The *Cryptococcus gattii* species complex in koalas: host-pathogen-environment interactions and molecular epidemiology

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Statement of authentication

This thesis is submitted to The University of Sydney in fulfillment of the requirements for the degree of Doctor of Philosophy.

The work presented in this thesis is original, to the best of my knowledge, except where acknowledged in the text.

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Summary

The *Cryptococcus gattii* species complex is a group of pathogenic basidiomycetous yeasts, closely related to the *C. neoformans* species complex. Together they comprise the aetiological agents of cryptococcosis. Members of the *C. gattii* species complex have a strong environmental association with decaying wood, and eucalypt tree hollows in Australia present an ideal environment for *C. gattii* VGI. Host mucosal colonisation and infection by the *C. gattii* species complex occurs subsequent to environmental exposure, most likely after the inhalation of reproductive basidiospores. In Australia, koalas (*Phascolarctos cinereus*) are known to exhibit a comparatively high prevalence of clinical and subclinical disease and nasal colonisation caused by *C. gattii* VGI, and occasionally *C. gattii* VGII. This, combined with their close and regular contact with eucalypts, makes them an ideal naturally-occurring model and sentinel for this disease, as well as a biological sampler for environmental members of the *C. gattii* species complex. Despite this potential, very little is known about cryptococcosis in free-ranging koala populations. This thesis will study aspects of cryptococcosis in koalas and other Australian animals, including: the environmental associations of *Cryptococcus* spp., diagnosis and treatment, prevalence of disease and colonisation in free-ranging populations, host-pathogen-environment interactions, and molecular epidemiology.

Section 1, the introduction and literature review, contextualises this thesis in the current body of knowledge and outlines the aims and overarching hypothesis, that '*disease is a random outcome of heavy environmental exposure*'.

In **section 2**, the ecological associations of *Cryptococcus* spp. in Australia are explored at a microbiome level using a robust amplicon-based next generation sequencing technique. The fungal community, or mycobiome, of Australian tree hollows was characterised and results were compared to culture-dependent methods for the detection of *Cryptococcus* spp. in environmental samples. Statistical analyses were also performed to determine if relationships between *Cryptococcus* and other fungal taxa exist in these tree hollows. The mycobiome was found to be comprised predominantly of fungi associated with ligneous breakdown. This was supportive of the assumption that the fungal community in tree hollows would be skewed towards wood degradation. The findings from culture-dependent and culture-independent methods were reasonably consistent, but discrepancies emphasised the

complementarity of these techniques. Culture remains advantageous in many scenarios due to the ability to perform downstream high-resolution genotyping of isolates, and the confirmation that an infectious yeast or spore was present in the sample. No associations between *Cryptococcus* and other fungal taxa were identified amongst the tree hollows studied, although some patterns of difference in relative abundance indicate that other relationships could be at play and further studies are warranted. This section also expanded the known environmental niches of the *C. gattii* species complex in Australia, with a further five tree species associations described.

Section 3 presents three publications that address clinical aspects of cryptococcosis in koalas, including its diagnosis and treatment, and the prevalence of subclinical disease and nasal colonisation in a free-ranging population. Firstly, **section 3.1** describes and characterises two unusual cases of cryptococcosis in related free-ranging koalas (a mother and joey) and discusses the pathogenesis, diagnosis and treatment of the disease in this context. Importantly, these were also sentinel cases for the case cluster investigated in section 4.1. The likely underlying cause of these cases was common exposure to a heavy environmental source of infection, and the successful treatment of the subclinical case is the first ever reported. The treatment of cryptococcosis in koalas is also discussed in detail, and recommendations are made for therapeutic protocols and monitoring.

Section 3.2 compares two serological tests for cryptococcal antigen detection, the latex cryptococcal antigen agglutination test (LCAT) and a lateral flow immunoassay (LFA), in order to validate the latter for use in koalas, along with cats and dogs. The LFA was confirmed as a highly useful screening test for cryptococcosis in all three species, but its variable specificity and positive predictive values indicate that positive results should be followed up by other confirmatory testing, such as an LCAT or mycological culture. It is likely that the LFA is more sensitive to cryptococcal antigen in these species and is thus detecting very low levels of antigenaemia (possibly indicative of heavy nasal colonisation or early exposure) that are generally not clinically relevant to the animal. This is particularly problematic in the koala, and indeed the specificity was poorest in this species. Despite this, its newly confirmed utility as a screening test is an important finding for the diagnosis of this disease, as the LFA is advantageous over the LCAT in its shelf-stability, ease of use and low cost per test strip. This makes it desirable for use 'cage-side' in veterinary hospitals and in remote field locations.

Section 3.3 then applies the LFA to a group of free-ranging koalas, while also screening for nasal colonisation. This sub-population was investigated due to the recent finding of a koala mortality attributable to cryptococcal pneumonia. The LFA proved ideal in this scenario, as it could be used rapidly in the field and provided evidence for early exposure to environmental *Cryptococcus* spp. This study was the first to systematically characterise cryptococcosis in any free-ranging population of koalas and identified a relatively low prevalence of both antigenaemia and nasal colonisation. This suggests that the higher prevalence of these phenomena seen in some captive populations is a consequence of the artificial conditions in captivity, namely the amplification of environmental *Cryptococcus* spp. from the high-density of koalas. The systematic exploration of this population also allowed for statistical analyses into individual koala and environmental factors that could increase the likelihood of colonisation or antigenaemia. No significant relationships could be identified, suggesting that these are random outcomes of host-pathogen-environment interactions in this population. An obvious trend was observed, however, between the abundance of *Eucalyptus camaldulensis* in the proximate environment and antigenaemia, and this requires further study. A significant relationship was noted between nasal colonisation and antigenaemia, which is intuitive considering that mucosal colonisation is a likely prerequisite to tissue invasion.

In **section 4**, one publication and three manuscripts are presented to explore the molecular epidemiology of cryptococcosis in Australian animals. **Section 4.1** describes a spatial and temporal case cluster of cryptococcosis, caused by *C. gattii* VGI, in a group of free-ranging koalas. This was investigated through the collection of disease isolates and the culture of nasal swabs (collected from koalas in both the hotspot area for cases of disease and the broader region) and environmental samples (from trees in the hotspot area). This revealed that koalas in this localised area were significantly more likely to be colonised than koalas from the broader region, and that *C. gattii* VGI was easy to isolate from trees within the same area (although likely not at an unusually high environmental prevalence). Multi-locus sequence typing and phylogenetic analysis of the resulting disease, colonising and environmental isolates indicated that no single, emergent outbreak strain was implicated in this cluster. It did, however, offer great insights into host-pathogen-environment interactions through its suggestion that a distinct genetic difference between most disease and

environmental isolates exists in this scenario. Whether this difference is due to rarer environmental isolates (potentially of higher virulence) being more likely to cause disease, microevolution occurring after contact with the host (during nasal colonisation), or by chance remains undetermined. Whole genome sequencing would be needed to address this further. Another pertinent finding of this study was the large number of sequence types, and broad distribution across the phylogenetic tree, of nasal colonising isolates. This indicates that koalas are in fact excellent biological samplers for environmental *C. gatti* VGI in a naturally-occurring, free-ranging context.

In **sections 4.2 and 4.3**, cases of cryptococcosis caused by *C. gattii* VGI in a captive bird and a domestic ferret were investigated. Environmental point sources of infection are usually very difficult to determine in cases of cryptococcosis, but the highly limited and known range of domicile of the hosts in both scenarios permitted a thorough investigation. This involved the collection of disease isolates, nasal swabs (section 4.3 only) and environmental samples. In both scenarios, only one site cultured positive, and multi-locus sequence typing provided further support for these as point sources of infection (and nasal colonisation, in section 4.3). These sections emphasise that any host species can offer pertinent insights into the pathogenesis of cryptococcosis.

In **section 4.4**, another case cluster of cryptococcosis in koalas is investigated. In this instance, the cluster involved a group of captive animals (spread across several co-owned facilities within the same region) and *C. gattii* VGII as the main culprit. Similar methods were used, including culture of nasal swabs and environmental samples and multi-locus sequence typing of the resulting isolates. In this case, whole genome sequencing was also performed on a representative sample of isolates to provide higher-resolution genotyping, as most isolates proved to be of an identical multi-locus sequence type. Through this, it was determined that *C. gattii* VGII was introduced to these facilities in eastern Australia through the transfer of a koala from Western Australia, where VGII is endemic. The results also indicated that the transfer of koalas between the related facilities in eastern Australia had spread *C. gattii* VGII into new environments, and that this molecular type may even be able to outcompete the local population of *C. gattii* VGI in the environment. This study proved definitively that colonised koalas can transfer members of the *C. gattii* species complex into new environments. This finding, and confirmation that this ability extends to *C. gattii* VGII,

has implications for the management and prevention of cryptococcosis in koalas and of possible future outbreaks in Australia. This study also provided the first evidence that environmental decontamination may be valuable to the management of cryptococcosis in captive animal environments.

Section 5 then discusses the major findings of this thesis in the context of the aims and hypothesis and suggests future directions for research.

This thesis has provided a body of evidence largely in support of the central hypothesis, that '*disease is a random outcome of heavy environmental exposure*', while also indicating that this relationship is highly complex and needs to be explored further, particularly in natural environments. Risk factors involved in the exposure to environmental members of the *C. gattii* species complex and the development of disease have been elucidated, while important clinical methodologies in diagnosis and treatment have been validated and discussed. The importance of animals in studying this environmentally-acquired disease has been emphasised through the confirmation that the koala acts as both a biological sampler and dispersal mechanism for environmental *Cryptococcus* spp. in Australia. Koalas and the other animal species discussed in this thesis have great potential as sentinels and naturally-occurring models for improving our understanding of the pathogenesis of cryptococcosis.

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Publications

Schmertmann, L.J., Danesi, P., Monroy-Nieto, J., Bowers, J., Engelthaler, D.M., Malik, R., Meyer, W. & Krockenberger, M.B. 2019. Jet-setting koalas spread *Cryptococcus gattii* VGII in Australia. *mSphere*, 4, e00216-19.

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Schmertmann, L.J., Malik, R., Meyer, W. & Krockenberger, M.B. Molecular epidemiology of cryptococcosis. Australian and New Zealand College of Veterinary Scientists: Science Week (Pathobiology chapter), 5th-7th July 2018. Gold Coast, Queensland, Australia.

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Schmertmann, L.J., Malik, R., Meyer, W. & Krockenberger, M.B. Unusual presentation of cryptococcosis in koalas: a case cluster in Port Stephens, New South Wales. Australian and New Zealand Mycology Interest Group meeting, 22nd February 2017. Adelaide, South Australia, Australia.

Abbreviations

Abbreviation	Meaning
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immunodeficiency syndrome
AMB	Amphotericin B
AT	Allele type
CFU	Colony-forming unit
CGB	L-canavanine glycine bromythymol blue
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
CYP	Cytochrome P450 monooxygenase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FeLV	Feline leukaemia virus
FFPE	Formalin-fixed paraffin-embedded
FIV	Feline immunodeficiency virus
GA	General anaesthesia
GXM	Glucuronoxylomannan
HIV	Human immunodeficiency virus
ITS	Internal transcribed spacer
KoRV	Koala retrovirus
LCAT	Latex cryptococcal antigen agglutination test
LFA	Lateral flow cryptococcal antigen immunoassay
MALDI-TOF	Matrix-assisted laser desorption ionisation time-of-flight
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MRI	Magnetic resonance imaging
NGS	Next generation sequencing
NSW	New South Wales
NT	Northern Territory
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
QLD	Queensland
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
ST	Sequence type
VIC	Victoria
WA	Western Australia
WGS	Whole genome sequencing
ZOTU	Zero-radius operational taxonomic unit

1. Introduction and literature review

The *Cryptococcus gattii* species complex comprises a group of saprophytic, environmental yeasts of the phylum Basidiomycota that occupy an environmental niche of decaying organic matter, most commonly in trees. The closely related *C. neoformans* species complex also comprises environmental basidiomycetous yeasts and occupies a similar ecological niche. Members of both species complexes can cause the severe mycotic disease cryptococcosis across a wide range of host species. The epidemiology of cryptococcosis caused by the *C. gattii* species complex began to be differentiated from the *C. neoformans* species complex following the HIV/AIDS outbreak, as it became apparent that disease caused by the *C. gattii* species complex was not strongly associated with severe immunosuppression in people, in contrast to disease caused by the *C. neoformans* species complex (Chen et al., 2014, Maziarz & Perfect, 2016). Both species complexes are primarily considered environmental organisms that opportunistically cause disease, possibly after exposure to a large inoculum and/or a particularly virulent strain.

Animals are assumed to be at the front line of exposure to *Cryptococcus* spp. in the environment, thus the role that domestic, captive and free-ranging animals can play as both sentinels and naturally-occurring models for disease caused by the *C. gattii* species complex should not be underestimated (Craig et al., 2002, Malik et al., 2011). In Australia, a wide variety of domestic animals and free-ranging or captive native species are known to be susceptible to cryptococcosis (Krockenberger et al., 2005, Malik et al., 2011). In particular, the koala (*Phascolarctos cinereus*) presents an interesting case study, as it exhibits a seemingly higher prevalence of disease than any other species and is readily exposed to one of the predominant environmental niches of the *C. gattii* molecular type VGI in Australia – eucalypt trees (Krockenberger et al., 2003). Therefore, this thesis focuses largely on koalas as a naturally-occurring model for cryptococcosis. This introduction will outline and review the *C. gattii* species complex and cryptococcosis, with emphasis on Australia and animal disease, by following the basic format of pathogen, environment and disease in the host.

