chromosome genes *AMHX*, *AMHY* and *CRSPY*. The genetic approach removes intensive handling techniques currently used to sex adult echidnas and is the first technique to determine the sex of juvenile echidnas. The PCR-based technique is quick, reliable and low cost, suitable for use on non-invasive samples such as hair, and has the ability to be used on echidna scats to investigate population structure in the wild. The genetic technique identified eight of 10 juvenile echidnas from Perth Zoo's captive breeding program as female and two as male, allowing keepers to take appropriate management actions and continue success of the breeding program. We see potential for this technique to be used for captive and wild echidna populations across Australia and in New Guinea to learn more about sex ratios in this iconic species.

Declaration of Competing Interests

We declare that we have no competing interests for this project.

Acknowledgements

Thanks to Prof. Steve Donnellan and Dr. Terry Bertozzi from the South Australian Museum for advice on the hair gDNA extraction protocol and for providing hair samples to test the protocol. TP is funded by the Research Training Program (RTP). DT-F is funded by the National Council of Science and Technology from Mexico (CONACYT). FG is an ARC research fellow. Thanks to Perth Zoo's Veterinary staff for collection and shipment of samples.

Author contributions

TP performed sample processing, laboratory work and co-wrote the manuscript. DT-F performed sample processing, laboratory work and co-wrote the manuscript. WXK performed sample processing and laboratory work. AF contributed to the design of the project. BT collected samples and advised on echidna biology. ET-A and SLL performed laboratory work. FG contributed to the design and supervision of the project as well as to the manuscript preparation and evaluation.

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Chapter Six: Summary

Echidnas are unique, enigmatic mammals that have fascinated scientists and the public alike for hundreds of years. Due to the cryptic lifestyle of echidnas and their Australia wide distribution it has been historically difficult to study them in the wild. Furthermore, echidnas are commonly held in captivity but have had issues with health, diet and breeding success. This thesis showcases how using multidisciplinary approaches can illuminate much about echidnas quickly and effectively. Here, I show that by combining public outreach with molecular biology, we can gather vast amounts of information on many populations of the short-beaked echidna (both wild and captive) across Australia.

EchidnaCSI is a nation-wide citizen science project where the public are asked to submit sightings of echidnas and collect echidna scats for molecular research. I led the design, implementation and day to day running of the project, which produced over 10,000 submissions of new sighting data and scat material, combined. EchidnaCSI successfully engaged a large cohort of Australians to aid in echidna research and is the largest and most geographically spread project to incorporate wildlife scat collection in citizen science, where DNA was successfully extracted and amplified (Chapter 2). This work demonstrates best practise and the feasibility to include scat material collection into citizen science protocols.

Scat samples collected by the public through EchidnaCSI allowed for analysis of more than 150 echidna scats from all major habitats to gain new insights into diet and gastric health. One of the major aims of this research was to use genomic methods on the scats to explore new avenues of echidna biology and health. To achieve this, I used 16S metabarcoding to explore the microbiome diversity and composition from a subset of the wild-collected echidna scats. This research is not only the first to characterise the echidna gut microbiome, but shows the extraordinary microbial variety that echidnas possess, across multiple environments within Australia. This microbiome work revealed that plants are likely a more significant part of echidnas' diet than is currently recognised and supports the hypothesis that echidnas should be recharacterised as 'insectivorous herbivores' (Chapter 3). Furthermore, by comparing the scats of wild animals to scats collected from echidnas held at Perth and Taronga Zoos, we revealed that captivity and different diets fed to echidnas significantly changed their gut microbiomes (Chapter 3). This research has provided vital insight into diet related microbiome changes in echidnas, which will aid in the continued development of new diets to ensure good gastric health in captive echidnas.

Echidnas on Kangaroo Island are very well-studied due to Dr Peggy Rismiller's work on the island for the past 30 years, characterising many important life-history and ecological traits of

echidnas and documenting conservation threats. EchidnaCSI had a large supporter base from Kangaroo Island, with dedicated individuals submitting many echidna sightings and scats over the past three years. This led us to be in a powerful position where we were able to assess changes in the gut microbiome of echidnas after the devastating bushfires that occurred over the 2019/2020 summer, due to already receiving echidna scats from prior to the fires. Not only did this research show that fire significantly affects echidna gut health and foraging behaviour but provided the first study to assess how bushfires impact the gut microbiome for any non-human mammal (Chapter 4). These results provide crucial information on Kangaroo Island echidnas who are listed as an urgent fire recovery species and highlights the importance of assessing microbial community changes in other native Australian animals affected by bushfires.

Lastly, for this research, I aimed to apply molecular tools for applications in developing better management strategies for echidnas in captivity, as they have historically had poor gut health and low breeding success. This was able to be achieved through strong collaborations formed at both Perth Zoo and Taronga Zoo. Along with assessing microbiome changes associated with different diets, we also developed a genetic sexing technique to determine the sex of juvenile echidnas born in zoos (Chapter 5). Due to our expertise in echidna genetics, especially sex chromosomes, we were able to collaboratively create this simple PCR-based technique and assess one of the largest captive-bred echidna populations, at Perth Zoo. These tools will continue to aid in the management of echidnas in zoos and aid in breeding efforts.

Future Directions

In this project, we have successfully applied molecular approaches to gain novel insights into echidna biology. The combination of community-based research and collaboration with zoos has allowed access to unique material to establish and apply molecular tools to determine the sex of echidnas and provides first steps towards a knowledge base about gut microbiome and health in wild and captive echidnas. As we have such a large and geographically diverse material set for echidnas, which continues to grow, there are many avenues we can take this research to further assess wild echidna populations. Firstly, as diet is still an unresolved area, genetic studies can be undertaken on the echidna scats to better understand the contents and diversity of the food sources echidnas are using. This can be achieved with two approaches: shotgun sequencing and metabarcoding. Shotgun sequencing can provide sequences from all DNA present in a scat sample, providing a powerful and non-discriminatory approach (Ang et al., 2020; Paula et al., 2016), which is ideal considering the uncertainty in echidna scat

composition. The added benefits of employing shotgun sequencing, is the ability to not only capture diet information, but DNA from the host animal (i.e. echidna) and the microbiome, simultaneously (Srivathsan et al., 2016). However, shotgun sequencing is expensive due to requiring a large sequencing depth per sample to capture the required information and relies on large computational power to analyse the data output (Hillmann et al., 2018). Alternatively, metabarcoding can allow the processing of hundreds of samples simultaneously as it requires PCR to target specific marker genes prior to sequencing (Barba et al., 2013). If we were to employ this with echidna scats, we would need to use multiple marker genes to target insects (COI), plants (trnL) and fungi (ITS) in order to capture and explore the full diversity of diet within each sample (Clarke et al., 2014; Srivathsan et al., 2015). These markers would also require testing, as many COI primers have been shown to differentially amplify specific orders of insects, e.g. the LepF1 primer will successfully amplify Lepidoptera (moths), Diptera (flies) and Hemiptera (true bugs) but not Isoptera (termites) or Orthoptera (grasshoppers) (Clarke et al., 2014).

The diversity of locations that echidna scats were collected from could also aid in population genetic studies. Echidnas are currently split into 5 subspecies; however, this is due to geographic and morphological differences rather than genetic analysis (Rismiller and Grutzner, 2019). Full mitochondrial genomes can be sequenced from animal scats (Ang et al., 2020), which would allow for a comprehensive population genetic analysis (Ingman and Gyllensten, 2006). Understanding echidna population genetics would not only inform on wild populations but could be used for determining the origin and heritage of captive echidnas, which is important for avoiding inbreeding.

Incorporating animal scats into citizen science has proven very effective for EchidnaCSI. Although the ability of participants to identify an echidna scat is aided by the scat's distinct appearance, we found the public very quick in their ability to learn to identify other animal scats when holding in-person scat identification workshops. Therefore, with clear communication, it is likely any animal scat could be incorporated into citizen science, which would greatly broaden the research potential for many projects. As the limitation in many wildlife genetic studies is the access to material, citizen science opens avenues for achieving broad-scale sample collection to enable larger assessments of wildlife through genomic approaches. Furthermore, as the genomic approaches are not species-specific, and has been proven to work effectively on non-invasively collected samples, the potential reach of this research is endless. EchidnaCSI achieved the research aims of providing sufficient samples to undertake genetic and microbial analyses. As a successful citizen science project, it also reached a significant amount of the

public to engage and educate on echidna biology and conservation. We hope to continue EchidnaCSI long into the future in order to continue monitoring wild echidna populations, with the aid of the public.

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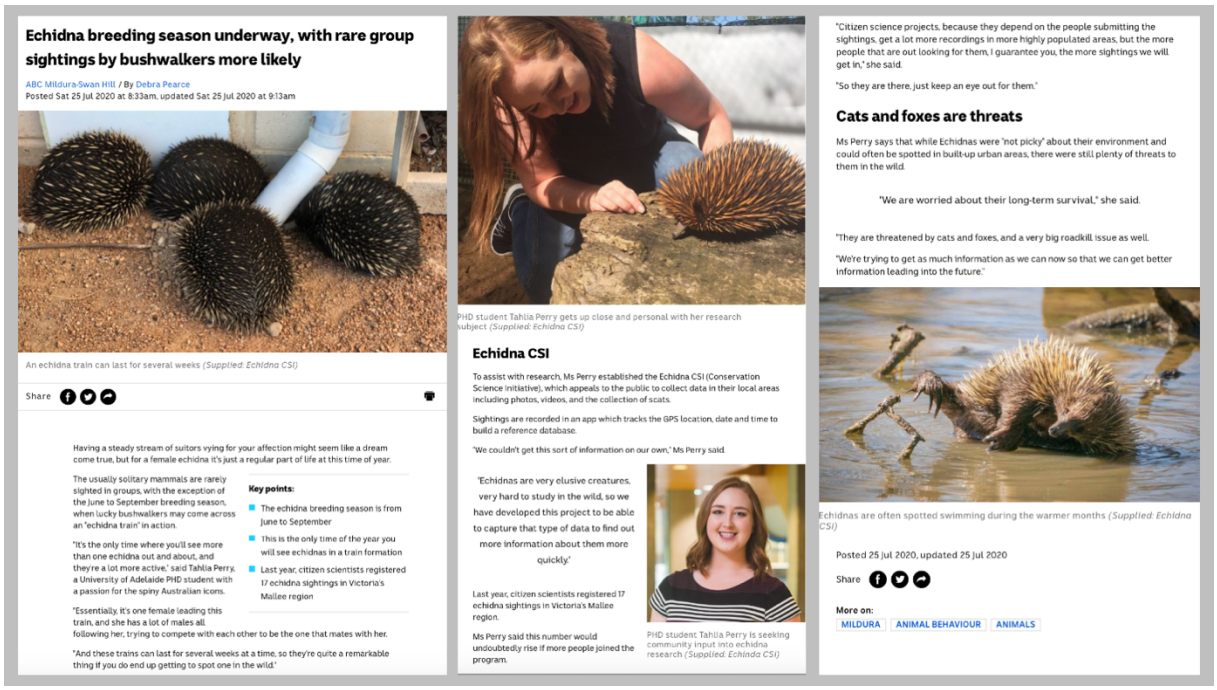
Appendix One:

Outreach

Scientific outreach and engagement has many benefits for society and to scientists themselves. Echidnas are extraordinary animals, but many aspects of their biology are unknown to most. The EchidnaCSI project has served as a brilliant platform for spreading awareness of the fascinating world of echidnas. I take great pride in sharing this information and entertaining stories that spark the interest of not only the general public, but other academics alike. In-person talks and workshops held over the past 3 years has led to a personal 'reach' of over 2500 people (see Appendix Table 1 below). This does not take into account the reach of other forms of traditional media; in 2017 and 2018 myself and my supervisor Prof. Frank Grützner appeared on popular children's TV shows 'Totally Wild' and 'Scope' and since 2017 I have provided more than 30 radio interviews for stations including ABC (National and Regional), 5AA, 2NM and Radio National. In 2020 I was featured in an ABC News article about echidnas during their mating season (Appendix Figure 1) as well as the cover article for November's Australian Geographic (Appendix Figure 2). Other forms of outreach include social media, where I run or oversee Facebook, Twitter and Instagram accounts for EchidnaCSI, which have a following of 4779, 1592 & 1181, respectively (as of the 10th of December 2020). Furthermore, I curate an e-newsletter that is sent to the email list of EchidnaCSI subscribers (currently sitting at 6273 as of the 10th of December; see Appendix Figure 3) to provide updates about the project and other relevant or interesting news. The excitement and wonder the wider community have shown for echidnas is inspiring and further embeds my passion for understanding and conserving these amazing creatures.