1.1 The pathogen

1.1.1 Taxonomy

Prior to 1970, all members of the *C. gattii* species complex were considered to be of a single species, *C. neoformans*. The early taxonomy of *C. neoformans* is convoluted and begins with the naming of the *Cryptococcus* genus by Kützing (1833), followed by the discovery of several similar yeast species classified under numerous genera in the following years, including one designated as *C. neoformans* (Vuillemin, 1901). Benham (1935) later determined that all belonged to the genus *Cryptococcus*. This eventually led to the proposal that all these species should fall under the name *C. neoformans* and that the disease they cause should be referred to as cryptococcosis (Benham, 1950).

Vanbreuseghem & Takashio (1970) created the first separation between the *C. gattii* and *C. neoformans* species complexes, introducing the term *C. neoformans* var. *gattii*, and the taxonomical history from this point onwards is presented in Table 1.1. Numerous taxonomical shifts occurred in the following decades, with *C. gattii* first appearing as a distinct species in 2002 (Kwon-Chung et al., 2002). The addition of the sexual teleomorph species, *Filobasidiella bacillispora* (Kwon-Chung, 1976b), persisted until January 2013, when the use of separate names for teleomorphs and anamorphs in fungal taxonomy was discontinued (Hawksworth, 2011). The taxonomy remained stable until the publication of a new proposal to divide *C. gattii* into five species (Hagen et al., 2015). This division was based on their separation into distinct genetic clades according to molecular typing techniques, namely amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) analysis (both methods are discussed further in section 1.1.5). The proposal by Hagen et al. (2015) was not universally accepted amongst the cryptococcal research community during discussion at the 10th International Conference on *Cryptococcus* and Cryptococcosis at Foz do Iguaçu, Brazil in March 2017. Due to concerns regarding ongoing taxonomic instability, the adoption of the term 'species complex' to encompass the *C. gattii* molecular types VGI, VGII, VGIII and VGIV was proposed as an interim solution (Kwon-Chung et al., 2017), and this nomenclature will therefore be used throughout this thesis.

TABLE 1.1 History of the taxonomy of the *C. gattii* species complex in chronological order, beginning from its first separation from *C. neoformans* in 1970.

Reference	Nomenclature
Vanbreuseghem & Takashio (1970)	*C. neoformans* var. *gattii*
Kwon-Chung (1976b)	*C. neoformans* var. *gattii* / *F. bacillispora*
Kwon-Chung et al. (1978)	*C. bacillisporus* / *F. bacillispora*
Kwon-Chung et al. (1982a)	*C. neoformans* var. *gattii* / *F. bacillispora*
Boekhout et al. (2001)	*C. bacillisporus* / *F. bacillispora*
Kwon-Chung et al. (2002)	*C. gattii* / *F. bacillispora*
Hagen et al. (2015)	*C. gattii* (VGI, AFLP4)
	C. deuterogattii (VGII, AFLP6)
	C. bacillisporus (VGIII, AFLP5)
	C. tetragattii (VGIV, AFLP7)
	C. decagattii (VGIV/VGIIIc, AFLP10)
Kwon-Chung et al. (2017)	*C. gattii* species complex (VGI-IV)

1.1.2 Geographical distribution

Members of the *C. gattii* species complex were classically considered tropical or sub-tropical pathogens, with some temperate distribution, including parts of Australasia (Kwon-Chung & Bennett, 1984). In the past 20 years, particularly following the ongoing North American Vancouver Island and Pacific Northwest *C. gattii* VGII outbreak (Craig et al., 2002, Hoang et al., 2004, Kidd et al., 2004), its association with temperate zones is unequivocal, and it is now considered to be widely distributed globally, albeit reasonably uncommon in some areas such as Europe (Baró et al., 1998, Chowdhary et al., 2012a, Hagen et al., 2012). Chen et al. (2014) performed a meta-analysis of *C. gattii* species complex strains from a multitude of studies worldwide to highlight the striking differences between the global distribution of certain *C.*

gattii molecular types, including a tendency for isolates from African studies to be predominantly *C. gattii* VGIV (a genotype very uncommon elsewhere in the world). In the Americas, VGII tends to dominate, while VGI is predominant in Asia, Australasia and Europe (Chen et al., 2014). These findings are outlined in Table 1.2.

TABLE 1.2 Geographical distribution of all isolations of the molecular types of the *C. gattii* species complex by continent, with molecular types ordered by their frequency of detection, based on meta-analysis data presented in two publications (Chen et al., 2014, Engelthaler & Meyer, 2017).

Continent		***C. gattii* molecular types**
Africa		VGIV, VGIII, VGII, VGI
Americas	South and Central	VGII, VGIII, VGI, VGIV
	North	VGII, VGIII, VGI, VGIV
Asia		VGI, VGII, VGIII, VGIV
Australasia		VGI, VGII, VGIII
Europe		VGI, VGII

Early speculation that the Vancouver Island outbreak may have arisen from Australia via the importation of eucalypts (particularly *Eucalyptus camaldulensis*) in the early 1900s was based on the genetic similarity between the comparatively low virulence Vancouver Island outbreak isolates and Australian isolates, with presumed *in situ* development of the hypervirulent outbreak strain (Fraser et al., 2005, Byrnes III et al., 2010). On the other hand, Ngamskulrungroj et al. (2009) noted that the greatest diversity of *C. gattii* species complex isolates globally was found in South America, and thus concluded that this was the most likely origin of the outbreak strains. This was further supported by phylogenetic and recombination analyses (Chowdhary et al., 2012b, Hagen et al., 2013). A theory of radiation from South America has progressively gained support, with slight shifts in the exact proposed area of origin (Souto et al., 2016) and observations that the patterns of global distribution could also be traced back to the supercontinent, Pangea, and continental drift (Casadevall et al., 2017, Engelthaler & Meyer, 2017).

In Australia, *C. gattii* VGI and VGII are the major molecular types (Sorrell et al., 1996a), with VGIII considered very rare (Firacative et al., 2016) and VGIV undetected. *C. gattii* VGI and VGII exhibit striking variation in their geographical distribution within Australia, with VGI predominant in eastern Australia, while VGII is comparatively more abundant in western and northern central Australia (Sorrell et al., 1996a, Chen et al., 1997a, Campbell et al., 2005, McGill et al., 2009). The Australian *C. gattii* VGII population exhibits relative clonality, with most strains found to be identical in one study using multi-locus sequence typing (MLST), grouping as type VGIIb (Carriconde et al., 2011). This same type was identified as one of the strains involved in the Vancouver Island outbreak (Kidd et al., 2004).

1.1.3 Virulence

The *C. gattii* and *C. neoformans* species complexes have many recognised and shared virulence traits which could also be considered advantageous for their environmental survival (Casadevall et al., 2003). The major traits are: thermotolerance, the polysaccharide capsule, intracellular survival, along with melanin, phospholipase and urease production.

There has been speculation that endothermy in mammals provided an evolutionary advantage over other non-mammalian species through natural resistance to most fungal diseases, with cryptococcosis providing one of relatively few exceptions (Casadevall, 2005). Most fungi are unable to grow at mammalian or avian physiological temperature, making the *C. gattii* and *C. neoformans* species complexes comparatively unusual in their ability to grow at up to 40°C, and thus capable of causing disease in these hosts (Casadevall, 2005, Perfect, 2006, Robert & Casadevall, 2009). This is an important quality for a mammalian pathogen to cause disease at central anatomic sites and would also allow the organism to survive and grow in the environment at high ambient temperatures.

The size of the polysaccharide capsule *in vitro* is considered the dominant virulence trait in the *C. neoformans* species complex (McClelland et al., 2006). In the *C. gattii* and *C. neoformans* species complexes, the capsule is comprised predominantly of glucuronoxylomannan (GXM) (Cherniak & Sundstrom, 1994) and contains antigenic structures that allow the designation of four serotypes and hybrids within the *C. neoformans*

(A and D) and *C. gattii* (B and C) species complexes (Wilson et al., 1968, Bennett et al., 1977), providing a historically useful means of differentiating between groups within the species complexes prior to the development of molecular typing techniques. The polysaccharide capsule likely provides protection from desiccation (Casadevall et al., 2018) and predation, e.g. by amoebae and paramecia, in the environment (Steenbergen et al., 2001, Frager et al., 2010, Casadevall et al., 2019). In the host, this also provides protection from phagocytosis by immune cells such as macrophages and components of the capsular antigen have been associated with immunomodulation (Vecchiarelli, 2000). If phagocytosed, the cryptococcal capsule can increase in size and provide further protection against oxidative stress and lysosomal breakdown, allowing for improved intracellular survival (Dykstra et al., 1977, Zaragoza et al., 2008). Vomocytosis, where live yeasts are expelled by macrophages, has been observed after intracellular survival likely due to protection from the capsule, and this is proposed as a 'Trojan horse' mechanism to facilitate penetration through the blood-brain barrier (Alvarez & Casadevall, 2006, Ma et al., 2006, Voelz et al., 2009). Exposure to mammalian or amoebae polar lipids, along with extracts from macrophages and mammalian serum, can also trigger capsule enlargement (Chrisman et al., 2011). Casadevall et al. (2018) reviewed the literature on the *C. neoformans* species complex capsule and noted that its size shows substantial variation both between strains and within the same strain, the latter in response to differences in culture media, *in vitro* versus *in vivo* isolates, or in disparate tissues during disease (lung versus brain).

The dark cell wall pigment present in the *C. gattii* and *C. neoformans* species complexes was first noted by Staib (1962) and later identified as melanin (Wang et al., 1995). Staib's differential media, *Guizotia abyssinica* seed extract agar (also known as niger seed agar or bird seed agar), remains a staple for the detection of *Cryptococcus* spp. from the environment (discussed further in section 1.2.3.1). Laccase is the enzyme responsible for melanin production in the *C. gattii* and *C. neoformans* species complexes (Williamson, 1994). Melanin has been shown to be a critical virulence factor in cryptococcal disease (Kwon-Chung et al., 1982b, Williamson, 1997). Its presence in cell walls is thought to protect cells from oxidative stress and harsh environmental conditions, such as ultraviolet radiation (Wang & Casadevall, 1994, Williamson, 1997, Casadevall et al., 2003).

Phospholipase is considered a critical virulence factor due to its ability to breakdown host cell membranes and therefore facilitate the penetration of yeasts through tissues, such as from an alveolus into the bloodstream (Chen et al., 1997b). Mutations to the phospholipase B (*PLB1*) gene lead to decreased virulence (Cox et al., 2001). Speculations regarding the benefits of phospholipase for the environmental survival of *Cryptococcus* spp. centre around its potential as a protectant against phagocytosis by amoebae and its use for nutritional purposes (Casadevall et al., 2003).

Urease production is an important virulence trait of the *C. gattii* and *C. neoformans* species complexes and provides a useful means to distinguish them from other fungal species (Cox et al., 2000, de Hoog et al., 2000). Urease causes alkalinisation, which could aid intracellular survival after phagocytosis, and may also play a role in the pathogenesis of central nervous system (CNS) invasion by permitting dissemination in macrophages (Olszewski et al., 2004, Fu et al., 2018).

Cryptococcal cell body gigantism, also referred to as giant or 'titan' cells, has been observed for some time in pulmonary cryptococcosis (Okagaki et al., 2010, Zaragoza et al., 2010). This adaptive change with polyploidy can impair phagocytosis and enhance dissemination (Crabtree et al., 2012, Okagaki & Nielsen, 2012). Most studies have centred around this phenomenon in the *C. neoformans* species complex, but there is some evidence that gigantism occurs also in the *C. gattii* species complex (Firacative et al., 2014).

Within the *C. gattii* species complex, variations in virulence have been observed between different molecular types. *C. gattii* VGII generally exhibits greater virulence than VGI; within VGII, VGIIa is considered more virulent than VGIIb (Fraser et al., 2005, D'Souza et al., 2011). However, this pattern may not extend to disease outcomes observed in patients (Galanis & MacDougall, 2010). A similar phenomenon was observed within VGIII (Byrnes III et al., 2011). Fernandes et al. (2016) also observed differences in thermotolerance and capsule size between different *C. gattii* molecular types. Other studies, however, have suggested that all *C. gattii* molecular types can exhibit a range of virulence (Firacative et al., 2014). A review by Chen et al. (2014) noted numerous inconsistencies in the published data, suggesting that such broad conclusions regarding virulence in relation to certain molecular types may be premature.

1.1.4 Reproduction

Broadly speaking, members of the *C. gattii* species complex reproduce by either asexual budding, haploid fruiting or sexual reproduction. Haploid fruiting involves a switch from asexual budding to the production of hyphae and basidiospores (the likely infectious propagule) and has been observed in mating type α and **a** isolates (designated by the mating type [*MAT*] locus) (Wickes et al., 1996, Tscharke et al., 2003, Hsueh et al., 2011). Sexual reproduction requires the fusion of cells, also leading to the production of hyphae and basidiospores, and was originally thought to occur as part of a bipolar mating system between α and **a** mating types (Kwon-Chung, 1976a, Hull & Heitman, 2002, Lengeler et al., 2002, Hsueh et al., 2011). Sexual reproduction has been induced under laboratory conditions but is yet to be observed in the environment (Kwon-Chung, 1975, Kwon-Chung & Bennett, 1978), although circumstantial evidence of environmental recombination indicates that it does occur (Saul et al., 2008). There is some evidence that fertility can also vary within molecular types of the *C. gattii* species complex (Ngamskulrungroj et al., 2008).