Appendix Table 1: List of outreach activities conducted during PhD

Date	Style	Event Organisation	Audience Demographic	Audience size (approx.)
2020	Invited Speaker	Australian Citizen Science Association Online Conference	Academic/Industry/ Government	100
2020	Online Lecture	Public Engagement in Science & Technology Adelaide Speaker Series	Academics	30
2019	Oral Presentation	USA Citizen Science Conference	Academics/Industry/ Government	50
2019	Invited Speaker	University of Adelaide EcoTourism Conference	Undergraduate Students	50
2019	Invited Speaker	Science in the Pub	General Public	40
2019	Invited Speaker	Local Government Association Young Leaders Workshop	Young Adults	30
2019	Invited Speaker	Pint of Science	General Public	150
2019	Poster Presentation	Lorne Genome Conference	Academics	200
2019	Workshop	SA Museum Citizen Science Showcase Week	Children & Parents	50
2018	Invited Speaker	Bright Sparks Science Club	Children (aged 5-11)	50
2018	Outdoor event	University of Adelaide Citizen Science Day	General Public (mix of children & adults)	150
2018	Invited Speaker	Modbury High School Women in STEM Day	High School Students (Years 8 & 9)	60
2018	Workshop	Morialta Conservation Park Miniblitz	General Public (families)	50
2018	Invited Speaker	Ingenuity Adelaide	High School Students (Years 8-12)	500
2018	Invited Speaker	'A Night of Science' In Naracoorte	General Public	100
2018	Oral Presentation	Australian Citizen Science Conference	Academics/Industry/ Government	60
2018	Oral Presentation	Natural Resources Management Conference	Academics/Industry/ Government	60
2018	Workshop	National Science Week at SA Museum	General Public (adults)	40
2018	Workshop	National Science Week at Victor Harbor	General Public (Adults/retirees)	60
2018	Workshop	National Science Week at Barossa	General Public (mix of children & adults)	40
2018	Invited Speaker	Friends of Onkaporinga Park	Adults/Retirees	40
2018	Invited Speaker	Trees for Life	Adults/Retirees	40
2017	Poster Presentation	BioInfoSummer Melbourne	Academics	150
2017	3 minute thesis competition	University of Adelaide	Academics	200
2017	Oral Presentation	International Mammalogical Congress	Academics	100
2017	Science communication competition	FameLab semi-final and final	General Public (adults)	100



Echidna breeding season underway, with rare group sightings by bushwalkers more likely

ABC Mildura-Swan Hill / By Debra Pearce
Posted Sat 25 Jul 2020 at 8:33am, updated Sat 25 Jul 2020 at 9:13am

An echidna train can last for several weeks *(Supplied: Echidna CSI)*

Share

Having a steady stream of suitors vying for your affection might seem like a dream come true, but for a female echidna it's just a regular part of life at this time of year.

The usually solitary mammals are rarely sighted in groups, with the exception of the June to September breeding season, when lucky bushwalkers may come across an 'echidna train' in action.

"It's the only time where you'll see more than one echidna out and about, and they're a lot more active," said Tahlia Perry, a University of Adelaide PHD student with a passion for the spiny Australian icons.

"Essentially, it's one female leading this train, and she has a lot of males all following her, trying to compete with each other to be the one that mates with her.

"And these trains can last for several weeks at a time, so they're quite a remarkable thing if you do end up getting to spot one in the wild."

Key points:

- The echidna breeding season is from June to September
- This is the only time of the year you will see echidnas in a train formation
- Last year, citizen scientists registered 17 echidna sightings in Victoria's Mallee region

PHD student Tahlia Perry gets up close and personal with her research subject *(Supplied: Echidna CSI)*

Echidna CSI

To assist with research, Ms Perry established the Echidna CSI (Conservation Science Initiative), which appeals to the public to collect data in their local areas including photos, videos, and the collection of scats.

Sightings are recorded in an app which tracks the GPS location, date and time to build a reference database.

"We couldn't get this sort of information on our own," Ms Perry said.

"Echidnas are very elusive creatures, very hard to study in the wild, so we have developed this project to be able to capture that type of data to find out more information about them more quickly."

Last year, citizen scientists registered 17 echidna sightings in Victoria's Mallee region.

Ms Perry said this number would undoubtedly rise if more people joined the program.

PHD student Tahlia Perry is seeking community input into echidna research *(Supplied: Echinda CSI)*

"Citizen science projects, because they depend on the people submitting the sightings, get a lot more recordings in more highly populated areas, but the more people that are out looking for them, I guarantee you, the more sightings we will get in," she said.

"So they are there, just keep an eye out for them."

Cats and foxes are threats

Ms Perry says that while Echidnas were "not picky" about their environment and could often be spotted in built-up urban areas, there were still plenty of threats to them in the wild.

"We are worried about their long-term survival," she said.

"They are threatened by cats and foxes, and a very big roadkill issue as well.

"We're trying to get as much information as we can now so that we can get better information leading into the future."

Echidnas are often spotted swimming during the warmer months *(Supplied: Echidna CSI)*

Posted 25 Jul 2020, updated 25 Jul 2020

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Appendix Figure 1: ABC News Article published on 25th July 2020. Here, I was interviewed in relation to the unusual sightings of echidna trains during breeding season (June – September) and how citizen science has led to new knowledge for wild populations.

TAHLIA PERRY SHOWS me an extraordinary photograph of an echidna, which is testament to the remarkable resilience of these animals in the face of bushfires. The picture, submitted to her citizen science project EchidnaCSI, shows an animal with its spines severed halfway along their length, giving it the appearance of having been trimmed into a flat-top hairstyle.

"The echidna had this weird thing, where it looked like its spines had been cut, but actually it had been caught in a controlled burn," explains Tahlia, who is based at the University of Adelaide. "It was a striking image…and our social media went pretty wild."

Often, rather than attempt to outrun a fire, echidnas simply burrow into the ground, or conceal themselves inside fallen logs. One of their many unique talents is that they can dig straight down on the spot, disappearing into the dirt in as little as a minute. They then enter a temporary hibernation-like state to conserve energy, and wait for the danger to pass. This 'torpor', which is also employed by echidnas in some cooler regions, sees them slow their heart rate, metabolism and temperature – breathing in and out as few as three times a minute. On rare occasions they may stay like this for weeks following a fire, only emerging once their insect prey has returned to the environment.

But the echidna in the image, snapped by citizen scientist Georgina Swan following a hazard reduction burn in bush in northern Sydney, clearly hadn't dug down far enough, and its spines were melted off. Tahlia explains that this might not have been painful for the animal, because the spines, being modified hairs, are not living tissue. Although that particular image was taken in August last year, just before the catastrophic Black Summer fire season got underway, pictures and reports emerged early this year of echidnas elsewhere with melted spines. In fact, along with wombats, which also take refuge underground, echidnas were some of the most commonly spotted mammal survivors emerging from the ashes of still-smouldering firegrounds.

Dr Peggy Rismiller, who has studied echidnas for 30 years from her base at the Pelican Lagoon Research and Wildlife Centre on Kangaroo Island (KI), says that when the fires blazed across the island in December and January, young echidnas were still in their nursery burrows, but during subsequent surveys she saw a number of them emerge unscathed. Adults dig in and manipulate dirt between their spines for insulation, she explains. Almost half of KI ultimately burnt, and, although some echidnas were no doubt killed, Peggy has found quite a few that bear no marks of having been through a fire, as well as "others whose spines melted because they didn't dig deep enough, but they have survived and are continuing their normal lives".

Under more usual fire conditions, right across Australia echidnas are able to survive better than other mammals. But with the Black Summer fires "being so intense and burning such a large area, we're unsure how many have survived", Tahlia says. Nevertheless, given that the platypus and the echidna – both egg-laying monotremes – are creatures that have been around for tens of millions of years, Peggy isn't surprised they are deft at navigating natural perils, such as bushfires.

Tahlia Perry runs the EchidnaCSI project, which is harnessing citizen scientists to rapidly fill in the missing pieces of the puzzle on these animals.

Echidnas vary slightly in appearance and biology across their huge range. Tassie echidnas, such as this one, have dense fur between the spines, which helps keep them warm during cooler months.

Monotremes are the "longest surviving" lineage of mammals, she says. "They were around with the dinosaurs and survived through greenhouse effects and ice ages, and if you've been around that long you know what you are doing." Found from the wet rainforests of the north, through the arid centre to the alpine uplands of the south-east, the short-beaked echidna is Australia's most widespread mammal. But perplexingly, its biology was almost completely unknown until about 30 years ago. Since then, populations on KI and Tasmania have been well studied by Peggy and University of Tasmania Associate Professor Stewart Nicol, unlocking many secrets about these enigmatic creatures. However, the echidnas on the Australian mainland have remained stubbornly elusive, something Tahlia is hoping to change.

"OF ALL THE Mammalia yet known, it seems the most extraordinary in its conformation," English zoologist George Shaw wrote of the platypus in 1799. The echidna's duck-billed cousin typically takes the crown as the world's weirdest mammal, but Australia's 'spiny anteater' certainly gives it a run for its money.

"Porcupine-like quills, a beak that's a bit like a cross between a bird and an anteater, a pouch like a marsupial, legs pointing in the direction that no other animal's legs point: they've got this very odd combination of features," says Jack Ashby, who specialises in Australian mammals and is manager of the University of Cambridge Museum of Zoology in the UK.

As most people who have encountered them in the wild know, echidnas have excellent defensive measures, not least of which is rolling up into a prickly ball to present a formidable and potentially painful challenge to a hungry dingo or wedge-tailed eagle.

"They were around with the dinosaurs and...if you've been around that long you know what you are doing."

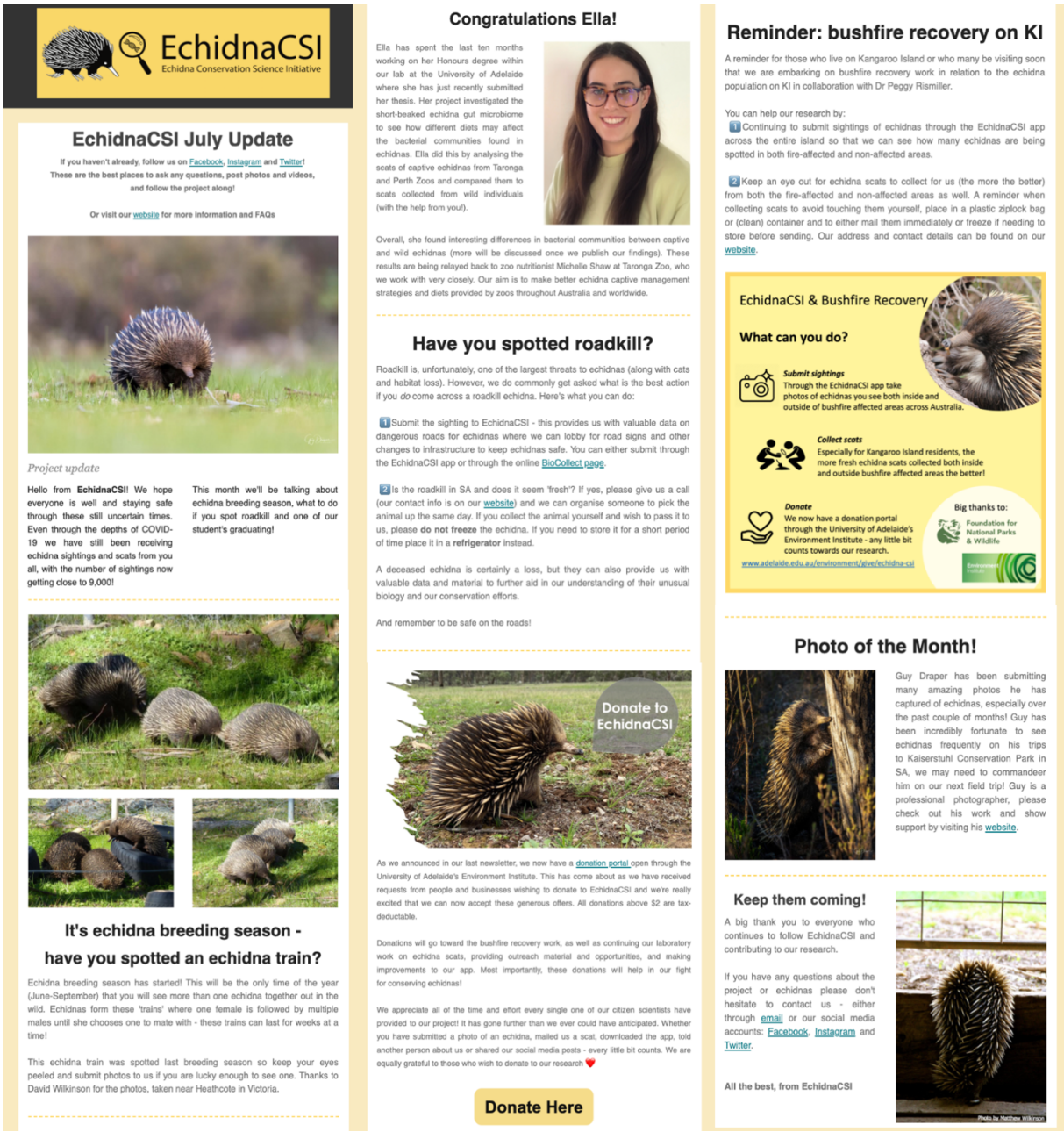
◄◄ This echidna, photographed halfway through last year by citizen scientist Georgina Swan and uploaded to the EchidnaCSI app, didn't dig down far enough during a hazard reduction burn in northern Sydney and had its spines melted off.

◄ Peggy Rismiller, on KI, who has been tracking echidnas in the wild for three decades, has spent more time observing these monotremes than anyone else in the world.

88 Australian Geographic

November - December 88

Appendix Figure 2: Australian Geographic cover story for November 2020. Interviewed to provide information on current knowledge of natural history and behaviour in echidnas, especially in relation to bushfires. The article also highlighted EchidnaCSI, including the knowledge gained from the project and a 'call to action' for readers to participate.



Appendix Figure 3: Example of one of the e-newsletters curated and sent to EchidnaCSI audience every 2 months. Topics covered include a project update, interesting information about echidna biology, news about EchidnaCSI team members and research outcomes, and a 'photo of the month' to highlight fantastic contributions to EchidnaCSI.