Most clinical and environmental isolates globally were classically found to be mating type α (Kwon-Chung & Bennett, 1978), although mating type **a** is found at greater frequency in South America (Escandón et al., 2006) and appears to be more common amongst environmental isolates than clinical isolates (Hsueh et al., 2011). In Australia, while most isolates are also mating type α, significant environmental populations of mating type **a** have also been found (Halliday et al., 1999, Saul et al., 2008). The preponderance of mating type α isolates globally led to the discovery of same-sex mating as a means of reproduction and recombination and the proposal that this mechanism played a role in the Vancouver Island outbreak (Fraser et al., 2005, Lin et al., 2005).

1.1.5 Genotyping

A wide variety of methods have been used over the past few decades to make genotypic distinctions between strains of the *C. gattii* species complex, allowing for separation into molecular types. Genotyping also allows for molecular epidemiological analyses of isolates in a global or local context. The genomes of the *C. gattii* and *C. neoformans* species complexes

separate into largely stable groups, which have remained consistent even with the introduction of increasingly discriminatory genotyping techniques.

Genotyping of the *C. gattii* and *C. neoformans* species complexes began with PCR fingerprinting, a relatively simple method involving the use of primers, such as M13 (derived from the wild-type phage M13) and simple repetitive oligonucleotides, such as $(GACA)_4$ and $(GTG)_5$, to generate amplifications of highly mutable and repetitive DNA sequences (Sidrim et al., 2010). The products of this amplification then undergo separation and visualisation by gel electrophoresis, and the patterns generated are distinct between the major molecular types of the *C. gattii* and *C. neoformans* species complexes (Meyer et al., 1993, Meyer & Mitchell, 1995, Meyer et al., 1999). Random amplification of polymorphic DNA (RAPD) analysis provided another early method of distinguishing between molecular types of the *C. gattii* species complex (Haynes et al., 1995, Sorrell et al., 1996b, Meyer et al., 1999). The disadvantages of both PCR fingerprinting and RAPD analysis primarily centre on their poor reproducibility between different laboratories (Sidrim et al., 2010).

AFLP analysis provides a higher resolution and increased specificity in detecting molecular types, separating the *C. gattii* and *C. neoformans* species complexes into 11 distinct clades (AFLP1 - AFLP11). These clades correspond with the eight molecular types (VGI – VGIV, VNI – VNIV) and allow for the identification of several inter-variety and interspecies hybrids (Boekhout et al., 2001, Hagen et al., 2010). AFLP provides a reliable and reproducible method of differentiating between molecular types and hybrids but involves numerous steps and reagents, thus increasing its complexity and cost (Sidrim et al., 2010).

RFLP analysis involves the double enzyme digestion of a PCR product. In the case of the *C. gattii* and *C. neoformans* species complexes, the *URA5* (orotidine monophosphate pyrophosphorylase) gene is most commonly used. Visualisation by gel electrophoresis against known standard strains then allows for the determination of the molecular type (Meyer et al., 2003). This method is again highly reproducible and reliable but is less sensitive than AFLP in the event of isolated mutations and can struggle to differentiate between some hybrids (Sidrim et al., 2010). Nevertheless, its simplicity, affordability and high reproducibility make it desirable as a reasonably fast and accessible method of determining molecular type.

A consensus MLST scheme for the *C. gattii* and *C. neoformans* species complexes was created by Meyer et al. (2009) involving the use of seven loci: six housekeeping genes *CAP59* (capsular-associated protein), *GPD1* (glyceraldehyde-3-phosphate dehydrogenase), *LAC1* (laccase), *PLB1*, *SOD1* (Cu, Zn superoxide dismutase) and *URA5*, three of which (*CAP59*, *LAC1* and *PLB1*) also code for virulence traits, along with the IGS1 region (ribosomal RNA intergenic spacer). A PCR amplification of each of these seven loci then undergoes sequencing of forward and reverse reads, which are then aligned and the contig sequence is assigned an allele type (AT) number using a consensus database, namely the Fungal MLST Database (http://mlst.mycologylab.org) (Meyer et al., 2009). The unique combination of AT numbers then allows for the designation of a sequence type (ST) number. Concatenated sequences can then be used for downstream phylogenetic analysis. MLST reliably distinguishes between the molecular types and allows for more fine-scale distinctions to be made between strains. MLST is widely used amongst the *C. gattii* species complex research community to characterise isolates, as it allows for direct comparisons at both a local and global level. Its major limitation is the potential for the highly conserved housekeeping genes to offer inadequate discriminatory power to distinguish between closely related strains (Sidrim et al., 2010).

The development of next generation sequencing (NGS) has made whole genome sequencing (WGS) increasingly available to researchers (Mardis, 2008). This has extended to the *C. gattii* species complex, with the first published study involving a comparison between the *C. gattii* VGI and VGII genomes (D'Souza et al., 2011). Gillece et al. (2011) expanded upon this, showing that WGS allowed for a higher discriminatory power between isolates than MLST, but also proving that the two methods were comparable in grouping isolates phylogenetically. WGS later provided both further evidence in support of the South American origins of the major Vancouver Island VGII outbreak strain, evidence of microevolution and further proof of the distinct molecular type lineages of the *C. gattii* species complex (Billmyre et al., 2014, Engelthaler et al., 2014, Farrer et al., 2015, Farrer et al., 2016). Another study showed that WGS could also be used for MLST analysis within the *C. neoformans* species complex (Chen et al., 2015). WGS is now a relatively standard tool in the cryptococcal research community, but its use remains somewhat hampered by the requirement for complex bioinformatics analyses and concerns regarding the standardisation of methods and appropriate storage and accessibility of large data sets (Farrer & Fisher, 2017).

1.2 The environment

1.2.1 Role and dispersal

As an environmentally-acquired pathogen and saprobe, understanding the role that members of the *C. gattii* species complex may play in the environment, along with the niche that they occupy, is critical to also understanding host exposure and eventual tissue invasion. Relatively little is known about the function of *Cryptococcus* spp. in the environment, but the strong association between members of the *C. gattii* species complex and decaying wood, including eucalypt tree hollows in Australia (Ellis & Pfeiffer, 1990), suggests that these yeasts may both contribute to this process while also using it as a source of nutrition and a growth substrate (Lazéra et al., 1998, Halliday & Carter, 2003). The detection of *C. gattii* VGI and VGII in air, soil, sea water and fresh water samples collected to investigate the Vancouver Island outbreak suggest that their environmental presence may be more complex than purely an association with trees and decaying wood (Kidd et al., 2007b).

A specific fungal microenvironment, or mycobiome, could play a role in allowing for the survival, growth and reproduction of environmental *Cryptococcus* spp., but there is no conclusive evidence yet in support of this theory. The isolation of the *C. gattii* species complex in insect frass (Kidd et al., 2003) could suggest that a relationship exists with mycoparasitic fungi of the same order as *Cryptococcus* spp. (Tremellales), such as *Tremella* spp. These fungi are also associated with wood detritus and insect frass and are capable of parasitising other wood-decaying fungi (Zugmaier et al., 1994, Findley et al., 2009). It appears likely that *Cryptococcus* spp. have diverged from a common ancestor with mycoparasitic fungi, but no longer exhibit this trait (Guého et al., 1993, Fell et al., 2000, Findley et al., 2009). In a mycobiome study of African trees, Vanhove et al. (2017) could not find any evidence of a relationship between the presence of *Cryptococcus* spp. and other fungi.

The environmental dispersal mechanisms of the *C. gattii* species complex, although presumably intended to allow the colonisation of new environments, are likely also critical in host exposure to these pathogens. Probably the most important dispersal mechanism is haploid fruiting, which allows for the production and dissemination of basidiospores in the absence of mating (Wickes et al., 1996, Tscharke et al., 2003). The *C. gattii* species complex

has also been detected in air samples collected under flowering *E. camaldulensis* trees, suggesting that flowering plants may present a method of dispersal (Ellis & Pfeiffer, 1990). Free-living birds could also play a role in the dispersal of members of the *C. neoformans* species complex, given their isolation from the cloacae of some birds of prey (Cafarchia et al., 2006b). Preliminary evidence of a similar phenomenon has been observed in the *C. gattii* species complex, with captive koalas exhibiting cryptococcal nasal or skin colonisation seemingly able to seed previously uncontaminated environments (Krockenberger et al., 2002b). One study offered strong evidence of anthropogenic environmental disturbances as a dispersal mechanism for *C. gattii* VGI and VGII, while also suggesting that domestic and wild animals could play a role (Kidd et al., 2007a). Water may also play a role in dispersal, with *C. gattii* VGII able to survive in sea water (at 4°C and room temperature) and distilled water (at room temperature) for a year (Kidd et al., 2007b). The polysaccharide capsule could provide critical buoyancy for this mode of dissemination (Vij et al., 2018).

1.2.2 Tree associations

Australia has long been central to our understanding of the environmental niche of the *C. gattii* species complex. Ellis & Pfeiffer (1990) described the first environmental isolation of the *C. gattii* species complex from *E. camaldulensis* trees in South Australia, and shortly thereafter also provided the first isolation from the same tree species in North America (Pfeiffer & Ellis, 1991). The same phenomenon was later observed in *E. camaldulensis* trees in a wide variety of locations worldwide, including India (Chakrabarti et al., 1997), Mexico (Argüero Licea et al., 1999), Egypt (Mahmoud, 1999), Brazil (Montenegro & Paula, 2000) and Colombia (Granados & Castañeda, 2005). These studies solidified the early opinion that the *C. gattii* species complex had a strong association with *E. camaldulensis*, and that its global presence is related to the exportation of eucalypts (later deemed unlikely, as discussed in section 1.1.2).

In Australia, the list of trees from which *C. gattii* VGI has been isolated now includes: *E. camaldulensis* (Ellis & Pfeiffer, 1990); *E. tereticornis* (Pfeiffer & Ellis, 1992); *E. bella* (Sorrell et al., 1996a); *E. blakelyi*, *E. gomphocephala* and *E. rudis* (Pfeiffer & Ellis, 1997); *Angophora costata* and *E. grandis* (Halliday et al., 1999); and *E. microcorys* and *Syncarpia glomulifera*

(Krockenberger et al., 2002b, Vilcins et al., 2002). This is further summarised in Table 2.1.1. It is now apparent that the *C. gattii* species complex, particularly VGI, can be associated with an expanding range of Australian tree species, although the strong association with *E. camaldulensis* and *E. tereticornis* (with mature trees frequently heavily colonised) appears to remain. It can be speculated that this occurs due to the propensity for these two species to drop branches and thus generate large hollows (Brooker & Kleinig, 2006) in which *C. gattii* VGI can thrive, due to the abundance of decaying wood substrate and protection from environmental conditions (Lazéra et al., 2000, Saul, 2009).

The Australian environmental niche of VGII remains largely cryptic (Chen et al., 1997a), with its first published environmental isolation in Australia from plant material in a sheep paddock in Western Australia (WA) (although it is unclear if this isolate was due to direct contamination from diseased sheep) (Sorrell et al., 1996a). Kidd et al. (2003) later detected *C. gattii* VGII in insect frass attached to an *E. tereticornis* tree in New South Wales (NSW) and referenced unpublished findings of prior VGII detections in *E. camaldulensis, E. miniata* and *E. tetrodonta.* This was later expanded to include isolations from heavily colonised *E. miniata, E. tetrodonta, Erythrophleum chlorostachys* and *Corymbia bella* trees in the Northern Territory (NT), but overall recovery rates from these tree species were low (Saul, 2009).

Globally, environmental isolates of the *C. gattii* species complex have been detected in a wide variety of trees including (but not limited to) Australian eucalypts. Examples of non-eucalypt tree associations outside of Australia include (with the molecular type noted, if reported): *Acacia* spp. in Argentina (VGI) (Refojo et al., 2009), Colombia (Granados & Castañeda, 2005) and India (VGI) (Randhawa et al., 2008); *Cassia grandis* in Brazil (Lazéra et al., 2000); *Ceratonia siliqua* in Spain (VGI) (Colom et al., 2012); *Ficus* spp. in Brazil (Lazéra et al., 2000); *Moquilea tomentosa* in Brazil (Lazéra et al., 1998); *Pinus* spp. in Colombia (Granados & Castañeda, 2005) and North America (VGII, VGIII) (Kidd et al., 2007b, Springer et al., 2014) *Pseudotsuga* spp. in Canada (VGII) (Kidd et al., 2004); *Syzygium cumini* in India (Randhawa et al., 2003) and *Terminalia catappa* in Colombia (Callejas et al., 1998). Soil associated with the base of trees (in the root zone) has also proven to be an environmental source of the *C. gattii* species complex, for example with *Pseudotsuga menziesii* var. *menziesii* (VGII) (Kidd et al., 2007b), *Liquidambar styraciflua* and *Pinus canariensis* trees (VGIII) (Springer et al., 2014) in parts of North America and *S. cumini* in India (VGI) (Randhawa et al., 2008).

1.2.3 Methods of detection and isolation

1.2.3.1 Culture-dependent

The primary method of the detection and isolation of members of the *C. gattii* species complex from environmental samples is by culture using differential and/or selective media. The classic media for this is Staib's bird seed agar (as mentioned in section 1.1.3), which incorporates a *G. abyssinica* seed extract that provides substrate for the production of melanin (Staib, 1962). Consequently, yeast colonies of the *C. gattii* and *C. neoformans* species complexes (along with some other *Cryptococcus* spp.) can be identified by the brown-colour-effect (Staib et al., 1987). Antibiotics were later introduced to bird seed agar to restrict bacterial growth (Shields & Ajello, 1966). Hopfer & Blank (1975) later confirmed that the need for the time-consuming extraction process from *G. abyssinica* seeds could be bypassed through the use of pure caffeic acid in the media. The presence of fast-growing filamentous fungi in environmental samples remains an ongoing problem for the isolation of *Cryptococcus* spp., as these fungi often overgrow the culture plates and likely out-compete the slower-growing yeasts. The addition of other compounds to bird seed agar may aid in inhibiting the growth of filamentous fungi (Pham et al., 2014, de Matos Castro e Silva et al., 2015). Nonetheless, bird seed agar remains the staple for environmental isolations of *Cryptococcus* spp. (Alvarez et al., 2013).

Once suspect cryptococcal growth is identified on a primary isolation plate, individual colonies then typically undergo further isolation on standard mycological media, such as Sabouraud's dextrose agar (SDA). At this stage, utilising the thermotolerance trait of the *C. gattii* species complex (by incubating plates at 37°C) can help to further rule out the accidental isolation of some other yeast species. Once a pure single colony isolate is obtained, DNA can be extracted, and genotyping techniques (as discussed in section 1.1.5) or barcoding (Schoch et al., 2012) (see 1.2.3.2) can subsequently be used to confirm the species and/or molecular type of the isolate. Classic biochemical and physiological methods of differentiation can also still be used to confirm that an isolate is a member of the *C. gattii* and *C. neoformans* species complexes, such as positive hydrolysis of urea, negative sugar fermentation, positive assimilation of most sugars and negative assimilation of nitrate (Ellis et al., 2007a). L-canavanine glycine bromothymol blue (CGB) agar can be used to differentiate between the *C.*

gattii and *C. neoformans* species complexes, with growth of the *C. gattii* species complex producing a blue colour change (indicating the assimilation of glycine). The *C. neoformans* species complex does not grow on CGB agar, and thus fails to cause a colour change (Kwon-Chung et al., 1982a). Serotyping can also distinguish between the two species complexes, and somewhat within them (Wilson et al., 1968, Meyer et al., 1999, Meyer et al., 2003), although the commercial kit for this test was discontinued and PCR is now widely used to determine the serotype when needed (Ito-Kuwa et al., 2007).

1.2.3.2 Culture-independent

Barcoding of fungi typically involves sequencing a PCR product of the internal transcribed spacer (ITS) region to distinguish between different species (Schoch et al., 2012). Amplicon-based metagenomics combines NGS technology with fungal barcoding of DNA directly extracted from samples, and thus allows for the culture-independent identification of fungi in environmental or clinical samples (Riesenfeld et al., 2004, Lindahl et al., 2013). This method does not rely on the visual identification of cryptococcal growth, is not affected by the presence of fast-growing filamentous fungi and allows for species to be considered within the context of the entire mycobiome (Lindahl et al., 2013). Its limitations include its need for high quality reference databases and complex bioinformatics analyses, questionable biological relevance (the presence of DNA does not necessarily equate to a live, infectious pathogen), unknown potential for PCR bias, and limited size of the amplicon (which can prevent more in-depth characterisation) (Santamaria et al., 2012, Kõljalg et al., 2013, Lindahl et al., 2013, Tedersoo et al., 2014, Nguyen et al., 2015a). This method has not yet been compared to culture-based methods as a means of detecting environmental *Cryptococcus* spp., but it has been known to provide discrepant, though perhaps complementary, results when compared to culture in other studies of environmental fungi (Romao et al., 2017).

1.3 Cryptococcosis - the disease

1.3.1 Host-pathogen-environment interactions

Exposure to members of the *C. gattii* species complex usually occurs due to their inhalation from the environment. The infectious propagule is not yet clearly defined but considered most likely to be basidiospores or poorly-encapsulated yeasts (Velagapudi et al., 2009, Maziarz & Perfect, 2016). Inhalation then results in the deposition of the propagules into the upper and/or lower respiratory tract (likely dependent on the host species) at which point they may be cleared by the host or go on to colonise the respiratory mucosa and/or invade tissue to cause subclinical or clinical disease. The outcome likely depends on the size of the inoculum, the individual strain/s, and the anatomy and susceptibility of the host (Krockenberger et al., 2010, Malik et al., 2011, Maziarz & Perfect, 2016). Prolonged exposure to an environment with a high environmental burden of *Cryptococcus* spp. is likely a risk factor in the development of colonisation and subclinical disease, as demonstrated in koalas (Krockenberger et al., 2002a), and will be discussed in more detail in section 1.3.4.1. In a rat model of cryptococcosis caused by the *C. gattii* species complex (using an intratracheal inoculation route), the infective dose was found to be a critical factor in pathogenesis, with doses below a certain threshold not initiating disease (Krockenberger et al., 2010).

After initial tissue invasion, eventual penetration into the bloodstream or lymphatics can lead to widespread haematogenous dissemination or spread to regional lymph nodes, respectively. At this stage, CNS disease often occurs, as the fungus appears to have a propensity to disseminate to this anatomical site (Chen et al., 2014, Maziarz & Perfect, 2016). Disseminated lesions are observed in a wide variety of other anatomical sites, but these are often smaller and are usually detected incidentally (Krockenberger et al., 2003). In animals, direct penetration of an invasive upper respiratory tract lesion through the cribriform plate of the ethmoid bone is also suspected as a means of initiating CNS cryptococcosis (Malik et al., 2011).

Consequently, the classic presenting signs of cryptococcosis tend to be indicative of respiratory or CNS disease, although this varies between different host species and possibly different genotypes or routes of infection. Although most cases of cryptococcosis due to the

C. gattii species complex in humans manifest as meningitis (Chen et al., 2014, Maziarz & Perfect, 2016), lung disease was the predominant manifestation of human disease in the Vancouver Island *C. gattii* VGII outbreak (Harris et al., 2011). A similar phenomenon was observed in humans with VGII disease in the NT, Australia (Jenney et al., 2004). Disease presentation and the primary site of lesions also varies between host species, for example, with domestic cats primarily presenting with upper respiratory tract disease and koalas exhibiting both upper and lower respiratory disease in relatively equal proportions (Krockenberger et al., 2003, Malik et al., 2011, Pennisi et al., 2013). This suggests that other factors, likely including host anatomy, local and systemic immunity and strain differences, are at play in the development of disease.

Direct inoculation has also been suggested as a route of infection in cases of primary cutaneous cryptococcosis where no other lesions can be detected (Christianson et al., 2003). Leão et al. (2011) offered a pertinent example of a patient with cutaneous cryptococcosis caused by *C. gattii* VGII, likely subsequent to traumatic injuries from barbed wire. Primary cutaneous disease is observed in some animal cases of cryptococcosis, including cats (Pennisi et al., 2013), birds (Malik et al., 2003) and koalas (Krockenberger et al., 2003). An increasing number of case reports of primary abdominal cryptococcosis in dogs (Tangeman et al., 2015) has led to the consideration of ingestion as another possible route of infection, while inoculation via the teat canal is the likely cause of cryptococcal mastitis seen in dairies (Pal & Mehrotra, 1983).

Zoonotic transmission of both the *C. gattii* and *C. neoformans* species complexes has been reported, but the supportive evidence for this phenomenon is weak. Two cases of cryptococcosis due to the *C. neoformans* species complex were purported zoonotic transmission events, with the human patients presumptively infected through exposure to the droppings of apparently healthy pet birds (Nosanchuk et al., 2000, Lagrou et al., 2005). Tsunemi et al. (2001) speculated that a human patient became infected with the *C. gattii* species complex after contact with koalas in Australia. None of these reports are supported by in-depth genotyping, and it appears more likely that both the humans and animals were in contact with a common environmental source of *Cryptococcus* spp.

1.3.1.1 Incubation period

The incubation period of cryptococcosis caused by the *C. gattii* species complex, or the time between first contact with a host and expression of clinical signs, is usually very difficult to determine, as the exact time of exposure to an environmental source is often impossible to determine. Travellers returning from areas associated with the Vancouver Island outbreak in North America that later developed disease caused by an outbreak strain (*C. gattii* VGIIa) provided a unique opportunity to study this, and an incubation period ranging from 2 to 11 months (with a median of 6 to 7 months) was established (MacDougall & Fyfe, 2006). Cases of cryptococcosis attributed to the *C. gattii* species complex were identified in Europe that likely exhibited several year incubation periods, or a state of dormancy, before clinical disease developed (Hagen et al., 2012). Other considerations of the incubation period are typically confined to individual case reports and are usually speculative, but nonetheless it is likely that it can vary greatly beyond the findings of MacDougall & Fyfe (2006). Some reports of cryptococcosis caused by the *C. gattii* species complex have speculated that incubation periods of less than one month (Tsunemi et al., 2001) or greater than seven years (Maccolini et al., 2017) are possible. An estimated three-year incubation period was recorded in one captive koala, based on its infection with a *C. gattii* VGII isolate and travel history from a VGII endemic area (WA to eastern Australia) (Saul, 2009).

The tendency for some hosts to exhibit sustained mucosal colonisation by members of the *C. gattii* species complex presents another difficulty, as the exact time of tissue invasion is problematic to determine. The koala is a pertinent example of this phenomenon (Connolly et al., 1999, Krockenberger et al., 2002a, Krockenberger et al., 2002b). This will be discussed further in sections 1.3.2 and 1.3.4.1.

1.3.2 Colonisation

Members of both the *C. gattii* and *C. neoformans* species complexes can colonise hosts, i.e. survive and/or proliferate on or in a host without causing clinical or subclinical disease. This typically occurs at the nasal mucosa (Malik et al., 1997b), but skin colonisation of koalas has also been documented (Krockenberger et al., 2002b) and lower airway colonisation is known

in humans (Maziarz & Perfect, 2016). The *C. neoformans* species complex may also be able to colonise the gastrointestinal tract of some species, with findings in birds of prey in Europe suggestive of this (Cafarchia et al., 2006b).

Numerous studies have documented cryptococcal nasal colonisation in cats, dogs, ferrets, humans, koalas and squirrels (Malik et al., 1997b, Connolly et al., 1999, Krockenberger et al., 2002b, Duncan et al., 2005b, Duncan et al., 2006, Morera et al., 2011, Kido et al., 2012, Danesi et al., 2014a). Attempts to resolve koala nasal colonisation by various forms of systemic and topical treatment have yielded inconclusive results (Kido et al., 2012). One study showed that groups of captive koalas living in heavily contaminated environments and exhibiting cryptococcal nasal colonisation were more likely to develop subclinical disease, but that progression to clinical disease was relatively rare (Krockenberger et al., 2002a). The point at which nasal colonisation can progress to subclinical, and perhaps clinical, disease remains unclear (Malik et al., 2011).

1.3.3 Subclinical disease

Subclinical cryptococcosis is primarily diagnosed by the detection of cryptococcal antigenaemia in asymptomatic patients. Human patients with HIV/AIDS and low CD4 T cell counts represent a cohort notoriously susceptible to *C. neoformans* species complex cryptococcal meningitis (Fries & Cox, 2011). Early screening can lead to the detection of circulating cryptococcal capsular antigen in asymptomatic human patients (Yuen et al., 1994, Feldmesser et al., 1996), with one study identifying this phenomenon three weeks prior to the onset of symptoms (French et al., 2002). The methods for such detection will be discussed later, in section 1.3.5.1. Cryptococcal antigen screening is now recommended for all at-risk HIV-positive patients (with low CD4 T cell counts) (World Health Organization, 2018). This is particularly important in areas with a high burden of cryptococcal meningitis, such as Sub-Saharan Africa (Rajasingham et al., 2012).

In animals, the primary example of subclinical cryptococcosis is the koala. Amongst some groups of captive koalas, over 50% of individuals can test positive for cryptococcal antigenaemia in the absence of clinical signs. Some of these koalas go on to develop clinical

disease while others can either remain persistently positive or the antigenaemia can resolve completely (Krockenberger et al., 2002a). This typically occurs in concert with a high prevalence of cryptococcal nasal colonisation and a heavy environmental presence of *Cryptococcus* spp. Although Krockenberger et al. (2002a) thoroughly explored this concept in captive koalas, only 14 free-ranging koalas were tested, and all were negative. A later study identified subclinical cryptococcosis in koalas at postmortem through the detection of small respiratory lesions (Krockenberger et al., 2003), while another koala with cryptococcosis was diagnosed incidentally while undergoing pre-shipment testing (Wynne et al., 2012). Cryptococcosis in koalas will be discussed further in section 1.3.4.1. Duncan et al. (2005b) identified that subclinical cryptococcosis can also affect cats and dogs. A follow-up study by the same authors found that, as in koalas, the antigenaemia either progressed to clinical disease, persisted or resolved spontaneously (Duncan et al., 2005a).