Appendix Two:

Conference Presentations & Awards

Conference Presentations

Perry, T., Invited Speaker for Australian Citizen Science Association Online Conference, October 2020: *Connections and Partnerships within EchidnaCSI*

Perry, T., Invited Speaker for University of Adelaide EcoTourism Conference October 2019: *Insights into the People Behind EchidnaCSI: How Can this Shape Our Engagement Approach?*

Perry T., Stenhouse A., Wilson, I., Rismiller, P., Grützner, G., Oral Presentation for USA Citzien Science Conference March 2019: *EchidnaCSI Uses Sample Collection and Sightings to Address Fundamental Questions in Echidna Biology and Conservation*

Perry, T., Wilson, I., Stenhouse, A., Rismiller P., Grützner, F., Oral Presentation for Natural Resources Management Conference May 2018: *EchidnaCSI: Using Citizen Science and Molecular Biology for Conservation*

Perry, T., Stenhouse, A., Wilson, I., Rismiller, P., Grützner, F., Oral Presentation for Australian Citizen Science Conference February 2018: *EchidnaCSI: A Forensic Approach to Help Echidna Conservation*

Perry, T., Toledo-Flores, D., Kang, WX., Ferguson, A., Tsend-Ayush, E., Lim, SL., Rismileer, P., Laming, B., Grützner, F., Oral Presentation for 12th International Mammalian Congress July 2017: *The Use of Non-invasive Genetic Sexing of Echidnas from Hair and Scat Samples for Captive Management and Conservation*

Awards

2019 Winner of Channel 7's SA Young Achiever Award in STEM
2018 Pitch It Clever National Finalist
2018 Best Talk Award at Australian Citizen Science Conference
2017 Best Poster Award at BioInfoSummer Melbourne
2017 3 Minute Thesis Winner for School of Biological Science, University of Adelaide
2017 FameLab Australia Finalist

Supplementary Material

Supplementary Material for Chapter 2

Table S1: Events that lead to a large increase of EchidnaCSI app downloads. Media = the media company that shared or wrote about EchidnaCSI. App downloads = sum of downloads for the 7 days after the event occurred. N/A = not applicable.

Date	Event	Media	Media Type	App downloads
4th Sep 2017	EchidnaCSI Launch	Channel 10 News, Channel 7 News, ABC News	TV and news articles	608
16th Jan 2018	CSIRO blog	CSIRO and Atlas of Living Australia	Blog article	487
21st Feb 2018	Guardian article	The Guardian	News article	836
9th Aug 2018	National Science Week	N/A	In person events	222
9th Jan 2020	Bushfire awareness	Facebook, Twitter, Instagram	Social media posts	206
24th July 2020	ABC article	ABC News	News article	125

Table S2: Survey answers depicting participants' changes in attitudes or behaviours due to their involvement in EchidnaCSI. Percentages of how participants ranked each question is below and separated between those that submitted data ('submitters' = S), to those that did not submit data but engaged in the project ('non-submitters' = NS). Percentages that are of particular interest and discussed in the text are underlined.

	What are your current views on the following statements?						How do you think your views to these statements have changed since following EchidnaCSI?					
Question	Disagree		Neither agree nor disagree		Agree		Decreased		Remained the same		Increased	
	S	NS	S	NS	S	NS	S	NS	S	NS	S	NS
Echidna conservation is important to me	0%	1%	1%	1%	99%	98%	0%	0%	58%	64%	42%	36%
The health of the environment is important to me	0%	0%	1%	0%	99%	100%	0%	0%	76%	78%	23%	22%
I take actions to protect or preserve the environment	0%	1%	3%	4%	97%	96%	0%	0%	73%	76%	26%	24%
Participating in citizen science is worth my time	0%	0%	8%	9%	92%	91%	1%	1%	49%	67%	50%	32%
I do what I can, but the environment is not my biggest concern	65%	66%	26%	27%	10%	7%	15%	12%	77%	82%	8%	6%

Table S3: Survey answers depicting participants' motivations for their involvement in EchidnaCSI, from those who had submitted data to EchidnaCSI. The questions are ranked from 'most important' to 'least important' based on the responses.

Question	Very Unimportant	Somewhat Unimportant	Neutral	Somewhat Important	Very Important
I want to contribute to wildlife conservation	4%	1%	2%	16%	76%
I like echidnas	5%	2%	6%	26%	62%
I enjoy contributing to original scientific research	4%	3%	7%	34%	52%
I want to learn more about echidnas	5%	2%	11%	36%	46%
The project is easy to participate in	3%	3%	16%	35%	43%
I like hearing news about the project's progress and outcomes	3%	4%	10%	42%	41%
It is easy to submit data	6%	2%	21%	31%	41%
I enjoy being a part of a community of like-minded people	5%	8%	23%	35%	29%
I find EchidnaCSI a valuable resource for teaching others	9%	12%	31%	25%	23%
I enjoy the time I spend with my family and/or friends when contributing to EchidnaCSI	12%	11%	36%	21%	20%
I like seeing recognition of my or other participants' contributions to the project	9%	13%	34%	26%	18%
I am interested in molecular biology	24%	19%	37%	15%	6%

Table S4: Survey answers depicting participants' motivations for their involvement in EchidnaCSI, from those who had not submitted data, but still engaged with EchidnaCSI. The questions are ranked from 'most important' to 'least important' based on the responses.

Question	Very Unimportant	Somewhat Unimportant	Neutral	Somewhat Important	Very Important
I like echidnas	4%	1%	4%	22%	69%
I want to contribute to wildlife conservation	2%	1%	4%	24%	69%
I want to learn more about echidnas	3%	3%	6%	33%	55%
I intend to submit data in the future	3%	2%	12%	38%	45%
I am excited to contribute to original scientific research	3%	3%	14%	37%	43%
I like hearing news about the project's progress and outcomes	2%	2%	10%	44%	42%
It is easy to submit data	3%	3%	28%	30%	37%
I enjoy being a part of a community of like-minded people	4%	6%	21%	44%	25%
I find EchidnaCSI a valuable resource for teaching others	6%	12%	34%	29%	19%
I like seeing recognition of my or other participants' contributions to the project	9%	13%	34%	29%	15%
I enjoy the time I spend with my family and/or friends when contributing to EchidnaCSI	13%	10%	47%	16%	14%
I am interested in molecular biology	16%	19%	37%	19%	8%

Supplementary File 1

Survey Questions

Start of Block: Demographics

Name:

_____

Email address (if registered through the EchidnaCSI app please enter the same email)

_____

What is your age?

- ○ 17 or under (1)
- ○ 18 - 24 years (2)
- ○ 25 - 34 years (3)
- ○ 35 - 44 years (4)
- ○ 45 - 54 years (5)
- ○ 55 - 64 years (6)
- ○ 65 - 74 years (7)
- ○ 75 - 84 years (8)
- ○ 85+ years (9)

What is your gender?

- ○ Male (1)
- ○ Female (2)
- ○ Prefer not to say (3)
- ○ Other (4) _____

What is your ethnicity? (Select all that apply)

- [ ] Asian (1)
- [ ] Black/African (2)
- [ ] Caucasian (3)
- [ ] Hispanic/Latinx (4)
- [ ] Indigenous Australian or Torres Straight Islander (5)
- [ ] Indian (6)
- [ ] Middle Eastern (7)
- [ ] Pacific Islander (8)
- [ ] Prefer not to answer (9)
- [ ] Other: (10) _____

What is your postcode?

_____

What is the highest level of education that you have completed?

- ○ Less than year 12 or equivalent (1)
- ○ Year 12 or equivalent (2)
- ○ Vocational qualification (3)
- ○ Associate diploma (4)
- ○ Undergraduate diploma (5)
- ○ Bachelor Degree (6)
- ○ Honours Degree (7)
- ○ Postgraduate diploma (8)
- ○ Master's Degree (9)
- ○ Doctorate (10)

What is your highest level of science education?

- ○ Year 10 or below (1)
- ○ Year 11 or 12 (2)
- ○ Bachelor Degree (3)
- ○ Honours Degree (4)
- ○ Master's Degree (5)
- ○ Doctorate (6)
- ○ Self directed learning (7)

What is your current employment status?

- ○ Employed full time (1)
- ○ Employed part time (2)
- ○ Employed casually (3)
- ○ Retired (4)
- ○ Student (5)
- ○ Unemployed (6)

If employed, what is your job title/description?

_____

Are you involved in any other volunteering?

- ○ No (1)
- ○ Yes (please specify) (2) _____

End of Block: Demographics

Start of Block: Level of activity/involvement with the project

How did you first hear about EchidnaCSI?

- ○ Word of mouth (1)
- ○ Radio (2)
- ○ TV (3)
- ○ Facebook (4)
- ○ Twitter (5)
- ○ Newspaper (6)
- ○ Presentation (7)
- ○ Conference (8)
- ○ University of Adelaide (9)
- ○ Other university (10)
- ○ Internet search (11)
- ○ Other: (12) _____

Have you promoted EchidnaCSI to anyone? (select all that apply)

- ☐ No (1)
- ☐ Yes, to family (2)
- ☐ Yes, to friends (3)
- ☐ Yes, to colleagues (4)
- ☐ Yes, online (e.g. through Facebook) (5)
- ☐ Yes, other (6) _____

How do you currently keep up with EchidnaCSI? (Select all that apply)

- [ ] Email Newsletter (1)
- [ ] Facebook (2)
- [ ] Twitter (3)
- [ ] Instagram (4)
- [ ] Email Communication (5)
- [ ] EchidnaCSI webpage (6)
- [ ] In-person talks (7)
- [ ] Other (8) _____

Have you submitted any data to EchidnaCSI?

- ○ Yes (1)
- ○ No (2)

End of Block: Level of activity/involvement with the project

Start of Block: If not submitted: motivations

If not, why not?

- [ ] Haven't seen an echidna or echidna scat (1)
- [ ] Have seen an echidna, but couldn't capture a photo (2)
- [ ] No longer interested in the project (3)
- [ ] Could not complete the submission process (4)
- [ ] Other (5) _____

How important are these factors in your interest in EchidnaCSI?

	Completely unimportant (1)	Somewhat unimportant (2)	Neutral (3)	Somewhat important (4)	Very important (5)
I like echidnas (1)	○	○	○	○	○
I care for echidna conservation (2)	○	○	○	○	○
I want to learn more about echidnas (3)	○	○	○	○	○
It is easy to submit data (4)	○	○	○	○	○
I intend to submit data in the future (5)	○	○	○	○	○
I value the goals of EchidnaCSI's research (6)	○	○	○	○	○
I am interested in molecular biology (7)	○	○	○	○	○
I am interested in citizen science (8)	○	○	○	○	○
I am interested in science in general (9)	○	○	○	○	○
I am excited to contribute to original scientific research (10)	○	○	○	○	○
I find EchidnaCSI a valuable resource for teaching others (11)	○	○	○	○	○
I want to contribute to wildlife conservation (12)	○	○	○	○	○

I enjoy the time I spend with my family and/or friends when contributing to EchidnaCSI (13)	○	○	○	○	○
I enjoy being a part of a community of like-minded people (14)	○	○	○	○	○
I am interested in learning how to identify echidna scats (15)	○	○	○	○	○
Other (16)	○	○	○	○	○

What are your current views on the following statements?

	Strongly disagree (1)	Somewhat disagree (2)	Neither agree nor disagree (3)	Somewhat agree (4)	Strongly agree (5)
Echidna conservation is important to me (1)	○	○	○	○	○
The health of the environment is important to me (2)	○	○	○	○	○
I take actions to protect or preserve the environment (3)	○	○	○	○	○
Participating in citizen science is worth my time (4)	○	○	○	○	○
I do what I can, but the environment is not by biggest concern (5)	○	○	○	○	○

How do you think your views to these statements have changed since following EchidnaCSI?

	Strongly decreased (1)	Somewhat decreased (2)	Remained the same (3)	Somewhat increased (4)	Strongly increased (5)
Echidna conservation is important to me (1)	○	○	○	○	○
The health of the environment is important to me (2)	○	○	○	○	○
I take actions to protect or preserve the environment (3)	○	○	○	○	○
Participating in citizen science is worth my time (4)	○	○	○	○	○
I do what I can, but the environment is not by biggest concern (5)	○	○	○	○	○

End of Block: If not submitted: motivations

Start of Block: If not submitted: future participation

How could the experience of participating in EchidnaCSI be improved to maintain your interest? (Select all that apply)

- [ ] Feedback about how your data is being used by scientists (1)
- [ ] More information on the process of the molecular analysis of echidna scats (2)
- [ ] Feedback about the results of molecular analysis of the echidna scats (3)
- [ ] More educational material available about echidnas and their conservation (4)
- [ ] More information about the scientists behind EchidnaCSI (5)
- [ ] Regular updates about the project (6)
- [ ] Regular updates about the data being submitted (7)
- [ ] Regular videos and photos of interesting echidna sightings (8)
- [ ] Regular in person meet-ups and educational workshops (9)
- [ ] Improved instructions on how to submit data (10)
- [ ] Other (please specify) (11)

_____

How would you most like to receive information from EchidnaCSI? (Select all that apply)

- [ ] Email newsletter (1)
- [ ] BioCollect website (2)
- [ ] EchidnaCSI webpage (3)
- [ ] Facebook (4)
- [ ] Twitter (5)
- [ ] Instagram (6)
- [ ] In-person events (7)
- [ ] In the app (8)
- [ ] Other (please specify): (9) _____

In 12 months time, do you anticipate being:

	Yes (1)	No (2)	Maybe (3)
Actively contributing data to Echidna CSI (1)	○	○	○
Staying up to date with the project (2)	○	○	○

If you have anything else you would like to share including stories about participating in EchidnaCSI or echidna encounters in general we would love to hear them:

_____

End of Block: If not submitted: future participation

Start of Block: Attitudes for citizen science - for non-submitters

Had you heard of 'citizen science' before following EchidnaCSI?