1.3.4 Range of host species

A broad range of host species, from protozoa and nematodes (Mylonakis et al., 2002, Malliaris et al., 2004) to humans and a wide variety of domestic and free-ranging animals, are susceptible to cryptococcosis (Malik et al., 2011, Chen et al., 2014). Some species of protozoa, invertebrates and rodents have also proven susceptible through their use in disease models, as reviewed by Sabiiti et al. (2012). More recently, zebra fish have also been used (Tenor et al., 2015). Rodents are also susceptible to naturally-occurring infection (Bauwens et al., 2004, Krockenberger et al., 2005, Payne et al., 2005, Singh et al., 2007). Cryptococcosis has also been documented in amphibians (Seixas et al., 2008), reptiles (McNamara et al., 1994, Hough, 1998) and birds (Malik et al., 2003). A huge number of eutherian mammalian species are susceptible to cryptococcosis, as reviewed thoroughly by Malik et al. (2011) and Singh et al. (2018). Reports of the disease in Australian marsupial species (particularly the koala) are also prevalent (Krockenberger et al., 2003, Krockenberger et al., 2005), and will be discussed further in section 1.3.4.1. In summary, this suggests that any host species could develop cryptococcosis, although some, such as domestic cats and koalas, appear comparatively more susceptible for reasons that are not well understood (Malik et al., 2011). Unlike in humans, where a link between *C. neoformans* species complex disease and immunocompromise is

well-described (Maziarz & Perfect, 2016), there is limited evidence that immunosuppression plays a role in animal cryptococcosis caused by the *C. gattii* or *C. neoformans* species complexes. Most studies have explored this issue indirectly looking for co-infection with feline immunodeficiency virus (FIV) or feline leukaemia virus (FeLV) in cats. A number of large studies have noted cryptococcosis in feline patients with FIV or FeLV co-infection but the prevalence has not been considered unusual and case outcomes were generally similar across all cats (Malik et al., 1992, O'Brien et al., 2004, O'Brien et al., 2006, Trivedi et al., 2011). A higher likelihood of treatment failure has been documented in FIV or FeLV-positive cats with cryptococcosis (Jacobs et al., 1997), however, and thus the relationship between cryptococcosis and immunosuppression in cats remains somewhat unclear. Therefore, the distinction between the *C. neoformans* species complex causing disease in the immunocompromised and the *C. gattii* species complex in the immunocompetent does not appear to apply to cats, as both species complexes are implicated across a range of patients (Malik et al., 1992, Malik et al., 2011, Trivedi et al., 2011). Indeed, this assumption is increasingly problematic in human patients (Lui et al., 2006, Maziarz & Perfect, 2016).

This thesis focuses on the koala, with two components encompassing avian and ferret cases. Thus, the literature regarding koala, avian and ferret cryptococcosis will be prioritised for further review.

1.3.4.1 Koalas and other Australian native mammals

Cryptococcosis in koalas was first reported by Backhouse & Bolliger (1960) and was quickly identified as an important infectious disease for this species (Backhouse & Bolliger, 1961, Bolliger & Finckh, 1962). Numerous case reports appeared in the literature over the following four decades (Gardiner & Nairn, 1964, Canfield et al., 1986, Canfield, 1987, Spencer et al., 1993, Malik et al., 1997a, Woods & Blyde, 1997, Makimura et al., 2002). Krockenberger et al. (2003) offered the first systematic insight into koala cryptococcosis, reviewing all 11 previously published cases and adding a further 32 to the literature. The major findings of this study were that the *C. gattii* species complex was responsible for all cases (in which a species identification could be obtained), respiratory disease (particularly pneumonia but often sinonasal disease, occasionally both) was present in over 70% of cases and CNS disease in

37%, while 30% of koalas exhibited lesions of widespread dissemination to various other anatomical sites. The authors also documented cases of localised disease, including two with only cutaneous lesions and one with only an inguinal lymph node apparently affected. Overall, Krockenberger et al. (2003) confirmed cryptococcosis as a significant but still uncommon infectious disease of koalas, but its prevalence, particularly amongst free-ranging koalas, remained undetermined. This was later explored in a mortality survey conducted as part of a BSc (vet) thesis by Stalder (2003). Overall, this indicated that approximately 3-4% of free-ranging koalas exhibited cryptococcal lesions at postmortem (Stalder, 2003, Krockenberger et al., 2005, Malik et al., 2011). Cases of cryptococcosis in captive koalas are periodically reported in the literature, particularly in animals in overseas zoological parks (Bercier et al., 2012, Wynne et al., 2012, Martínez-Nevado et al., 2017, Hanaki et al., 2018), and are often managed but not reported in Australia (M.B. Krockenberger, pers. comm.). *C. neoformans* var. *grubii* was implicated as the aetiological agent in one case (involving a captive koala in Spain) (Martínez-Nevado et al., 2017). All other reported cases in koalas have been attributed to the *C. gattii* species complex.

As previously mentioned, captive koalas frequently exhibit cryptococcal nasal and skin colonisation (Connolly et al., 1999, Krockenberger et al., 2002b, Kido et al., 2012). Critically, Krockenberger et al. (2002b) also noted that colonised captive koalas were able to both amplify the amount of environmental *Cryptococcus* spp. and seed it into previously uncontaminated environments, although this observation was not underpinned by in-depth molecular analysis. In the same study, it was hypothesised that the koala sinonasal cavity could act as an ideal biological sampler and offer insights into environmental members of the *C. gattii* species complex in Australia. Cryptococcal nasal colonisation was also found to extend to koalas held in captivity overseas (Kido et al., 2012). Contrary to the findings of Krockenberger et al. (2002b), where the majority of colonising isolates were members of the *C. gattii* species complex, Kido et al. (2012) detected mostly *C. neoformans* species complex isolates. Another Australian study also found a high proportion of *C. neoformans* species complex isolates (Connolly et al., 1999). Subclinical disease in captive koalas was defined by Krockenberger et al. (2002a) using latex cryptococcal antigen agglutination testing (LCAT), a serological diagnostic test that was successfully trialled by Malik et al. (1996b) in a few koalas and will be further described in section 1.3.5.1. A case of subclinical disease was hinted at in

a report by Makimura et al. (2002), where a koala exported to Japan later died of cryptococcosis that was presumably acquired in Australia, and another case was successfully identified through pre-shipment testing of a captive individual (Wynne et al., 2012). Despite attempts to detect cryptococcal colonisation (Krockenberger et al., 2002b) and asymptomatic antigenaemia (Krockenberger et al., 2002a) in small numbers of free-ranging koalas, this has not been reported to date and there have been no systematic studies of this issue. It therefore remains possible that both occur because of the artificial amplification of the *C. gattii* species complex in captivity, rather than occurring in nature.

The host-pathogen-environment interactions involved in the comparatively higher prevalence of cryptococcosis in koalas and the propensity for them to be infected or colonised by members of the *C. gattii* species complex remains unclear, but their close and frequent contact with eucalypts has long been speculated to play an important role (Krockenberger et al., 2003). *E. camaldulensis* and *E. tereticornis* are commonly used feed trees for koalas (Lee et al., 1988), and have strong associations with *C. gattii* VGI (Ellis & Pfeiffer, 1990, Pfeiffer & Ellis, 1992). Koalas are not generally considered to enter or use eucalypt hollows but there are occasional anecdotal reports, including two recent observations of this behaviour (both occurring in summer in the Liverpool Plains region of NSW) (J. Lemon, pers. comm.; A. Rus & A. Kan, pers. comm.). There are, however, no systematic studies on this behaviour and how often it occurs. The Liverpool Plains region suffered a notorious heatwave in which an estimated 25% of the koala population was killed (Lunney et al., 2012). With eucalypt tree hollows exhibiting relatively stable internal temperatures (Saul, 2009), it is reasonable to assume that koalas in this area could use tree hollows for shelter on extremely hot days.

Krockenberger et al. (2005) summarised cryptococcosis in other native Australian animals and suggested that it is much more sporadic than in the koala. Many other native Australian animals regularly use eucalypts and their hollows for nesting and shelter (Goldingay, 2009), and so it remains unclear as to why a similarly high prevalence of disease is not seen in these species. Brushtail possums *(Trichosurus vulpecula*) in the Sydney region were opportunistically tested for cryptococcal nasal colonisation and antigenaemia, and all were negative (M.B. Krockenberger, unpublished data). Many reports of cryptococcosis in Australian native wildlife remain unpublished (thus difficult to access), are not peer reviewed, have minimal case details and/or do not definitively identify the causative organism, making

systematic review difficult. Published reports encompass monotremes (Ladds, 2009), numbats (Gaynor et al., 1990), dasyurids (Holz, 2008), bandicoots and bilbies (Lynch, 2008), wombats (Reece & Hartley, 1994) possums and gliders (Booth, 1994, Reece & Hartley, 1994), macropods (Canfield & Hartley, 1992, Vaughan et al., 2007, Vogelnest & Portas, 2008) and rodents (Payne et al., 2005). Most of these reports concern captive animals, meaning that our understanding of the prevalence of cryptococcosis in free-ranging Australian wildlife is extremely limited.

There is no evidence that immunocompromise or co-infection plays a role in cryptococcosis in koalas (Krockenberger et al., 2003). Koala retrovirus (KoRV) co-infection could potentially predispose koalas to develop symptomatic cryptococcosis. There are many known variants of KoRV, with some (KoRV-A) considered endogenous and having no known immune impacts, while exogenous variants (such as KoRV-B) have been associated with alterations to the immune system, chlamydiosis and lymphoma/leukaemia (Xu et al., 2013, Maher & Higgins, 2016, Waugh et al., 2017). KoRV-A has been found in the genome of 100% of koalas in some areas of eastern Australia (Simmons et al., 2012), but KoRV-B is presumed to be at a lower prevalence (Waugh et al., 2017). The relationship between KoRV infection and cryptococcosis is unknown. Cryptococcosis and KoRV co-infection has been reported in one captive koala in Japan, but it is unclear which variant/s the authors tested for (Hanaki et al., 2018). There have been some studies into the major histocompatibility complex (MHC) in the koala, and a reasonable diversity of MHC II has been shown in many populations (Jobbins et al., 2012, Lau et al., 2014b). Some MHC II variants and other immune genes have been associated with the development of chlamydiosis (Lau et al., 2014a, Johnson et al., 2018). It is possible that similar factors could be involved in cryptococcosis in koalas, but this remains untested. Anatomy could also play a role in the propensity of koalas to develop colonisation and upper respiratory tract cryptococcosis. Hemsley et al. (2013) noted the presence of a large ventral conchal sinus that was deemed likely to greatly slow airflow through the nasal passages, potentially allowing more time for inhaled particles, such as cryptococcal basidiospores, to adhere to the mucosa. The same study also found that the complexity of the nasal turbinates was greatly reduced in koalas compared to most mammals, which could explain why koalas have an equal, if not greater, tendency to develop lower respiratory tract disease (Krockenberger et al., 2003), as less filtering of inspired gases might facilitate

deposition of infectious propagules in the lower airways. As an aside, the same phenomenon might explain how cetaceans develop cryptococcosis (Gales et al., 1985), potentially from low concentrations of *Cryptococcus* spp. in seawater (Kidd et al., 2007b) .

1.3.4.2 Birds

Avian cryptococcosis was first reported by Griner & Walch (1978) and sporadic case reports followed until all 11 published cases, and 15 new cases, were presented and thoroughly reviewed by Malik et al. (2003). Parrots accounted for most of the reports, with a few cases in Columbiformes, kiwis (*Apteryx mantelli*) and domestic poultry also noted. Most cases presented with upper respiratory tract disease, including lesions of the nasal cavity and sinuses. Invasive lesions that had extended from the respiratory tract into neighbouring tissues, such as the beak and subcutis of the infraorbital region, were also observed (Malik et al., 2003). Localised cutaneous lesions, pneumonia, air sac disease and lesions of dissemination in the brain, kidney, bone, along with other visceral organs were occasionally observed. Malik et al. (2003) also noted that most cases in Australia were attributable to the *C. gattii* species complex and speculated that cases caused by the *C. neoformans* species complex (mostly in North America and Europe) may have involved immunocompromised individuals. This was based on the observation that widespread dissemination to central anatomical sites mostly occurred in cases involving the *C. neoformans* species complex. The core body temperature of birds has generally been considered too high to allow for the growth of *Cryptococcus* spp., and this was also proposed as a reason for upper respiratory lesions at more peripheral sites being the primary presentation (Malik et al., 2003). A study of the *C. neoformans* species complex, however, found that it was able to proliferate at avian core body temperature, but that avian macrophages were capable of suppressing its growth, and this may explain the rarity of disseminated disease in birds (Johnston et al., 2016).