- ○ Yes (1)
- ○ No (2)

Are you more likely to be involved in other citizen science projects since following EchidnaCSI?

- ○ Yes (1)
- ○ No (2)

Are you already participating in any other citizen science projects?

- ○ No (1)
- ○ Yes (please specify) (2) _____

If yes, did you join the project/s before or after being involved in EchidnaCSI?

- ○ Before (1)
- ○ After (2)
- ○ Unsure (3)

End of Block: Attitudes for citizen science - for non-submitters

---

Start of Block: Level of involvement: if submitted data

Have you used the EchidnaCSI app to submit data?

- ○ Yes (1)
- ○ No (2)

Have you used the BioCollect website to submit data?

- ○ Yes (1)
- ○ No (2)

How many sightings of echidnas have you submitted? (Best estimation if unsure)

- ○ 0 (1)
- ○ 1 (2)
- ○ 2 (3)
- ○ 3 (4)
- ○ 4 (5)
- ○ 5-9 (6)
- ○ 10-19 (7)
- ○ 20-49 (8)
- ○ 50+ (9)

How many scats have you submitted? (Best estimation if unsure)

- ○ 0 (1)
- ○ 1 (2)
- ○ 2 (3)
- ○ 3-9 (4)
- ○ 10-19 (5)
- ○ 20-49 (6)
- ○ 50+ (7)

When do you submit echidna sightings/scats? (Select all that apply)

- ☐ At home (1)
- ☐ On holidays (2)
- ☐ When out driving (3)
- ☐ Taking walks (4)
- ☐ While working (5)
- ☐ When actively searching (6)
- ☐ Other (7) _____

Who are you with when you submit sightings? (Select all that apply)

- [ ] Myself (1)
- [ ] Partner (2)
- [ ] Children (3)
- [ ] Grandchildren (4)
- [ ] Parent (5)
- [ ] Grandparent (6)
- [ ] Friend (7)
- [ ] Colleague (8)
- [ ] Other (9) _____

Do you actively seek out echidnas for this project?

- ○ Yes, and I **only** see them when I seek them out (1)
- ○ Yes, but I **also** see them opportunistically (2)
- ○ Yes, but I **only** see them opportunistically (3)
- ○ No (4)

Do you actively seek out echidna scats for this project?

- ○ Yes, and I **only** see them when I seek them out (1)
- ○ Yes, but I **also** see them opportunistically (2)
- ○ Yes, but I **only** see them opportunistically (3)
- ○ No (4)

End of Block: Level of involvement: if submitted data

Start of Block: Attitudes towards citizen science

Had you heard of 'citizen science' before participating in EchidnaCSI?

- ○ Yes (1)
- ○ No (2)

Are you more likely to be involved in other citizen science projects since participating in EchidnaCSI?

- ○ Yes (1)
- ○ No (2)

Are you already participating in any other citizen science projects?

- ○ No (1)
- ○ Yes (please specify) (2) _____

If yes, did you join the project/s before or after being involved in EchidnaCSI?

- ○ Before (1)
- ○ After (2)
- ○ Unsure (3)

End of Block: Attitudes towards citizen science

Start of Block: Motivations

	How important are these factors to your participation in EchidnaCSI?				
	Completely unimportant (1)	Somewhat unimportant (2)	Neutral (3)	Somewhat important (4)	Very important (5)
I like echidnas (1)	○	○	○	○	○
I care for echidna conservation (2)	○	○	○	○	○
I want to learn more about echidnas (3)	○	○	○	○	○
It is easy to submit data (4)	○	○	○	○	○
I value the goals of EchidnaCSI's research (5)	○	○	○	○	○
I am interested in molecular biology (6)	○	○	○	○	○
I am interested in citizen science (7)	○	○	○	○	○
I am interested in science in general (8)	○	○	○	○	○
The project is easy to participate in (9)	○	○	○	○	○
I enjoy contributing to original scientific research (10)	○	○	○	○	○
I find EchidnaCSI a valuable resource for teaching others (11)	○	○	○	○	○
I want to contribute to wildlife conservation (12)	○	○	○	○	○

I enjoy being outdoors (13)	○	○	○	○	○
I care about the environment (14)	○	○	○	○	○
I enjoy the time I spend with my family and/or friends when contributing to EchidnaCSI (15)	○	○	○	○	○
I enjoy being a part of a community of like-minded people (16)	○	○	○	○	○
I am interested in learning how to identify echidna scats (17)	○	○	○	○	○

What are your current views on the following statements?

	Strongly disagree (1)	Somewhat disagree (2)	Neither agree nor disagree (3)	Somewhat agree (4)	Strongly agree (5)
Echidna conservation is important to me (1)	○	○	○	○	○
The health of the environment is important to me (2)	○	○	○	○	○
I take actions to protect or preserve the environment (3)	○	○	○	○	○
Participating in citizen science is worth my time (4)	○	○	○	○	○
I do what I can, but the environment is not by biggest concern (5)	○	○	○	○	○

How do you think your views to these statements have changed since following EchidnaCSI?

	Strongly decreased (1)	Somewhat decreased (2)	Remained the same (3)	Somewhat increased (4)	Strongly increased (5)
Echidna conservation is important to me (1)	○	○	○	○	○
The health of the environment is important to me (2)	○	○	○	○	○
I take actions to protect or preserve the environment (3)	○	○	○	○	○
Participating in citizen science is worth my time (4)	○	○	○	○	○
I do what I can, but the environment is not by biggest concern (5)	○	○	○	○	○

End of Block: Motivations

Start of Block: Future participation/improving future experience

How would you most like to receive information from EchidnaCSI? (Select all that apply)

- ☐ Email newsletter (1)
- ☐ BioCollect website (2)
- ☐ Grutzner Lab website (3)
- ☐ Facebook (4)
- ☐ Twitter (5)
- ☐ Instagram (6)
- ☐ In-person events (7)
- ☐ Other (please specify): (8) _____

How could the experience of participating in EchidnaCSI be improved to maintain your participation? (Select all that apply)

- ☐ Feedback about how your data is being used by scientists (1)
- ☐ More information on the process of the molecular analysis of echidna scats (2)
- ☐ Feedback on the results of molecular analysis of echidna scats (3)
- ☐ More educational material available about echidnas and their conservation (4)
- ☐ More information about the scientists behind EchidnaCSI (5)
- ☐ Regular updates about the project (6)
- ☐ Regular updates about the data being submitted (7)
- ☐ Regular videos and photos about interesting echidna sightings (8)
- ☐ Regular in person meet-ups and educational workshops (9)
- ☐ Improved instructions on how to submit data (10)
- ☐ Other (please specify) (11) _____

In 12 months time, do you anticipate being:

	Yes (1)	No (2)	Maybe (3)
Actively contributing data to Echidna CSI (1)	○	○	○
Staying up to date with the project (2)	○	○	○

If you have anything else you would like to share, including stories about participating in EchidnaCSI or echidna encounters in general, we would love to hear them:

_____

End of Block: Future participation/improving future experience

Supplementary Material for Chapter 3

Table S1: Information for faecal samples collected from wild echidnas. Location consists of state and then nearest suburb; Location = State followed by suburb sample was collected in; DOC = date of collection.

Sample ID	Latitude	Longitude	Location	Season	Breeding season	DOC	Land use	Anthropogenic biomes	Climate class	Land cover
170901SA1	-33.988	138.614	SA;Watervale	Spring	Yes	1/9/17	Cropping	Populated croplands	Temperate	Annual crops and highly modified pastures
170916SA1	-36.970	140.370	SA;Lucindale	Spring	Yes	16/9/17	Urban intensive uses	Populated croplands	Temperate	Built-up
170917SA1	-35.180	138.790	SA;Meadows	Spring	Yes	17/9/17	Modified pastures	Residential rainfed croplands	Temperate	Annual crops and highly modified pastures
170917VIC1	-37.689	143.997	VIC;Lal Lal	Spring	Yes	17/9/17	Rural residential	Populated croplands	Temperate	Native forests and woodlands
170920SA1	-35.750	137.690	SA;Kangaroo Island	Spring	Yes	20/9/17	Cropping	Populated croplands	Temperate	
170920SA2	-35.067	138.915	SA;Mount Barker	Spring	Yes	20/9/17	Modified pastures	Mixed settlements	Temperate	Annual crops and highly modified pastures
170927SA1	-35.740	137.640	SA;Kangaroo Island	Spring	Yes	27/9/17	Modified pastures	Populated croplands	Temperate	
170928SA2	-34.880	138.720	SA;Montacute	Spring	Yes	28/9/17	Nature conservation	Urban	Temperate	Native forests and woodlands
171001SA4	-30.451	139.090	SA;Gammon Ranges	Spring	No	1/10/17	Nature conservation	Remote rangelands	Grassland	Native forests and woodlands
171002SA1	-35.600	137.580	SA;Kangaroo Island	Spring	No	2/10/17	Cropping	Populated croplands	Temperate	
171007SA1	-35.740	137.640	SA;Kangaroo Island	Spring	No	7/10/17	Modified pastures	Populated croplands	Temperate	
171008SA4	-35.740	137.640	SA;Kangaroo Island	Spring	No	8/10/17	Modified pastures	Populated croplands	Temperate	
171011SA1	-35.750	137.690	SA;Kangaroo Island	Spring	No	11/10/17	Cropping	Populated croplands	Temperate	
171012SA1	-34.901	138.772	SA;Montacute	Spring	No	12/10/17	Urban intensive uses	Residential woodlands	Temperate	Native grasslands and minimally modified pastures
171101NSW1	-34.618	149.708	NSW;Middle Arm	Spring	No	1/11/17	Modified pastures	Populated rangelands	Temperate	Native forests and woodlands

171101NSW2	-34.618	149.708	NSW;Middle Arm	Spring	No	1/11/17	Modified pastures	Populated rangelands	Temperate	Native forests and woodlands
171101NSW3	-34.422	150.139	NSW;Canyonleigh	Spring	No	1/11/17	Other minimal uses	Populated rangelands	Temperate	Native forests and woodlands
171104SA1	-35.248	138.798	SA;Paris Creek	Spring	No	4/11/17	Modified pastures	Residential rainfed croplands	Temperate	Native forests and woodlands
171104SA3	-35.248	138.798	SA;Paris Creek	Spring	No	4/11/17	Modified pastures	Residential rainfed croplands	Temperate	Native forests and woodlands
171105SA1	-33.987	138.613	SA;Watervale	Spring	No	5/11/17	Cropping	Populated croplands	Temperate	Annual crops and highly modified pastures
171108NSW1	-34.618	149.707	NSW;Middle Arm	Spring	No	8/11/17	Modified pastures	Populated rangelands	Temperate	Native forests and woodlands
171109NSW1	-34.616	149.709	NSW;Middle Arm	Spring	No	9/11/17	Modified pastures	Populated rangelands	Temperate	Native forests and woodlands
171111VIC1	-38.469	144.897	VIC;Cape Schanck	Spring	No	11/11/17	Urban intensive uses	Populated rangelands	Temperate	
171111VIC2	-38.470	144.897	VIC;Cape Schanck	Spring	No	11/11/17	Urban intensive uses	Populated rangelands	Temperate	
171119SA1	-35.648	138.187	SA;Deep Creek	Spring	No	19/11/17	Nature conservation	Populated woodlands	Temperate	Native forests and woodlands
171119SA2	-35.647	138.180	SA;Deep Creek	Spring	No	19/11/17	Nature conservation	Populated woodlands	Temperate	Native forests and woodlands
171201SA1	-33.289	136.736	SA;Yalanda	Summer	No	1/12/17	Other minimal uses	Remote croplands	Grassland	Native shrublands and heathlands
171201SA2	-33.289	136.728	SA;Yalanda	Summer	No	1/12/17	Cropping	Remote croplands	Grassland	Annual crops and highly modified pastures
171212SA1	-34.032	140.757	SA;Uia Riverland	Summer	No	12/12/17	Water	Remote rangelands	Grassland	Native forests and woodlands
171226VIC1	-37.716	145.304	VIC;Christmas Hills	Summer	No	26/12/17	Modified pastures	Residential rangelands	Temperate	Annual crops and highly modified pastures
171227WA1	-31.906	116.688	WA;St Ronans	Summer	No	27/12/17	Cropping	Remote croplands	Temperate	Annual crops and highly modified pastures
171231VIC1	-37.674	145.187	VIC; Neerim Rise	Summer	No	31/12/17	Plantations	Urban	Temperate	Native grasslands and minimally modified pastures
180118SA1	-35.566	138.533	SA;Waitpinga	Summer	No	8/1/18	Modified pastures	Populated croplands	Temperate	Annual crops and highly modified pastures
180119SA1	-35.555	139.249	SA;Narrung	Summer	No	19/1/18	Modified pastures	Remote croplands	Grassland	Ephemeral and Permanent Water Features
180204VIC1	-38.887	145.951	VIC;Tarwin Lower	Summer	No	4/2/18	Modified pastures	Populated rangelands	Temperate	Annual crops and highly modified pastures

180215QLD1	-26.260	152.792	QLD;Mothar Mountain	Summer	No	16/2/18	Other minimal uses	Residential woodlands	Subtropical	Native forests and woodlands
180225SA1	-35.348	138.792	SA;Finnis	Summer	No	25/2/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180225SA2	-35.348	138.792	SA;Finnis	Summer	No	25/2/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180303NSW1	-34.449	149.499	NSW;Crookwell	Autumn	No	3/3/18	Modified pastures	Residential rainfed croplands	Temperate	Annual crops and highly modified pastures
180309VIC1	-38.597	143.182	VIC;Cooriemungle	Autumn	No	9/3/18	Grazing of native pastures	Remote croplands	Temperate	Native grasslands and minimally modified pastures
180310SA1	-37.052	140.803	SA;Struan	Autumn	No	10/3/18	Urban intensive uses	Remote croplands	Temperate	Annual crops and highly modified pastures
180313SA1	-35.011	138.629	SA;Glenalta	Autumn	No	13/3/18	Urban intensive uses	Urban	Temperate	Native shrublands and heathlands
180313SA3	-35.011	138.629	SA;Glenalta	Autumn	No	13/3/18	Urban intensive uses	Urban	Temperate	Native shrublands and heathlands
180323SA1	-35.441	138.387	SA;Wattle Flat	Autumn	No	23/3/18	Modified pastures	Populated croplands	Temperate	
180330SA1	-35.565	139.209	SA;Narrung	Autumn	No	30/3/18	Modified pastures	Remote croplands	Temperate	Annual crops and highly modified pastures
180331NSW2	-34.750	150.496	NSW;Kangaroo Valley	Autumn	No	31/3/18	Other minimal uses	Remote woodlands	Temperate	Native forests and woodlands
180404SA1	-34.510	139.359	SA;Sedan	Autumn	No	4/4/18	Cropping	Remote croplands	Grassland	Annual crops and highly modified pastures
180407SA1	-35.351	138.793	SA;Finnis	Autumn	No	7/4/18	Other minimal uses	Populated croplands	Temperate	Annual crops and highly modified pastures
180407VIC1	-37.103	144.219	VIC;Campbells Creek	Autumn	No	7/4/18	Other minimal uses	Residential rainfed croplands	Temperate	Native forests and woodlands
180411VIC1	-38.312	144.078	VIC;Gherang	Autumn	No	11/4/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180411VIC2	-38.312	144.078	VIC;Gherang	Autumn	No	11/4/18	Other minimal uses	Populated croplands	Temperate	Native forests and woodlands
180412VIC1	-38.329	141.552	VIC;Portland West	Autumn	No	12/4/18	Modified pastures	Remote croplands	Temperate	Annual crops and highly modified pastures
180417QLD1	-22.110	145.195	QLD;Upper Cornish Creek	Autumn	No	17/4/18	Nature conservation	Remote rangelands	Grassland	Native shrublands and heathlands
180418NSW1	-33.331	151.270	NSW;Somersby	Autumn	No	18/4/18	Modified pastures	Populated woodlands	Temperate	Native shrublands and heathlands
180419SA1	-35.352	138.794	SA;Finnis	Autumn	No	19/4/18	Other minimal uses	Populated croplands	Temperate	Annual crops and highly modified pastures

180420SA1	-35.351	138.794	SA;Finnis	Autumn	No	20/4/18	Other minimal uses	Populated croplands	Temperate	Annual crops and highly modified pastures
180422SA1	-30.448	139.031	SA;Gammon Ranges	Autumn	No	22/4/18	Nature conservation	Remote rangelands	Grassland	Native forests and woodlands
180503QLD1	-15.471	145.259	QLD;Cooktown	Autumn	No	3/5/18	Urban intensive uses		Tropical	
180505SA1	-33.982	138.620	SA;Watervale	Autumn	No	5/5/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180520NSW1	-35.221	149.344	NSW;Wamboin	Autumn	No	20/5/18	Rural residential	Populated rangelands	Temperate	Annual crops and highly modified pastures
180530SA1	-34.597	139.342	SA;Sedan	Autumn	No	30/5/18	Modified pastures	Populated croplands	Grassland	Native forests and woodlands
180612SA1	-30.138	136.898	SA;Arid Recovery	Winter	Yes	12/6/18	Grazing of native pastures	Remote rangelands	Desert	Native grasslands and minimally modified pastures
180620SA3	-35.096	139.076	SA;Monarto	Winter	Yes	20/6/18	Nature conservation	Populated croplands	Grassland	Annual crops and highly modified pastures
180621SA1	-35.350	138.789	SA;Finnis	Winter	Yes	21/6/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180623SA3	-34.545	135.918	SA;Louth Bay	Winter	Yes	23/6/18	Mining and waste	Populated croplands	Temperate	Native grasslands and minimally modified pastures
180627SA1	-30.134	139.399	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180627SA2	-30.137	139.394	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180627SA3	-30.134	139.387	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180627SA4	-30.132	139.396	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native shrublands and heathlands
180627SA5	-30.133	139.396	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native shrublands and heathlands
180627SA6	-30.133	139.397	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180627SA7	-30.134	139.387	SA;Arkaroola	Winter	Yes	27/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180628SA1	-30.135	139.397	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180628SA2	-30.139	139.395	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180628SA3	-30.140	139.395	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands

180628SA4	-30.141	139.396	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180628SA5	-30.145	139.398	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180628SA6	-30.145	139.398	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180628SA7	-30.163	139.426	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180628SA8	-30.163	139.427	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180628SA9	-30.162	139.437	SA;Arkaroola	Winter	Yes	28/6/18	Grazing of native pastures	Remote rangelands	Grassland	Native forests and woodlands
180701QLD1	-27.564	152.007	QLD;Withcott	Winter	Yes	1/7/18	Rural residential	Populated rangelands	Temperate	Native grasslands and minimally modified pastures
180711WA1	-16.423	125.046	WA;King Leopold Ranges	Winter	Yes	9/7/18	Grazing of native pastures	Remote rangelands	Tropical	Native shrublands and heathlands
180712SA2	-35.347	138.791	SA;Finnis	Winter	Yes	12/7/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180715SA1	-26.462	132.029	SA;Umuwa	Winter	Yes	15/7/18	Managed resource protected areas	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180716SA1	-26.462	132.029	SA;Umuwa	Winter	Yes	16/7/18	Managed resource protected areas	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180716SA2	-26.462	132.029	SA;Umuwa	Winter	Yes	16/7/18	Managed resource protected areas	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180718SA1	-26.462	132.029	SA;Umuwa	Winter	Yes	18/7/18	Managed resource protected areas	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180718SA2	-26.462	132.029	SA;Umuwa	Winter	Yes	18/7/18	Managed resource protected areas	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180719SA2	-26.462	132.029	SA;Umuwa	Winter	Yes	19/7/18	Managed resource protected areas	Remote rangelands	Grassland	Native grasslands and minimally modified pastures
180721SA1	-34.973	138.641	SA;Urrbrae	Winter	Yes	21/7/18	Nature conservation	Urban	Temperate	Native forests and woodlands
180725SA1	-30.373	136.844	SA;Arid Recovery	Winter	Yes	25/7/18	Nature conservation	Remote rangelands	Desert	Native grasslands and minimally modified pastures
180726QLD1	-25.083	152.547	QLD;Woodgate	Winter	Yes	26/7/18	Grazing of native pastures	Inhabited treeless and barren lands	Subtropical	
180729SA1	-30.373	136.844	SA;Arid Recovery	Winter	Yes	29/7/18	Nature conservation	Remote rangelands	Desert	Native grasslands and minimally modified pastures
180729SA2	-30.373	136.844	SA;Arid Recovery	Winter	Yes	29/7/18	Nature conservation	Remote rangelands	Desert	Native grasslands and minimally modified pastures

180729SA3	-34.964	138.648	SA;Glen Osmond	Winter	Yes	29/7/18	Mining and waste	Urban	Temperate	Native forests and woodlands
180729VIC1	-37.280	144.289	VIC;Denver	Winter	Yes	29/7/18	Other minimal uses	Remote rangelands	Temperate	Native forests and woodlands
180730NSW1	-28.810	149.869	NSW;Garah	Winter	Yes	30/7/18	Grazing of native pastures	Remote rangelands	Subtropical	Native forests and woodlands
180809SA1	-35.265	138.870	SA;Strathalbyn	Winter	Yes	9/8/18	Nature conservation	Residential rainfed croplands	Temperate	Annual crops and highly modified pastures
180812NSW1	-28.688	153.581	NSW;Cooper's Shoot	Winter	Yes	12/8/18	Rural residential	Residential woodlands	Subtropical	Native grasslands and minimally modified pastures
180813SA1	-35.349	138.790	SA;Finnis	Winter	Yes	13/8/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180813SA2	-35.349	138.790	SA;Finnis	Winter	Yes	13/8/18	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
180824VIC1	-36.112	146.816	VIC;West Wodonga	Winter	Yes	24/8/18	Modified pastures	Populated croplands	Temperate	Annual crops and highly modified pastures
180831VIC1	-36.723	141.950	VIC;Natimuk	Winter	Yes	18/8/31	Cropping	Residential rainfed croplands	Temperate	Native forests and woodlands
180903SA1	-34.899	138.773	SA;Montacute	Spring	Yes	3/9/18	Irrigated horticulture	Residential woodlands	Temperate	Horticultural trees and shrubs
180911SA1	-34.117	140.808	SA;Murtho	Spring	Yes	11/9/18	Irrigated horticulture	Populated rangelands	Grassland	Annual crops and highly modified pastures
180912SA1	-34.901	138.872	SA;Lobethal	Spring	Yes	12/9/18	Modified pastures	Residential woodlands	Temperate	Annual crops and highly modified pastures
180915SA1	-35.160	138.559	SA;Onkaparinga	Spring	Yes	15/9/18	Nature conservation	Urban	Temperate	Horticultural trees and shrubs
180925SA1	-35.739	137.636	SA;Kangaroo Island	Spring	Yes	25/9/18	Modified pastures	Populated croplands	Temperate	
180927SA1	-30.373	136.844	SA;Arid Recovery	Spring	Yes	27/9/18	Nature conservation	Remote rangelands	Desert	Native grasslands and minimally modified pastures
180930NSW1	-30.517	151.738	NSW;Armidale	Spring	Yes	30/9/18	Modified pastures	Mixed settlements	Temperate	Annual crops and highly modified pastures
181012VIC1	-37.646	149.700	VIC;Wingan River	Spring	No	12/10/18	Nature conservation	Remote woodlands	Temperate	Native forests and woodlands
181016NSW1	-36.440	148.556	NSW;Crackenback	Spring	No	16/10/18	Other minimal uses	Populated rangelands	Temperate	Native forests and woodlands
181016NSW2	-34.279	146.045	NSW;Griffith	Spring	No	16/10/18	Urban intensive uses	Mixed settlements	Grassland	Built-up
181018SA1	-30.373	136.844	SA;Arid Recovery	Spring	No	8/10/18	Nature conservation	Remote rangelands	Desert	Native grasslands and minimally modified pastures

181025SA1	-33.289	138.262	SA;Huddlestone	Spring	No	25/10/18	Other minimal uses	Remote croplands	Temperate	Annual crops and highly modified pastures
181026SA1	-33.289	138.261	SA;Huddlestone	Spring	No	26/10/18	Other minimal uses	Remote croplands	Temperate	Annual crops and highly modified pastures
181027SA1	-33.287	138.262	SA;Huddlestone	Spring	No	27/10/18	Other minimal uses	Remote croplands	Temperate	Annual crops and highly modified pastures
181027SA2	-33.287	138.261	SA;Huddlestone	Spring	No	27/10/18	Other minimal uses	Remote croplands	Temperate	Annual crops and highly modified pastures
181028SA1	-33.289	138.260	SA;Huddlestone	Spring	No	28/10/18	Other minimal uses	Remote croplands	Temperate	Annual crops and highly modified pastures
181028SA2	-33.289	138.262	SA;Huddlestone	Spring	No	28/10/18	Cropping	Remote croplands	Temperate	Native forests and woodlands
181107QLD1	-26.319	148.757	QLD;Eumamurrin	Spring	No	7/11/18	Cropping	Remote rangelands	Subtropical	Native grasslands and minimally modified pastures
181107QLD2	-26.319	148.757	QLD;Eumamurrin	Spring	No	7/11/18	Cropping	Remote rangelands	Subtropical	Native grasslands and minimally modified pastures
181115SA2	-35.114	139.265	SA;Murray Bridge	Spring	No	15/11/18	Urban intensive uses	Mixed settlements	Grassland	Native forests and woodlands
181125SA1	-35.567	138.482	SA;Waitpinga	Spring	No	25/11/18	Cropping	Populated croplands	Temperate	Annual crops and highly modified pastures
181231VIC1	-38.344	146.769	VIC;Willung South	Summer	No	31/12/18	Modified pastures	Wild woodlands	Temperate	Annual crops and highly modified pastures
190103QLD1	-27.462	152.935	QLD;The Gap	Summer	No	3/1/19	Rural residential	Urban	Subtropical	Native forests and woodlands
190126VIC1	-38.330	146.759	VIC;Willung South	Summer	No	26/1/19	Modified pastures	Wild woodlands	Temperate	Native grasslands and minimally modified pastures
190312ACT1	-35.354	149.085	ACT;Chifley	Autumn	No	12/3/19	Urban intensive uses	Urban	Temperate	Built-up
190402VIC1	-36.846	144.469	VIC;Eppalock	Autumn	No	2/4/19	Nature conservation	Populated croplands	Temperate	Native forests and woodlands
190402VIC2	-36.846	144.471	VIC;Eppalock	Autumn	No	2/4/19	Nature conservation	Populated croplands	Temperate	Native forests and woodlands
190424SA1	-35.350	138.791	SA;Finnis	Autumn	No	24/4/19	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
190424SA2	-35.350	138.791	SA;Finnis	Autumn	No	24/4/19	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
190424SA3	-35.350	138.791	SA;Finnis	Autumn	No	24/4/19	Modified pastures	Populated croplands	Temperate	Native forests and woodlands
190427SA1	-35.555	139.249	SA;Narrung	Autumn	No	27/4/19	Modified pastures	Remote croplands	Grassland	Ephemeral and Permanent Water Features