A handful of reports of avian cryptococcosis have followed since the 2003 review. Notably, an outbreak in psittacine birds at a Brazilian aviary caused by the *C. gattii* species complex was reported (Raso et al., 2004). Many of the birds had upper and lower respiratory tract disease, and dissemination to the viscera was common. The cause of the outbreak could not be identified. One case in a parrot (with a fluctuant cutaneous mass) was described as

part of the Vancouver Island outbreak investigation (Lester et al., 2004), while Krockenberger et al. (2005) mention a further five cases in Australian psittacine birds (with no case details provided). An unusual case of *C. gattii* species complex cryptococcosis occurred in a parrot where the primary and presenting lesion was a cryptococcal osteomyelitis of the humerus (Molter et al., 2014). The authors speculated that, as a pneumatic bone, infection could occur here directly via an aerosol, or haematogenously (cryptococcal lesions were also reported in the trachea). *C. gattii* VGIIa was the culprit in another case with classic upper respiratory tract disease and widespread disseminated lesions (Maccolini et al., 2017). These cases further reinforce the observations that cases are mostly seen in psittacine birds, upper respiratory tract lesions account for the majority of cases and that both the *C. gattii* and *C. neoformans* species complexes can be associated with disseminated disease (Malik et al., 2003, Raso et al., 2004, Maccolini et al., 2017).

Many species of native Australian parrots use eucalypt tree hollows for shelter and nesting (Goldingay, 2009), and this could present an exposure risk to *C. gattii* VGI (and possibly VGII, but its niche remains largely cryptic) for some avian species. Little is known about the prevalence of cryptococcosis in free-ranging birds in Australia, or indeed anywhere worldwide. Therefore, it can only be speculated that the hollow-dwelling tendencies of some parrots could play a role in infection.

1.3.4.3 Ferrets

Cryptococcosis in domestic ferrets (*Mustela putorius furo*) is reasonably well-documented but uncommon, with the first published report in 1954 (Skulski & Symmers, 1954) and with Malik et al. (2002), Morera et al. (2011) then Wyre et al. (2013) later collectively reviewing the 16 published cases. Two cases in WA were reported that were not included in these reviews (McGill et al. 2009), and Morera et al. (2014) later added another two cases to the literature. Ferrets are susceptible to members of both the *C. gattii* and *C. neoformans* species complexes, with the aetiological agent in the reported cases divided roughly equally between the two (Morera et al., 2011, Wyre et al., 2013). Sex differences have been noted amongst the reported cases, with more male ferrets (13) than females (3) affected (based on the publications where sex is reported) (Wyre et al., 2013). Nearly half of the published cases

have been reported in Australian ferrets (Lewington, 1982, Malik et al., 2000, Malik et al., 2002, McGill et al., 2009), with the rest divided amongst North America and Europe (Wyre et al., 2013), although this may simply reflect global patterns of ferret ownership and/or research groups with an interest in the disease in this host species.

Respiratory disease is the most common manifestation of cryptococcosis in ferrets, with both the upper and lower respiratory tracts affected. Nasal discharge, mass lesions or facial asymmetry, sneezing, coughing and dyspnoea are all common clinical signs of ferret cryptococcosis (Malik et al., 2002, Morera et al., 2011, Wyre et al., 2013). Neurological disease (Greenlee & Stephens, 1984) and lymphadenopathies (Malik et al., 2002, Hanley et al., 2006) have also been reported. Cases presenting with cutaneous (Malik et al., 2002), ocular (Morera et al., 2011, Ropstad et al., 2011), gastrointestinal (Hanley et al., 2006, Eshar et al., 2010) and musculoskeletal disease (Malik et al., 2002) also occur.

Immunosuppression and co-morbidities could play a role in some cases of ferret cryptococcosis, with one case having both a history of recent treatment with systemic corticosteroids and suffering from congestive heart failure (Greenlee & Stephens, 1984), one with lymphoma (Erdman et al., 1992) and another that tested positive for Aleutian virus (Morera et al., 2014). There is speculation that ferrets may be more susceptible to cryptococcosis than some other species based on their behavioural tendencies [often sniffing, digging and exploring in dirt and confined spaces (Boyce et al., 2001)] and propensity to develop various upper respiratory tract ailments, collectively known as ‘snuffles’, (Lewington, 2007, Johnson-Delaney & Orosz, 2011) which present co-morbidities that could affect their local and systemic immune responses.

1.3.5 Diagnosis

An early presumptive diagnosis of cryptococcosis is often obtained through the direct visualisation of suspect organisms (e.g. in cytological smears from needle aspirates, cerebrospinal fluid (CSF), nasal washings or bronchoalveolar lavage fluid specimens) in patients with consistent clinical signs, such as respiratory or neurological disease (Chen et al., 2014, Sykes & Malik, 2014, Maziarz & Perfect, 2016). Confirmation of the diagnosis can then

be achieved, and the possibility of invasive disease caused by other pathogenic yeasts ruled out, by specific serological antigen testing and/or mycological culture (Sykes & Malik, 2014, Maziarz & Perfect, 2016). In a veterinary context, the initial visualisation of organisms is typically achieved through the cytopathological evaluation of aspirates or smears taken from masses and/or exudates (using a rapid Romanowsky-type stain). Histopathology (with standard haematoxylin and eosin [H & E] staining, or variably fungal specific stains such as mucicarmine or periodic acid Schiff) is also often used, but has a longer turnaround time (Malik et al., 1992, Blanshard & Bodley, 2008, Pennisi et al., 2013, Sykes & Malik, 2014).

Culture of cryptococcal organisms directly from lesions, blood or CSF remains the gold standard for diagnosis as it allows for the definitive phenotypic and genotypic identification of the causative organism and permits downstream antifungal susceptibility testing (Chen et al., 2014, Sykes & Malik, 2014). Culture of *Cryptococcus* spp. can involve the use of differential and/or selective media such as bird seed agar (useful when sampling normally contaminated sites such as the nasal cavity) or CGB agar, but standard mycological media such as SDA are primarily used (as mentioned in section 1.2.3.1) (Kwon-Chung et al., 1982a, Staib et al., 1987). Culture also allows for other identification procedures to be performed, such as MALDI-TOF mass spectrometry (Firacative et al., 2012). Culture results must be interpreted in the context of the clinical signs and be supported by other diagnostic tests, as a positive culture could simply indicate mucosal colonisation in some samples (e.g. nasal swabs) (Malik et al., 1997b, Connolly et al., 1999, Maziarz & Perfect, 2016).

1.3.5.1 Serological testing

In animals, the LCAT remains one of the most frequently employed serological tests for cryptococcal antigen detection. This test uses latex particles coated with a monoclonal antibody that targets the capsular polysaccharide (GXM), resulting in a visible agglutination reaction in the presence of cryptococcal antigen (Merdian Bioscience, 2016). It is well-validated (particularly in humans, cats, dogs and koalas) and considered highly sensitive and specific for cryptococcosis (Temstet et al., 1992, Malik et al., 1996b). False results are largely avoided if pronase pre-treatment and heat inactivation steps are adhered to (Gray & Roberts, 1988, Malik et al., 1996b). The LCAT can also be used to determine the antigen titre, which

has proven to be a useful tool to monitor response to treatment in cats and dogs (Malik et al., 1996b), and can be used to detect subclinical disease in cats, dogs and koalas (Krockenberger et al., 2002a, Duncan et al., 2005b). The major limitations of this test include its requirement for an experienced technician and laboratory equipment, prolonged turnaround time, and the risk of false results due to cross-reactivity, contaminants or uncommon varieties of the *C. gattii* and *C. neoformans* species complexes (such as hybrids) (Heelan et al., 1991, Blevins et al., 1995, Wilson et al., 2011, Rickerts et al., 2015, Tone et al., 2016).

A lateral flow immunoassay (LFA) for cryptococcal capsular antigen, developed in 2010 (CrAg® LFA, IMMY, Norman, Oklahoma, USA), revolutionised the diagnosis of cryptococcosis in humans (particularly in the developing world) through its use as a simple, inexpensive point-of-care diagnostic test (Jarvis et al., 2011, Lindsley et al., 2011, Kozel & Bauman, 2012). This test incorporates two monoclonal antibodies (to cover all serotypes of the *C. gattii* and *C. neoformans* species complexes) that bind to cryptococcal GXM and are then wicked along the test strip to interact with immobilised antibodies on the test line. Here, the antibody-antigen complexes form a sandwich to produce a visible positive result (Kozel & Bauman, 2012, IMMY, 2016). Originally intended for use with serum, plasma and CSF samples, it has since also proven useful for whole blood and urine (Jarvis et al., 2011, Huang et al., 2015, Williams et al., 2015, Vidal et al., 2016, Brito-Santos et al., 2017). The test is shelf-stable, has a rapid turnaround time, requires minimal equipment and is simple to interpret (Kozel & Bauman, 2012). The LFA is proven to be more sensitive than the LCAT to lower levels of antigen both in clinical specimens and GXM-spiked saline (McMullan et al., 2012, Gates-Hollingsworth & Kozel, 2013, Boulware et al., 2014), which has led to its use as a screening test for asymptomatic cryptococcal antigenaemia in high-risk human patients (Rajasingham et al., 2012). It can detect all molecular types and hybrids of the *C. gattii* and *C. neoformans* species complexes (Kozel & Bauman, 2012, Gates-Hollingsworth & Kozel, 2013, Rickerts et al., 2015), and has relatively few false-positive (Rivet-Dañon et al., 2015, Dubbels et al., 2017) and false-negative results reported (all hypothesised to have been caused by the prozone, or 'high-dose hook' effect) (Lourens et al., 2014, Lee et al., 2018). The LFA has been validated by many clinical diagnostic laboratories around the world for use in humans (Jarvis et al., 2011, Lindsley et al., 2011, McMullan et al., 2012, Boulware et al., 2014, Huang et al., 2015). The requirement for using multiple test strips to determine a semi-quantitative antigen titre may

prove problematic in a veterinary context, where there are typically more cost constraints. Nevertheless, it shows great potential (particularly as a screening test) for use in veterinary hospitals, field research and diagnostic laboratories, but remains unvalidated in animals. Several similar immunochromatography methods have been developed more recently by other manufacturers, most notably the CryptoPS (BioSynex®, Illkirch-Graffenstaden, France) and the StrongStep® (Liming Bio, Nanjing, China). The CryptoPS showed excellent specificity in all sample types but variable sensitivity (particularly in serum), although it shows promise as a useful test for the screening of urine (Temfack et al., 2018). The StrongStep® exhibited good results in CSF samples, but its performance in plasma samples proved less than desirable (Mpoza et al., 2018). Further studies are required to determine the reliability and utility of these and other newly developed cryptococcal antigen assays (Rajasingham et al., 2019), particularly in animals.

1.3.5.2 Adjunct diagnostic tools

Diagnostic imaging can be an adjunct diagnostic tool, as although it cannot definitively confirm cryptococcosis, many techniques can help to identify and characterise the disease in the context of the patient's clinical signs (particularly in the event of mass lesions). Ultrasonography, radiography and endoscopy are often used in animals and although these methods can identify some mass lesions and accumulations of abnormal fluid, they do not provide a tissue for cytology, histology and/or culture, which is ultimately required to make a definitive diagnosis of cryptococcosis. Ultrasonography and endoscopy, however, also provide opportunities to accurately collect samples for further diagnostic testing, such as aspirates or punch biopsies (Sykes & Malik, 2014, Martínez-Nevado et al., 2017). Advanced cross-sectional imaging techniques, including computed tomography (CT) and magnetic resonance imaging (MRI), are frequently used in human patients thought to have cryptococcosis, and are often especially helpful for evaluating the nasal cavity, middle ear (CT) and CNS (MRI) (Chen et al., 2014, Maziarz & Perfect, 2016). There are many examples of the successful use of advanced imaging in referral veterinary settings (Woods & Blyde, 1997, Sykes et al., 2010, Kwiatkowska et al., 2011, Bercier et al., 2012, Siak et al., 2015, Martínez-Nevado et al., 2017), and although they are considered highly useful tools in the diagnosis of

cryptococcosis (Sykes & Malik, 2014), these technologies are not accessible to many general practice veterinarians and patients due to their high cost. General anaesthesia (GA) is usually required to perform CT scans or MRI studies in animals, which can introduce an added element of cost and risk to the investigation, for example GA may have exacerbated CNS disease in one koala (Martínez-Nevado et al., 2017).

CSF collection and analysis, although routinely performed in humans for antigen testing, culture and/or India ink visualisation of cryptococcal organisms (Chen et al., 2014, Maziarz & Perfect, 2016), is generally not recommended in animals unless other diagnostic tests have proven inconclusive, due to the risk of cerebellar tonsil herniation (Pennisi et al., 2013, Sykes & Malik, 2014). Nevertheless, there are many reports of its collection during the work-up of animal cases of cryptococcosis (Sykes et al., 2010, Kwiatkowska et al., 2011), but in two cases (both localised sinonasal disease in koalas) it yielded no evidence of cryptococcal disease (Woods & Blyde, 1997, Wynne et al., 2012). Sometimes CSF is collected from animals in which cryptococcosis was not initially considered as a differential diagnosis, and yet its proper analysis yields a definitive diagnosis of cryptococcosis; this happens most often in regions where the disease is uncommon (R. Malik, pers. comm.). CSF collection in companion animals is typically performed in a referral hospital setting under GA, and ideally MRI or CT is undertaken first to assess the risk for cerebellar tonsil herniation. Because of the high cost and risks associated with GA, advanced imaging and CSF collection, it is prudent to perform serological testing for cryptococcal antigen before embarking on these procedures (Sykes & Malik, 2014). Should the patient be antigen positive, the animal's owner and attending veterinarian can then determine whether the risk and expense of further investigation is justified.