190430NSW1	-32.378	149.702	NSW;Cook's Gap	Autumn	No	30/4/19	Rural residential	Remote rangelands	Temperate	Native forests and woodlands
190430NSW2	-29.250	150.751	NSW;Coolatai	Autumn	No	30/4/19	Urban intensive uses	Remote rangelands	Temperate	Native forests and woodlands
190502NSW1	-29.021	151.935	NSW;Tenterfield	Autumn	No	2/5/19	Modified pastures	Remote rangelands	Temperate	Native forests and woodlands
190502NSW2	-29.021	151.935	NSW;Tenterfield	Autumn	No	2/5/19	Modified pastures	Remote rangelands	Temperate	Native forests and woodlands
190502NSW3	-29.021	151.935	NSW;Tenterfield	Autumn	No	2/5/19	Modified pastures	Remote rangelands	Temperate	Native forests and woodlands
190508SA1	-31.272	138.388	SA;Parachilna	Autumn	No	8/5/19	Grazing of native pastures	Remote rangelands	Desert	Native shrublands and heathlands
190508SA2	-31.275	138.375	SA;Parachilna	Autumn	No	8/5/19	Grazing of native pastures	Remote rangelands	Desert	Native shrublands and heathlands
190508SA3	-31.275	138.375	SA;Parachilna	Autumn	No	8/5/19	Grazing of native pastures	Remote rangelands	Desert	Native shrublands and heathlands
190523QLD1	-27.846	150.155	QLD;Southwood	Autumn	No	23/5/19	Grazing of native pastures	Remote rangelands	Subtropical	Native forests and woodlands
190601NSW1	-29.237	152.013	NSW;Sandy Flat	Winter	Yes	1/6/19	Modified pastures	Remote rangelands	Temperate	Annual crops and highly modified pastures
190603SA1	-31.274	138.381	SA;Parachilna	Winter	Yes	3/6/19	Grazing of native pastures	Remote rangelands	Desert	Native shrublands and heathlands
190603SA3	-31.274	138.381	SA;Parachilna	Winter	Yes	3/6/19	Grazing of native pastures	Remote rangelands	Desert	Native shrublands and heathlands
190610SA1	-35.256	138.728	SA;McHarg Creek	Winter	Yes	10/6/19	Other minimal uses	Populated woodlands	Temperate	Annual crops and highly modified pastures
190727WA2	-26.515	114.098	WA;Hamelin Pool	Winter	Yes	27/7/19	Grazing of native pastures	Remote rangelands	Grassland	Native shrublands and heathlands
190801WA1	-27.525	114.529	WA;Eurardy	Winter	Yes	1/8/19	Grazing of native pastures	Remote rangelands	Grassland	Native shrublands and heathlands
190801WA2	-27.605	114.693	WA;Eurardy	Winter	Yes	1/8/19	Grazing of native pastures	Remote rangelands	Grassland	Native shrublands and heathlands
190812NSW1	-33.583	149.263	NSW;Blayney	Winter	Yes	12/8/19	Modified pastures	Residential rainfed croplands	Temperate	Native forests and woodlands
190812NSW2	-33.583	149.263	NSW;Blayney	Winter	Yes	12/8/19	Modified pastures	Residential rainfed croplands	Temperate	Native forests and woodlands
190813WA1	-16.081	124.469	WA;Derby West Kimberley	Winter	Yes	13/8/19	Other minimal uses		Tropical	Native grasslands and minimally modified pastures
190817SA1	-34.947	138.691	SA;Greenhill	Winter	Yes	17/8/19	Other minimal uses	Mixed settlements	Temperate	Built-up

190822WA1	-16.688	125.246	WA;King Leopold Ranges	Winter	Yes	22/8/19	Grazing of native pastures	Remote rangelands	Tropical	Native shrublands and heathlands
190903NSW1	-34.847	149.085	NSW;Lade Vale	Spring	Yes	3/9/19	Modified pastures	Remote croplands	Temperate	Annual crops and highly modified pastures
190903TAS1	-43.097	147.964	TAS;Fortescue	Spring	Yes	3/9/19	Nature conservation	Inhabited treeless and barren lands	Temperate	
190930QLD1	-27.317	152.796	QLD;Cedar Creek	Spring	Yes	30/9/19	Other minimal uses	Populated woodlands	Subtropical	Native grasslands and minimally modified pastures

Table S2: Information for faecal samples collected in captivity. UMD = Updated Meat Diet; DOC = Date of Collection; [a] = technical replicate.

Sample ID	Echidna Name	Diet	Sex	Zoo	Season	Breeding Season	DOC
BL231018	Blue	Meat	Female	Perth	Spring	No	23/10/18
BL241018	Blue	Meat	Female	Perth	Spring	No	24/10/18
BL251018	Blue	Meat	Female	Perth	Spring	No	25/10/18
CH240818	Chindi	Meat	Female	Perth	Winter	Yes	24/8/18
CH250818	Chindi	Meat	Female	Perth	Winter	Yes	25/8/18
CH260818	Chindi	Meat	Female	Perth	Winter	Yes	26/8/18
CO230218	Cojine	Meat	Male	Perth	Summer	No	23/2/18
CO2302181[a]	Cojine	Meat	Male	Perth	Summer	No	23/2/18
CO2302182[a]	Cojine	Meat	Male	Perth	Summer	No	23/2/18
CO2302183[a]	Cojine	Meat	Male	Perth	Summer	No	23/2/18
CO240218	Cojine	Meat	Male	Perth	Summer	No	24/2/18
CO250218	Cojine	Meat	Male	Perth	Summer	No	25/2/18
CO2502181[a]	Cojine	Meat	Male	Perth	Summer	No	25/2/18
CO2502182[a]	Cojine	Meat	Male	Perth	Summer	No	25/2/18
CO2502183[a]	Cojine	Meat	Male	Perth	Summer	No	25/2/18
GR231018	Green	Meat	Female	Perth	Spring	No	23/10/18
GR241018	Green	Meat	Female	Perth	Spring	No	24/10/18
GR251018	Green	Meat	Female	Perth	Spring	No	25/10/18
JI240718	Jilba	Meat	Female	Perth	Winter	Yes	24/7/18
JI250718	Jilba	Meat	Female	Perth	Winter	Yes	25/7/18
JI260718	Jilba	Meat	Female	Perth	Winter	Yes	26/7/18
KA240718	Kain	Meat	Male	Perth	Winter	Yes	24/7/18
KA250718	Kain	Meat	Male	Perth	Winter	Yes	25/7/18

KA260718	Kain	Meat	Male	Perth	Winter	Yes	26/7/18
MI241018	Mila	Meat	Female	Perth	Spring	No	24/10/18
MI251018	Mila	Meat	Female	Perth	Spring	No	25/10/18
MI261018	Mila	Meat	Female	Perth	Spring	No	26/10/18
MO230718	Moa	Meat	Female	Perth	Winter	Yes	23/7/18
MO240718	Moa	Meat	Female	Perth	Winter	Yes	24/7/18
MO250718	Moa	Meat	Female	Perth	Winter	Yes	25/7/18
NY260218	Nyingarn	Meat	Male	Perth	Summer	No	26/2/18
NY270218	Nyingarn	Meat	Male	Perth	Summer	No	27/2/18
MSSBE175	Snorky	UMD	Female	Taronga	Spring	No	17/10/19
MSSBE181	Rose	UMD	Female	Taronga	Spring	No	17/10/19
MSSBE193	Bristle	UMD	Male	Taronga	Autumn	No	3/5/19
MSSBE197	Bali	UMD	Female	Taronga	Winter	Yes	7/8/19
MSSBE202	Rex	UMD	Male	Taronga	Spring	Yes	18/9/19
MSSBE111	Bristle	Vetafarm	Male	Taronga	Spring	No	21/11/19
MSSBE140	Leroy	Vetafarm	Female	Taronga	Summer	No	13/12/18
MSSBE187	Bristle	Vetafarm	Male	Taronga	Autumn	No	24/4/19
MSSBE210	Bali	Vetafarm	Female	Taronga	Winter	Yes	19/7/19
MSSBE61	Spike	Vetafarm	Female	Taronga	Summer	No	6/2/17
MSSBE62	Spike	Vetafarm	Female	Taronga	Summer	No	7/2/17
MSSBE63	Spike	Vetafarm	Female	Taronga	Summer	No	11/2/17
MSSBE205	Bali	Wombaroo	Female	Taronga	Spring	Yes	18/9/19
MSSBE224	Ganyi	Wombaroo	Female	Taronga	Summer	No	16/12/19
MSSBE225	Ganyi	Wombaroo	Female	Taronga	Summer	No	17/12/19
MSSBE226	Ganyi	Wombaroo	Female	Taronga	Summer	No	18/12/19
MSSBE235	Jindi	Wombaroo	Male	Taronga	Summer	No	5/12/19
MSSBE236	Gemma	Wombaroo	Female	Taronga	Summer	No	9/12/19

Table S3: Ingredients of the four different diets fed to echidnas in captivity.

Ingredients			
Perth Zoo's Meat Diet	**Taronga Zoo's Updated Meat Diet**	**Taronga Zoo's Vetafarm Diet**	**Taronga Zoo's Wombaroo Diet**
Beef, lean fine mince	Beef, lean fine mince	Meat Meal (Kangaroo)	Meat Meal (Kangaroo)
Microcrystalline cellulose	Microcrystalline cellulose	Corn	Soy and protein isolates, cellulose
Egg, hardboiled	Egg, hardboiled	Roughage (straw)	Processed cereals (wheat and rice)
Banana	KER bone food	Potato starch	Vegetable oil, Omega 3 & 6 fatty acids
Pentavite with iron	Pentavite with iron	Minerals (calcium, chloride, cobalt, copper, iodine, iron, magnesium, manganese, phosphorous, potassium, sodium, sulphur, selenium and zinc)	Minerals (calcium, chloride, cobalt, copper, iodine, iron, magnesium, manganese, phosphorous, potassium, sodium, sulphur, selenium and zinc)
Calcium carbonate	Calcium carbonate	Vegetable oils and organic acids	Amino acids (lysine, methionine, taurine)
Mealworms	KER nano E	Amino acids (arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, taurine, threonine, tryptophan, tyrosine & valine)	Vitamins: A, B1, B2, B3, B5, B6, B9, B12, C, D3, K, biotin & choline
		Natuzyme multi-enzyme Actigen® dried yeast prebiotic	Natuzyme multi-enzyme Actigen® dried yeast prebiotic

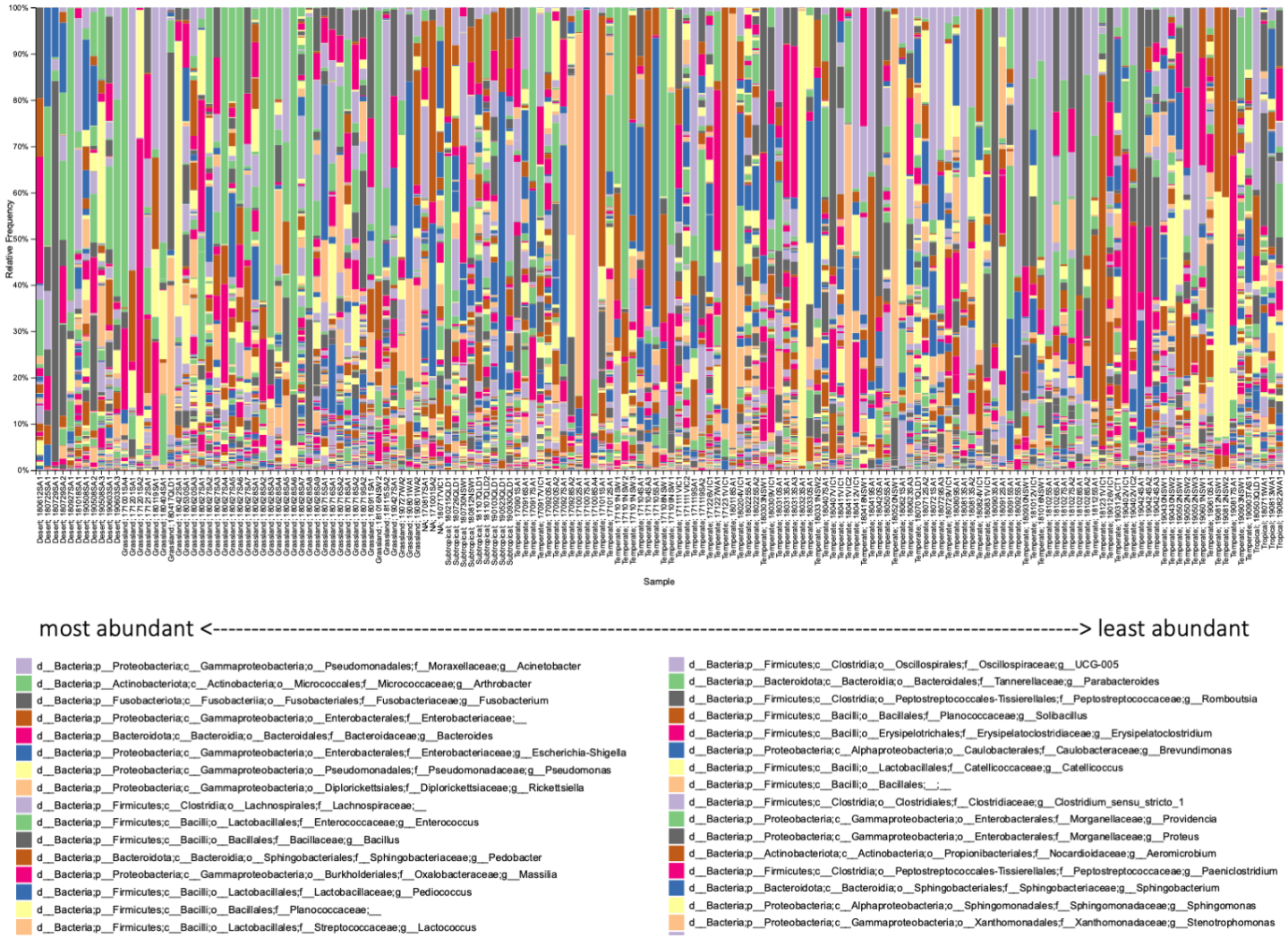


Figure S1: Taxonomy bar plots of relative frequency of bacteria present in all wild echidna scats at the genus level. Samples are labelled and organised by their sample ID and climate class (Table S1). The top 32 genera and families present are included in the legend, however only the most abundant are easily visualised; as bar colours repeat, the legend is labelled with most abundant taxa on the left to least abundant taxa on the right. d = domain; p = phyla; c = class; o = order; f = family; g = genus.

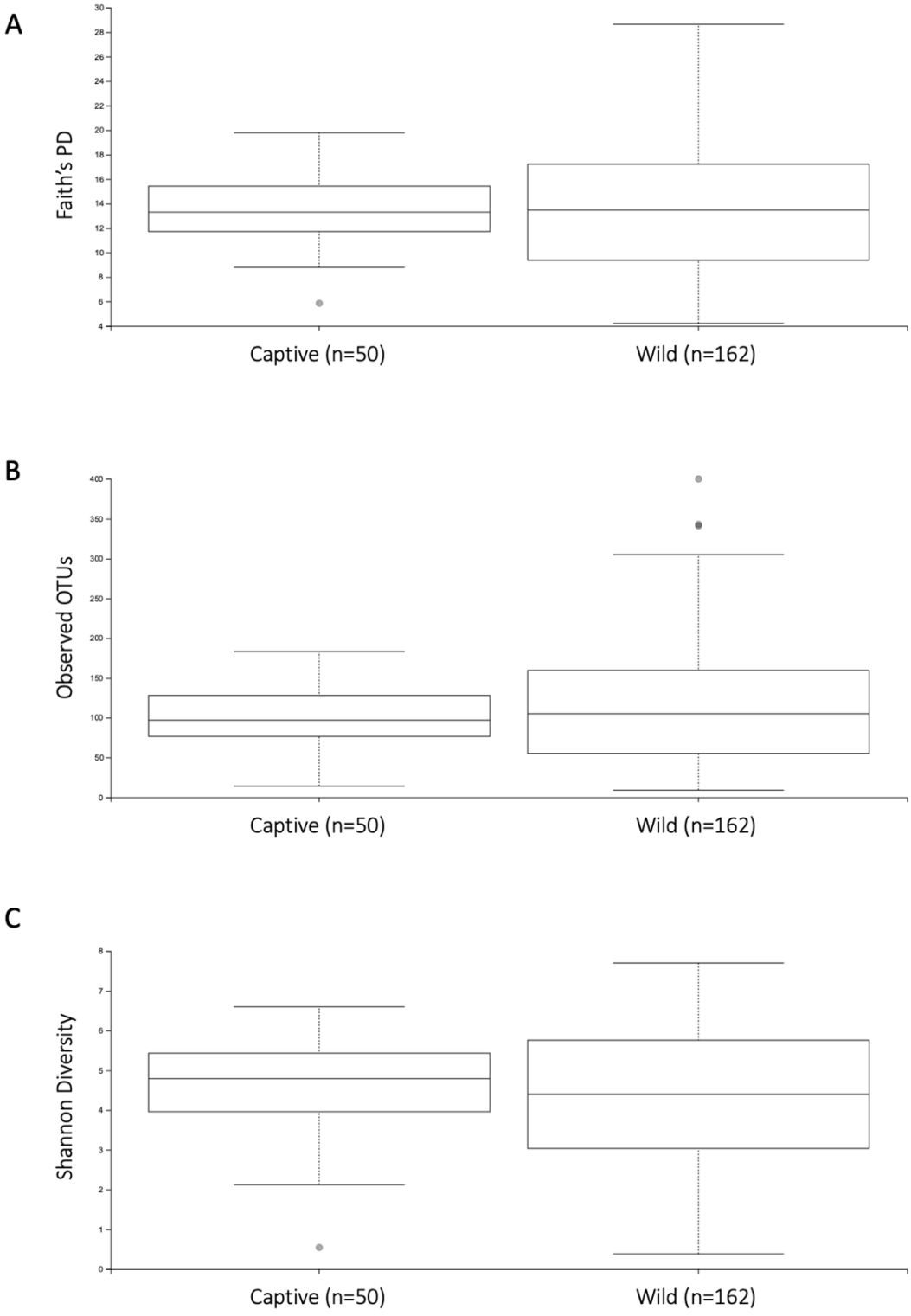


Figure S2: Alpha diversity analyses of gut microbiomes from samples collected in captivity compared to those collected in the wild. Whisker-box plots depict the following metrics: A) Faith's phylogenetic diversity (Faith's PD); B) Observed ASVs; C) Shannon's Diversity index (captive). Horizontal lines indicate median values, upper and lower bounds represent the 25th and 75th percentiles, and top and bottom whiskers indicate maximum and minimum values. Outliers are shown as grey circles. No significance was observed for any of these diversity metrics.

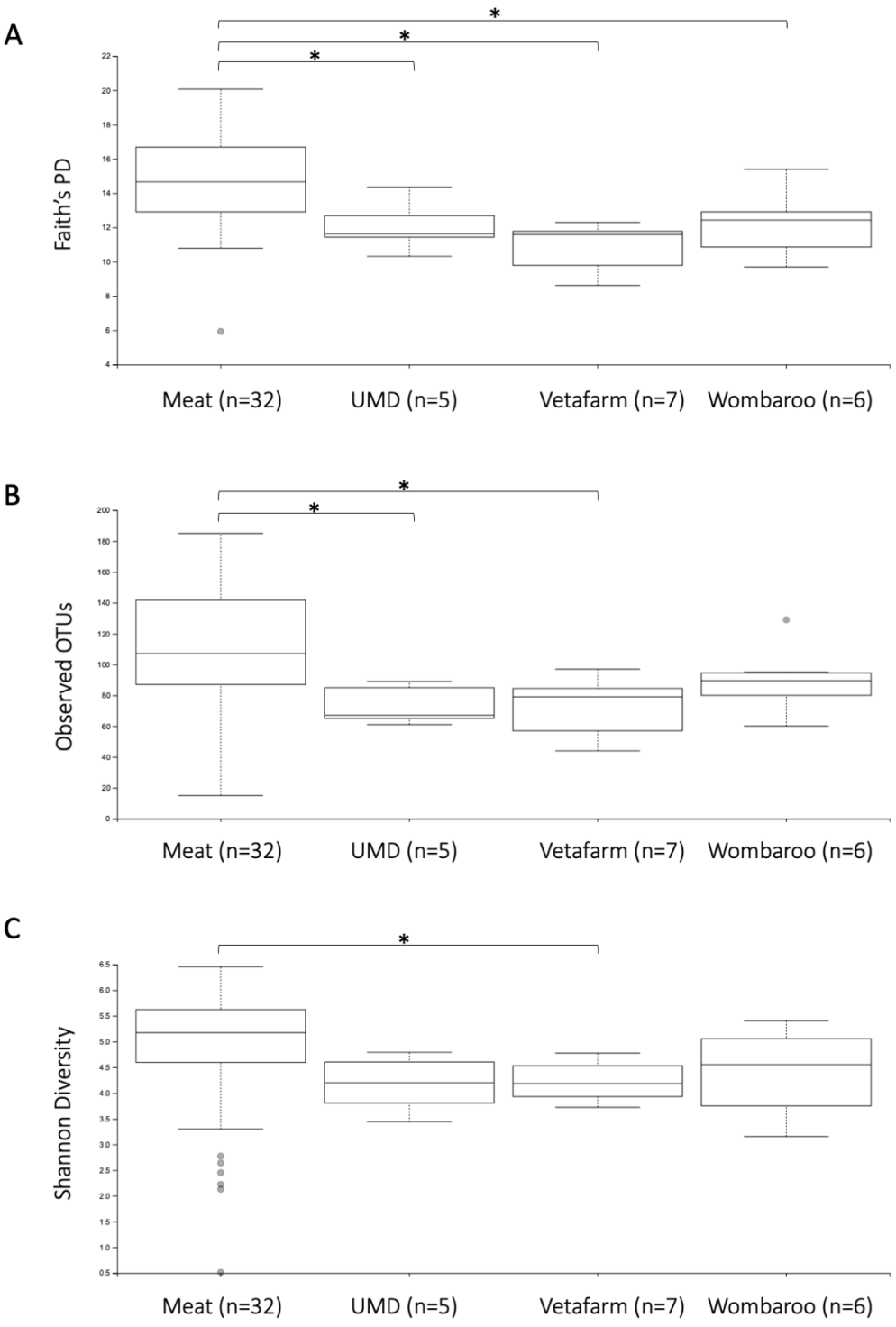


Figure S3: Alpha diversity analyses of gut microbiomes from samples collected in captivity of echidnas fed four different diets. Whisker-box plots depict the following metrics: A) Faith's phylogenetic diversity (Faith's PD); B) Observed ASVs; C) Shannon's Diversity index. Horizontal lines indicate median values, upper and lower bounds represent the 25th and 75th percentiles, and top and bottom whiskers indicate maximum and minimum values. Outliers are shown as grey circles. * = significance ($p<0.05$); UMD = Updated Meat Diet.

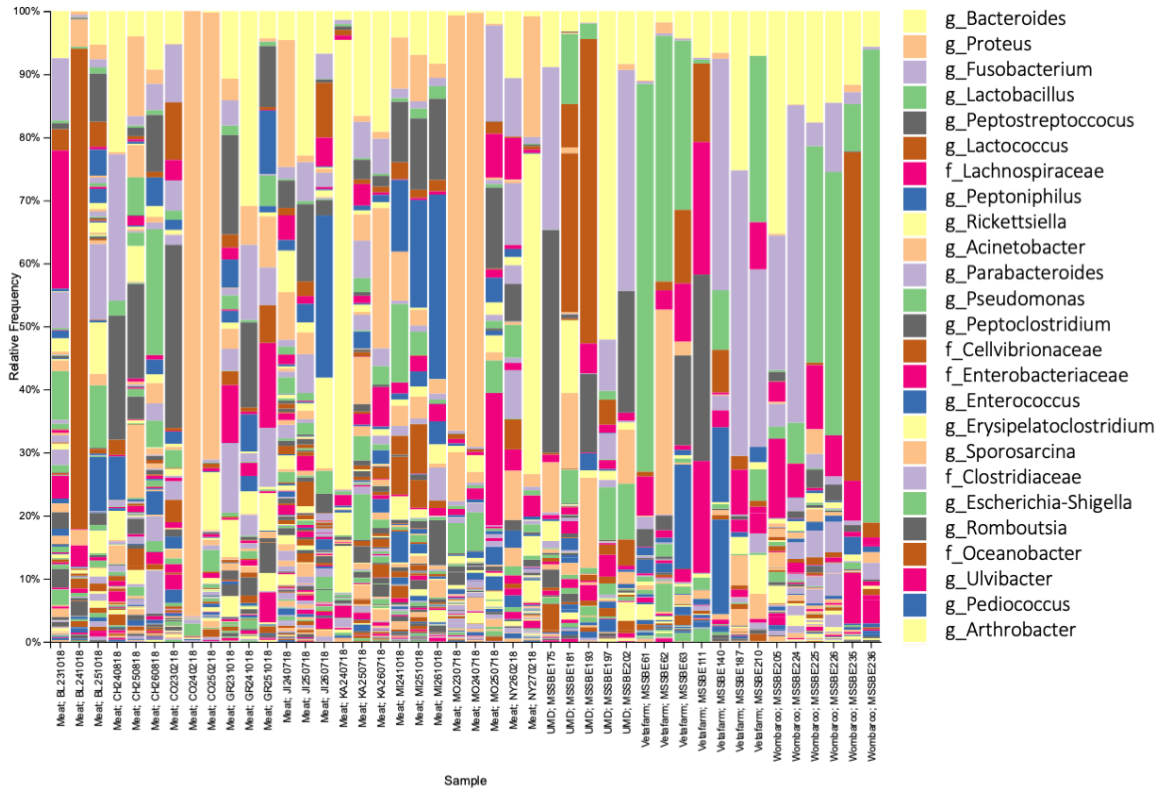
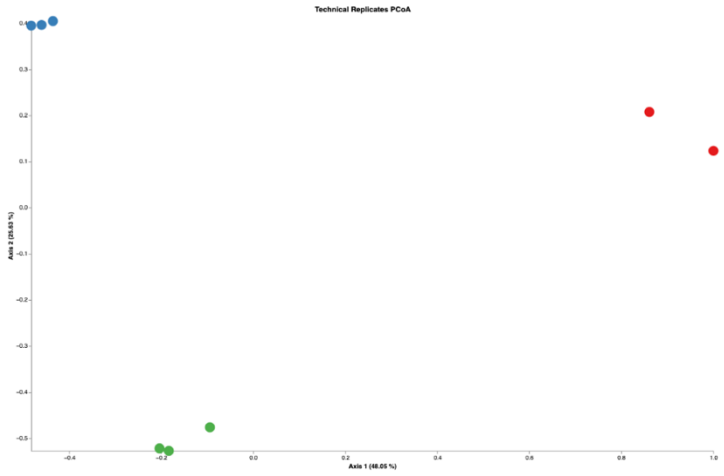


Figure S4: Taxonomy bar plots of relative frequency of bacteria present in all captive echidna scats at the genus level. Samples are labelled and organised by their diet and sample ID (Table S2). The top 25 genera and families present are included in the legend, however only the most abundant are easily visualised; as bar colours repeat, the legend is labelled with most abundant taxa on top to least abundant taxa on bottom. f = family; g = genus; UMD = Updated Meat Diet.