In free-ranging animals, diagnoses are often made at postmortem examination, thus appropriate samples for antigen testing and mycological culture may not have been collected (especially when the ante-mortem diagnosis of cryptococcosis had not been suspected). In these cases, immunohistochemistry can be used to confirm which species complex is implicated in a suspect cryptococcal lesion observed during histopathological examination of tissues (Krockenberger et al., 2001, Krockenberger et al., 2003). The extraction of DNA from formalin-fixed paraffin-embedded (FFPE) tissues can also often allow for PCR amplification of the ITS region and barcoding analysis (Meason-Smith et al., 2017).

1.3.6 Treatment

In humans, the antifungal treatment guidelines for cryptococcosis are well-described and follow the steps of induction, consolidation and maintenance (or suppression) (Perfect et al., 2010, World Health Organization, 2018). For the induction phase, the recommended treatment is generally intravenous amphotericin B (AMB) and oral (or intravenous) flucytosine for a period of 2-6 weeks, with the exact time depending on the clinical status of the patient, the type of disease present and sometimes also the causative organism (Perfect et al., 2010, Chen et al., 2013). A two-week induction protocol is the current recommendation for HIV-infected patients, and this ideally involves a week of AMB and flucytosine combination therapy followed by a week of high-dose fluconazole (World Health Organization, 2018). The consolidation phase then typically involves a high dose of fluconazole given as a monotherapy for approximately eight weeks, ideally using therapeutic monitoring to ensure optimal blood levels are obtained (Perfect et al., 2010, World Health Organization, 2018). Fluconazole is continued in the maintenance phase, but the dose is roughly halved. This phase continues typically for 6-12 months (but can be much longer). Itraconazole (or another triazole) can be substituted for fluconazole if there is resistance or it is not well tolerated by the patient (Perfect et al., 2010, Mourad & Perfect, 2018, World Health Organization, 2018).

The treatment of cryptococcosis in animals should ideally approach these guidelines, but often this is not possible. For example, flucytosine can be difficult to source, cost-prohibitive, problematic to administer (due to 6-8 hourly dosing) and associated with drug reactions in some species (particularly dogs), making it uncommonly used (Sykes & Papich, 2014). The administration of intravenous AMB can also be difficult due to the requirement for prolonged hospitalisation, which can make the process cost-prohibitive and logistically difficult for some animals, such as koalas (and other wildlife species) or fractious cats and dogs (Malik et al., 1996a). The ideal protocol in all animal species involves the initial use of AMB, to a cumulative dose of approximately 20-40 mg/kg, and the addition of an oral triazole antifungal (most commonly fluconazole, but itraconazole is also often used).

After AMB treatment is ceased, triazole monotherapy should be continued until the animal shows full resolution of all clinical signs and until cryptococcal antigenaemia is no longer detectable. It is generally recommended that treatment continues at least one month

beyond a negative result on a serological cryptococcal antigen test, and that follow-up serological testing is performed to confirm that the animal remains antigen-negative. This can take months to years, and sometimes results in life-long treatment (Malik et al., 1996b, Blanshard & Bodley, 2008, Pennisi et al., 2013, Sykes & Malik, 2014). Decreases in sequential antigen titres tend to indicate a favourable response to treatment, although they do not always reflect the clinical status of the patient (with the fall in the LCAT typically lagging behind a favourable clinical response) (Malik et al., 1996b). In the event of a failure to respond to treatment (i.e. a lack of clinical response and/or persistence or increase from the baseline antigen titre), antifungal susceptibility testing and therapeutic drug concentration monitoring are recommended to determine if resistance or an ineffective dosing regimen are the underlying cause (Sykes & Malik, 2014).

In animals, the dose of the oral triazole is often not adjusted [as it is in humans from the consolidation to maintenance phase (Perfect et al., 2010, World Health Organization, 2018)] unless evidence from therapeutic drug level monitoring, antifungal susceptibility testing or the condition of the patient suggest that this is required (Sykes & Malik, 2014, Sykes & Papich, 2014). Antifungal drugs, adjunct therapies and the peculiarities of treatment in different animal species (focusing on koalas, birds and ferrets) will be discussed further in this section.

1.3.6.1 Azoles

Azole antifungals are fungistatic inhibitors of fungal membrane synthesis that remain a cornerstone of the treatment of cryptococcosis. Fluconazole and itraconazole are triazoles widely used in a variety of animal species, likely due to their affordability, ease of oral administration and larger scale reports of their successful use, particularly in cats and dogs (Malik et al., 1992, Medleau et al., 1995, O'Brien et al., 2006, Sykes & Malik, 2014, Sykes & Papich, 2014). Fluconazole exhibits greater CNS penetration than itraconazole (Felton et al., 2014) and is generally cheaper. The lower cost is attributable to reliable generic formulations being readily available, and the ability of compounding pharmacists to offer a consistent product formulated to the exact size of the patient and/or presented in a formulation that is palatable to animals. Fluconazole is generally exceptionally well tolerated and has few

reported adverse effects, resulting in it often being the first choice for treatment in all animal species, including birds (Sykes & Malik, 2014). Itraconazole has become increasingly popular due to more stable plasma levels at once daily dosing (Sykes & Malik, 2014), which is logistically preferable in many animal patients. Problems with the absorption of compounded formulations (Molter et al., 2014, Renschler et al., 2018) and fewer generic options (particularly in liquid form), however, often make this drug much more expensive than fluconazole. Although both fluconazole and itraconazole are usually well tolerated in animals, inappetence, anorexia and vomiting can occur with either, and it is recommended that liver function is monitored regularly during treatment due to the potential for hepatotoxicity (particularly during itraconazole therapy) (Sykes & Papich, 2014).

As mentioned previously, either drug may be selected initially and given in conjunction with AMB, particularly in cases of severe cryptococcosis (which could be defined as disease that is extensively invasive, disseminated and/or in which the CNS is involved), for an induction period (Sykes & Malik, 2014). Although not ideal, triazole monotherapy (with no AMB induction period) can also be suitable to treat some milder cases in humans and animals, particularly if subclinical or localised, minimally-invasive cryptococcosis is present (Sykes & Malik, 2014, Mourad & Perfect, 2018). For example, mild to moderate pulmonary disease (with no CNS disease) in humans can be treated with fluconazole monotherapy (Perfect et al., 2010). Likewise, uncomplicated cryptococcal rhinosinusitis in cats and koalas can often be successfully treated by long courses of high dose fluconazole, with therapeutic monitoring to ensure the serum fluconazole concentration is at least twice or even four-times the minimum inhibitory concentration (MIC) of the isolate (R. Malik, pers. comm.). Evidence in cats suggests that if presented in a timely manner for veterinary attention, many respond well to fluconazole or itraconazole alone (particularly if there is no dissemination or CNS involvement) (O'Brien et al., 2006).

Several other azole antifungals are occasionally used to treat cryptococcosis. Ketoconazole tends to be avoided due to an increased risk of side effects, such as hepatotoxicity (Sykes & Papich, 2014), but examples of its successful use have been reported, including one in a koala (Woods & Blyde, 1997). It was favoured by many Canadian veterinarians treating *C gattii* VGII cases seen as part of the Vancouver Island outbreak (Lester et al., 2004). Ketoconazole is no longer commercially available in oral formulations in

Australia, but it can be sourced by compounding pharmacists and it continues to be reported as a treatment option in cats (Pennisi et al., 2013). Other triazole antifungals, such as voriconazole and posaconazole, are expensive and have not been thoroughly validated in animals. Voriconazole has potential as a useful treatment option in dogs but has been associated with neurological abnormalities in cats (including ataxia and visual deficits), probably due to excessive blood concentrations as a result of not using therapeutic monitoring (Quimby et al., 2010, Sykes & Papich, 2014). Voriconazole might have a place in managing fluconazole and/or itraconazole resistance, as it has good penetration of the CNS (Felton et al., 2014). Posaconazole exhibits a broad spectrum of antifungal activity, is generally very well tolerated and is available in a palatable liquid formulation (suitable for cats and small dogs) (Sykes & Papich, 2014) or as a sustained release tablet (relatively cost effective for medium and large dogs) (R. Malik, pers. comm.). It remains much more expensive than fluconazole, however, and has found no special utility for treating human cryptococcosis (Mourad & Perfect, 2018). It shows some potential as a treatment for cryptococcosis in animals, but there is very limited data regarding its use (Sykes & Papich, 2014, Kendall & Papich, 2015, Gharibi et al., 2017).

1.3.6.2 Amphotericin B

AMB is a polyene antifungal, thought to be fungicidal at high doses, that binds irreversibly to sterols in the fungal cell membrane (thus creating channels and allowing for leakage of ions). It is the second pillar of treatment for cryptococcosis in animals (Malik et al., 1996a, Sykes & Papich, 2014). Although only detectable at low concentrations in the brain and CSF after intravenous administration, AMB still achieves clinical resolution in humans and animals with cryptococcal meningitis (O'Brien et al., 2006, Felton et al., 2014, Sykes & Malik, 2014, Sykes & Papich, 2014, Stott et al., 2018). It is available in several preparations, most notably as AMB deoxycholate or in lipid formulations (including liposomal and lipid complex). AMB deoxycholate is associated with higher risks of nephrotoxicity and other adverse effects compared to lipid formulations, and thus is usually not considered the gold standard for human patients but remains an important option in animals due to its utility in a subcutaneous administration protocol (discussed further in the following paragraph). AMB

deoxycholate is also typically more cost effective, which makes it an ideal choice in many veterinary settings and in the developing world (Sykes & Papich, 2014, Mourad & Perfect, 2018, World Health Organization, 2018). AMB deoxycholate is likely the most commonly used form in Australian veterinary hospitals (R. Malik, pers. comm.). Liposomal AMB is recommended for use in humans wherever possible due to a decreased risk of nephrotoxicity and other adverse effects (Mourad & Perfect, 2018), but it is rarely used in animals due to its cost. The most common lipid formulation used intravenously in animals is AMB lipid complex, largely because it is more cost effective that the liposomal form. The risk of adverse effects for AMB lipid complex typically falls between the deoxycholate and liposomal formulations (Sykes & Papich, 2014).

Addition of AMB to an azole treatment protocol is recommended in most cases of animal cryptococcosis where there is severe invasive disease, disseminated disease or CNS involvement (Sykes & Malik, 2014), but it also can be used for subclinical disease in koalas (Govendir et al., 2016), particularly if the antigen titre fails to change in response to protracted azole monotherapy (M.B. Krockenberger, pers. comm.). The cumulative dose requirement is not well characterised but is thought to range from approximately 20-40 mg/kg (Malik et al., 1996a, Pennisi et al., 2013), given over many months. Short breaks can be taken during therapy if there is any evidence of impaired kidney function, as any AMB-related nephrotoxicity is transient if detected promptly (Sykes & Papich, 2014). The risk of nephrotoxicity and its expense (primarily due to the need for slow intravenous administration using a fluid pump, and/or the higher cost of lipid formulations) present limitations to the use of AMB in animals. Malik et al. (1996a) circumvented some of these concerns by instead administering AMB deoxycholate subcutaneously (lipid formulations cannot be used this way). This involved the AMB being administered two to three times weekly (to cats and dogs with cryptococcosis) as a subcutaneous bolus in a 0.45% saline and 2.5% dextrose solution. The same technique has been adapted for koalas (Govendir et al., 2016).

1.3.6.3 Other treatments

Flucytosine presents a useful treatment alternative to azoles in cats but is generally not used in dogs due to an association with toxic epidermal necrolysis (Panciera & Bevier, 1987, Malik

et al., 1996c, Sykes & Malik, 2014). Monotherapy with flucytosine is not recommended because of the rapid emergence of mutational resistance during a course of therapy, but when given in concert with AMB there is synergy (Beggs, 1986, Schwarz et al., 2003). This combination is considered a highly effective treatment protocol and is the gold standard for human patients (Mourad & Perfect, 2018, World Health Organization, 2018). The various downsides of flucytosine therapy in animals (high cost, limited access, requirement for dosing 3-4 times a day and minor risks of bone marrow dyscrasias) mean that it is rarely used, mainly in cats with CNS cryptococcosis (Sykes & Papich, 2014). Terbinafine presents another treatment option for cryptococcosis and can lead to good outcomes in some animals, but its use is generally reserved for cases of azole resistance, or where other antifungal drugs have failed to resolve clinical signs (Pennisi et al., 2013, Sykes & Malik, 2014).

Symptomatic and supportive care is an important aspect of treatment, potentially including fluid therapy, nutritional support (e.g. assisted feeding), seizure management and oxygen supplementation (Sykes & Malik, 2014). Short term anti-inflammatory corticosteroid use has also been reported to improve outcomes for cats and dogs with CNS cryptococcosis during preliminary therapy (when the sudden release of fungal antigens can cause transient deterioration in neurological function) (Sykes et al., 2010). Surgical removal of accessible lesions or debulking (in conjunction with antifungal therapy) is also recommended in some patients (Malik et al., 2002, Malik et al., 2003, Bercier et al., 2012, Pennisi et al., 2013), but medical treatment alone is considered capable of resolving even large cryptococcal lesions (Sykes & Malik, 2014).