A



B

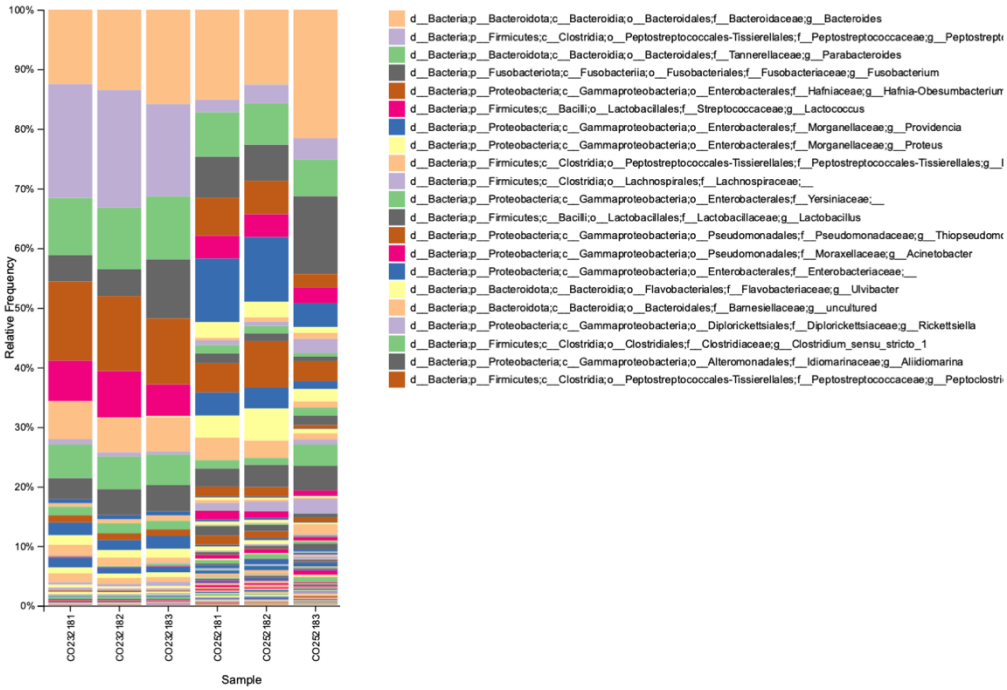


Figure S5: Technical triplicates confirm daily variation is true biological phenomenon and not due to technical issues. A) PCoA plot of unweighted UniFrac distances showing tight clustering of triplicates extracted from two samples collected two days apart from the same echidna: CO23218 (blue) and CO25218 (green); red samples indicate negative controls (no template 16S PCRs that were sequenced to capture potential contamination). B) Taxonomy bar plots showing relative frequencies of bacteria present technical triplicates at the genus and family level. Samples are labelled by their sample ID (Table 2). The top 21 genera and families present are included in the legend, however only the most abundant are easily visualised; as bar colours repeat, the legend is labelled with most abundant taxa on top to least abundant taxa on bottom.

Supplementary Material for Chapter 5

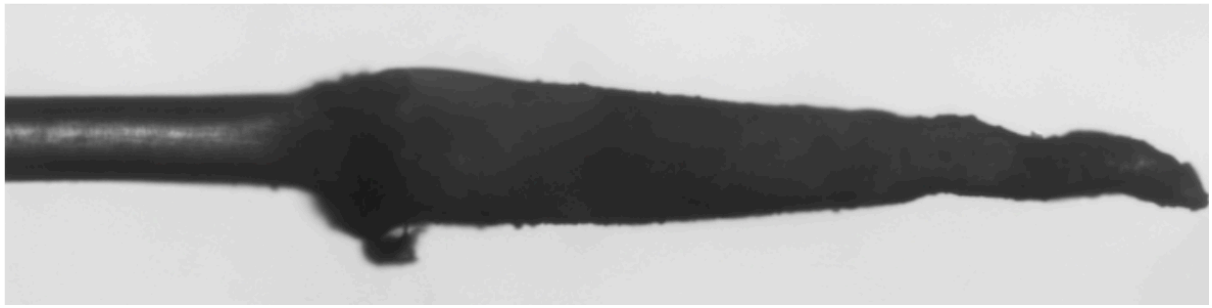


Figure S1: Image of echidna hair follicle. Echidnas have a distinct conical shaped hair follicle in comparison to the characteristic bulb shaped follicle in humans. For this protocol, the hair shaft was removed from the follicle so that only the follicle was used for DNA extraction.

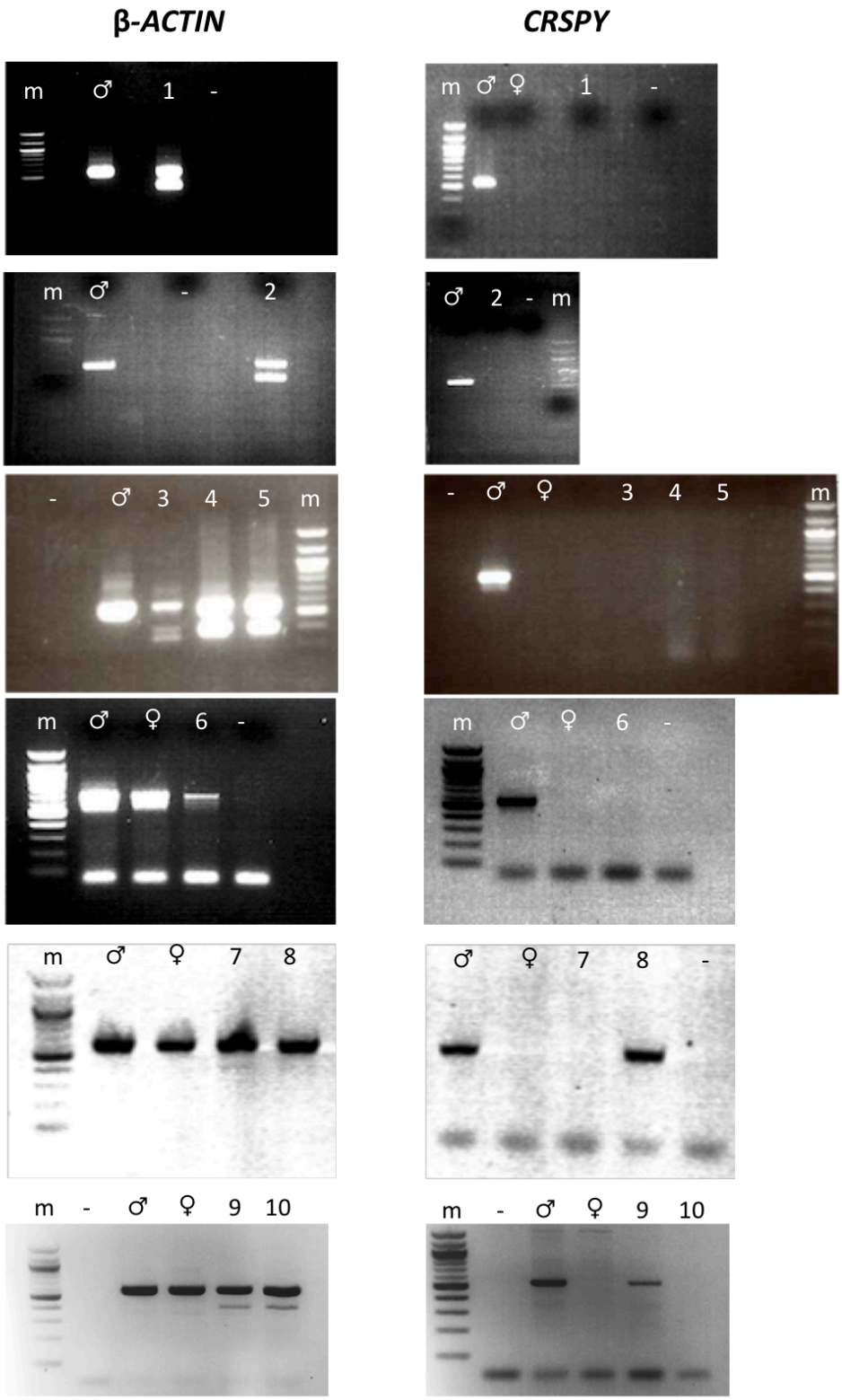


Figure S2: Original gel images relating to Figure 3. For Figure 3, gel images were cropped to appear in an easier to read figure describing the sex of echidnas via PCR. The original gel images are shown here for the amplification of β-actin (*ACTB*) and *CRSPY* for all samples analysed in this paper (ID 1-10). Again, showing that Echidnas 8 and 9 are male and all remaining are female. ♂ = known male, ♀ = known female, - = negative control. β-*ACTIN* is positive control indicating that genomic extraction was successful for all individuals. Number denotes the ID for echidna DNA used in PCR (see Table 1). 100 bp ladder is used as size marker (m). Both *CRSPY* and *ACTB* are approximately 600bp in size.

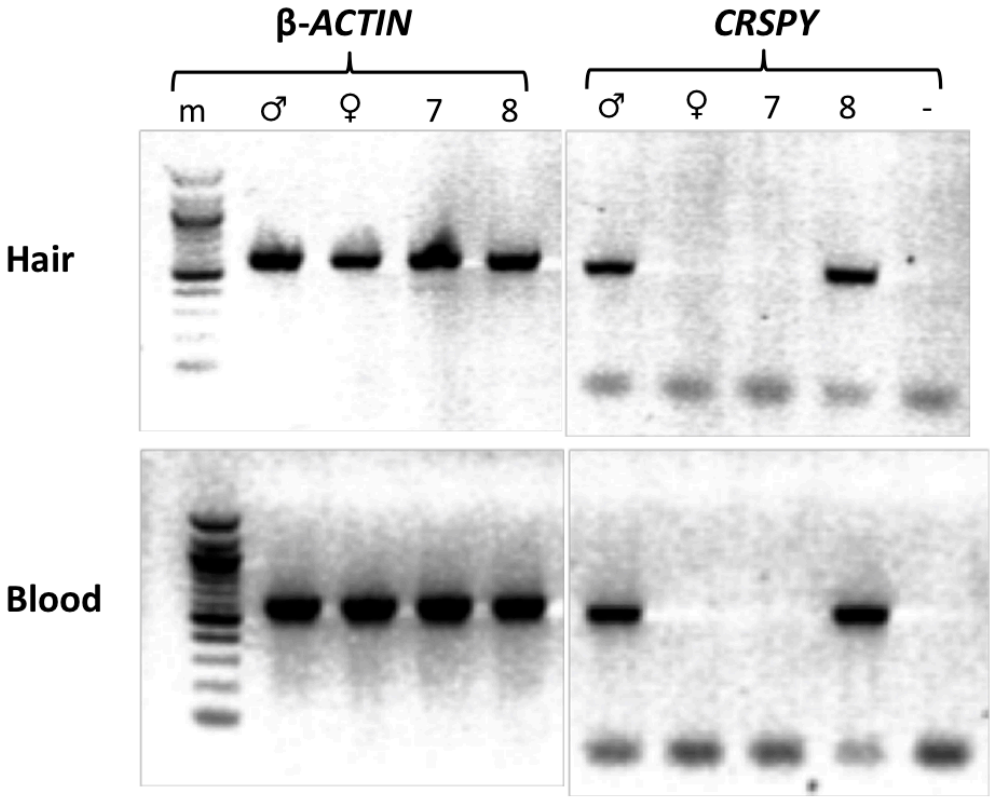


Figure S3: Confirming sex of two echidnas with PCR of *CRSPY* on gDNA extracted from blood. Amplification of *CRSPY* gives a single band in males only. Single band amplified for echidna ID 8 shows it is male; no amplification in Echidna 7 indicates it is a female. This is consistent with the amplification pattern from gDNA extracted from hair follicles. ♂ = known male, ♀ = known female, - = negative control. β-actin (*ACTB*) is positive control indicating that genomic extraction was successful for all individuals. 100 bp ladder is used as size marker (m). Both *CRSPY* and *ACTB* are approximately 600bp in size.