1.3.6.4 Koalas, birds and ferrets

Koalas with cryptococcosis present unique problems in relation to implementing practical therapy. Studies have shown that koalas require large doses of xenobiotics, including antifungal agents. For example, roughly double the dose of oral fluconazole (on a mg/kg basis, when compared to cats and dogs) is required for koalas to reach adequate plasma concentrations (Black et al., 2014, Govendir et al., 2016). Thus, extrapolating dose rates from companion animal species will lead to ineffective therapy (Govendir, 2018). This pattern of low bioavailability and/or high hepatic biotransformation for orally administered agents is not

unique to antifungals and is reflected amongst other drugs (xenobiotics) administered orally to koalas, including non-steroidal anti-inflammatories and antibiotics (Griffith et al., 2010, Kimble et al., 2013). The exact mechanism for this impaired bioavailability is unknown but may reflect poor absorption due to the huge amounts of particulate material in the koala gastrointestinal tract, which could potentially act as a 'lipid sink' (Blanshard & Bodley, 2008). This does not, however, explain the koala's higher requirement for many drugs administered parenterally (Griffith et al., 2010, Kimble et al., 2013, Black et al., 2014, Govendir et al., 2016). Enhanced hepatic metabolism in koalas (hypothesised to be related to their diet of eucalypt leaves, which contain high concentrations of plant secondary metabolites) is also considered likely to be involved, with oral bioavailability especially impacted by an accentuated first-pass effect (Pass & Brown, 1990). Several studies have found that cytochrome P450 monooxygenase (CYP) activity is enhanced in koalas, as reviewed by Govendir (2018). A recent study of the koala genome characterised an expansion of the CYP pathways when compared to other species and revealed that these had high hepatic expression (suggestive of their role in detoxifying eucalypts and likely also other xenobiotics) (Johnson et al., 2018). Other factors that can hamper the treatment of koalas include difficulties in their hospitalisation and the maintenance of intravenous access, along with their tendency to present late in the course of disease, as will be discussed further in section 1.3.7.2.

Nevertheless, some protocols have been suggested for koalas. Originally, these were based largely on empirical observations during attempted therapy and a few published reports. These reports led to the recommendation of either itraconazole orally at 100 mg per koala once daily (approximately 10-20 mg/kg, depending on the individual koala's body weight) or fluconazole orally at 50-100 mg (approximately 10-20 mg/kg) twice daily, combined with AMB administered subcutaneously [according to the protocol devised by Malik et al. (1996a)] at 0.5 mg/kg twice weekly in 350 mL 0.45% NaCl and 2.5% dextrose (Blanshard & Bodley, 2008). This recommendation was later adjusted to oral fluconazole 20-25 mg/kg twice daily and subcutaneous AMB 0.7-0.8 mg/kg twice weekly (Govendir et al., 2016), although the clinical utility of this regimen and the pharmacokinetics of this dose of fluconazole when given in concert with AMB have not been documented. Further studies into the bioavailability of other antifungals (particularly itraconazole) and systematic reviews to

assess the optimal treatment for koala cryptococcosis are still needed, including a retrospective study into the large number of unpublished cases.

One study also reported the treatment of subclinical cryptococcosis in three koalas using a protocol of oral fluconazole 10 mg/kg twice daily, but the outcome for these individuals was unclear (Govendir et al., 2016). As mentioned in section 1.3.6.2, AMB is sometimes added to the treatment protocol for subclinical disease in koalas, but this is usually reserved for cases where the antigen titre does not decline as expected during azole monotherapy. Subclinical cryptococcosis in koalas has been successfully managed, although results of retrospective case series are yet to be collated and presented as a peer-reviewed publication (M.B. Krockenberger, pers. comm.).

Although the antifungal therapy of cryptococcosis in koalas is largely restricted to the use of fluconazole or itraconazole (often with subcutaneous AMB), one case was successfully treated with ketoconazole (Woods & Blyde, 1997), posaconazole shows some promise as a possible treatment (Gharibi et al., 2017), and alternative methods of AMB administration have also been reported with mixed success. An AMB protocol that alternated between the use of subcutaneous, intravenous and intra-lesional AMB (along with fluconazole or itraconazole orally) contributed to the successful treatment of one koala with a sinonasal mass lesion (Wynne et al., 2012). There have also been two reports of the use of AMB in a nebulised form for presumptive nasal cryptococcosis, but both were unsuccessful (Kido et al., 2012, Hanaki et al., 2018). Surgical excision or debulking of lesions is generally uncommon in koalas but has been reported (Bercier et al., 2012).

The treatment of avian cryptococcosis has not been systematically reported, with dose ranges and protocols largely extrapolated from a handful of case reports (where protocols were developed empirically) and anecdotal information. Fluconazole monotherapy is often used as a first-line treatment at a dose of 10-15 mg/kg twice daily (or potentially 20-25 mg/kg once daily, as twice daily administration can be difficult to achieve in some birds given husbandry constraints), and this is the current recommendation of some experts with experience in treating this disease (R. Malik, pers. comm.). Itraconazole (approximately 10 mg/kg once daily) or potentially compounded ketoconazole (approximately 5-20 mg/kg per day) can be substituted if these drugs prove easier to access, are better tolerated or there is proven resistance to fluconazole. Terbinafine, flucytosine and compounded voriconazole eye

drops (for ocular cryptococcosis) have also been used as part of combination protocols, with mixed success (Malik et al., 2003, Molter et al., 2014, Schunk et al., 2017). Intra-lesional and nebulised AMB likely represent useful adjunct treatments for avian cryptococcosis (Malik et al., 2003, Molter et al., 2014). Intravenous AMB administration has also been reported (Schunk et al., 2017) but is not well-characterised, although all methods of AMB administration are likely difficult in most avian patients (probably requiring GA). Nevertheless, AMB should be considered for cases of severe disease, or where there is a failure of response to other treatments. Surgical excision or debulking of lesions can also present an important treatment option for avian cryptococcosis, particularly considering the propensity for lesions to often be located superficially and the paucity of information regarding the use of antifungal drugs in avian species (Malik et al., 2003).

Richardson & Perpiñán (2017) reported that oral itraconazole monotherapy (25-33 mg/kg once daily) is the treatment of choice for cryptococcosis in ferrets. This is generally supported by an early case series (Malik et al., 2002) and a later review (Wyre et al., 2013). Itraconazole doses of 10-15 mg/kg once daily can also lead to good outcomes and are often better tolerated by ferrets (with less anorexia and inappetence observed) (R. Malik, pers. comm.). Itraconazole is likely preferred over fluconazole in ferrets due to a greater ease of accurately dosing these small patients, as liquid formulations or capsules (which can be opened and easily divided into small doses) are readily available. Fluconazole can also be used as a monotherapy with a reported recommended dose of 10 mg/kg once daily (Richardson & Perpiñán, 2017), but twice daily administration at a dose of 10-15 mg/kg is likely to be more successful (R. Malik, pers. comm.). AMB is rarely used, most likely because of the difficulty of administration in this species (due to issues of size and temperament) and the paucity of published reports. Both subcutaneous and intravenous administration of AMB would likely require GA and prolonged hospitalisation in this species, which could quickly become cost-prohibitive in many cases. AMB was administered intravenously to one ferret that later succumbed to disease (Eshar et al., 2010), and a dose rate of 0.4-0.8 mg/kg once daily for a week has been recommended (Richardson & Perpiñán, 2017). Further research into effective dose ranges for antifungal drugs and protocols for the treatment of cryptococcosis are required in all species discussed in this section.

1.3.6.5 Antifungal resistance

Given the relatively limited antifungal arsenal, resistance presents a potential problem for the treatment of cryptococcosis (Mourad & Perfect, 2018). Luckily, most isolates from the *C. gattii* and *C. neoformans* species complexes in Australia are susceptible to AMB, flucytosine, itraconazole and fluconazole (Ellis et al., 2007b, Chen et al., 2014). The lack of breakpoints for the *C. gattii* and *C. neoformans* species complexes presents a problem in classifying susceptibility and resistance, with epidemiological cut-off values the current alternative (Lockhart et al., 2017). In the *C. gattii* species complex, intrinsic resistance is usually considered the main mechanism (Varma & Kwon-Chung, 2010, Cheong & McCormack, 2013) but acquired resistance (during a course of therapy) has also been reported (Sykes et al., 2017). This can be duplicated in the laboratory by gradually increasing fluconazole concentration in growth media (Varma & Kwon-Chung, 2010). Most reports of resistance involve triazole antifungals (mainly fluconazole), but resistance to AMB and flucytosine (particularly if used as a monotherapy) are also occasionally reported (Vermes et al., 2000, Thompson III et al., 2009, Chen et al., 2014, Smith et al., 2015).

There is evidence that members of the *C. gattii* species complex exhibit more antifungal resistance, especially to fluconazole, than those of the *C. neoformans* species complex (Varma & Kwon-Chung, 2010). Differences in antifungal susceptibility may also exist within each species complex, with VGII found to have higher MICs for flucytosine and triazoles (especially fluconazole) than other *C. gattii* molecular types (Chong et al., 2010). In the same study, a similar difference was noted in the *C. neoformans* species complex, with VNII isolates exhibiting lower MICs for fluconazole than VNI isolates. When Chen et al. (2014) reviewed the literature on antifungal susceptibilities and resistance between the two species complexes and within the *C. gattii* species complex, inconsistencies were noted between the findings of many studies worldwide (some of which were likely skewed by the inclusion of large numbers of environmental isolates). These observations have also led to the conclusion that antifungal susceptibility testing is often unnecessary unless there is evidence of a failure to respond to treatment (Chen et al., 2014, Sykes & Malik, 2014).

The major recognised mechanisms for acquired azole resistance in *Cryptococcus* spp. are mutations or over-expressions of the *ERG11* gene (which encodes a target for azoles) or

over-expression of plasma membrane proteins that 'pump' azoles out of fungal cells (Chen et al., 2014). Hetero-resistance, an intrinsic mechanism where a sub-population of a strain typically considered susceptible to an antifungal can transiently express resistance (Ferreira & Santos, 2017), has also been reported in both the *C. gattii* and *C. neoformans* species complexes, particularly to fluconazole (Mondon et al., 1999, Varma & Kwon-Chung, 2010). This was noted as the likely cause treatment failure in a feline case of cryptococcosis caused by *C. gattii* VGIII (Sykes et al., 2017). Intrinsic resistance is considered to be a more common mechanism than acquired resistance in cryptococcosis (Cheong & McCormack, 2013).

1.3.7 Management and prognosis

1.3.7.1 Management

The management and prevention of cryptococcosis in animals has not been systematically explored, and at present is largely based on anecdotal evidence. In captive or domestic settings, decontamination of the environment using disinfectants could theoretically be beneficial if there is a known reservoir of environmental *Cryptococcus* spp. and/or at-risk animals (Tell et al., 1997). Krangvichain et al. (2016) found bleach and a quaternary ammonium compound used at standard dilutions to be effective fungicides, when used on a mixture of sterilised pigeon droppings and *C. neoformans* var. *grubii* in a laboratory setting. The decontamination and disinfection of enclosure 'furniture' in captive scenarios is often recommended for koalas (M.B. Krockenberger, pers. comm.), but there is no systematic or published evidence to indicate if this is effective.

Another common management strategy for cryptococcosis in captive koalas is regular screening for antigenaemia, and this approach is used in Australia, especially prior to the shipping and export of koalas overseas (Australian Government, 2009). One published case of early sinonasal disease was successfully picked up on a pre-shipment screening test in the USA (Wynne et al., 2012), while another reported case may have been prevented by testing prior to export (Makimura et al., 2002). Hence there is evidence that this is an effective strategy, and it can potentially allow for the treatment of subclinical cryptococcosis before

progression of disease (Govendir et al., 2016), especially considering that transport and new surroundings can cause stress and thus potentially some amount of immunosuppression.

1.3.7.2 Prognosis

In animals, the prognosis is generally considered good to guarded, regardless of the exact causative species complex or molecular type. In one study that followed case outcomes in cats and dogs with cryptococcosis, 76% of cats and 55% of dogs were treated successfully (O'Brien et al., 2006). Given that some strains of *C. gattii* VGII appear to exhibit comparatively higher MICs for some antifungals and greater virulence, cases involving this molecular type are often considered more cautiously and poorer outcomes are sometimes reported (for example, in animals in WA, where VGII is endemic) (Lester et al., 2004, McGill et al., 2009, Malik et al., 2011). Within the *C. neoformans* species complex, there is evidence that genotype can predict clinical outcome in humans in Africa (Wiesner et al., 2012). Despite this, resource-limited settings and delayed diagnosis are generally considered more important prognostic indicators than genotype in human patients (Maziarz & Perfect, 2016). Overall, early diagnosis is also considered the most important prognostic factor in animals, along with prompt and aggressive antifungal therapy (Krockenberger et al., 2003, Blanshard & Bodley, 2008, Sykes & Malik, 2014). In cats with early diagnosis and sustained assiduous treatment, including good compliance with drug therapy, the prognosis is often favourable (Pennisi et al., 2013). Localised disease is also associated with better clinical outcomes in birds and ferrets (Malik et al., 2003, Wyre et al., 2013), while CNS involvement is generally associated with a poorer prognosis in cats, dogs and koalas (Blanshard & Bodley, 2008, Sykes et al., 2010). A similar phenomenon has been observed in immunocompetent humans with CNS disease, where delayed diagnosis (typically due to cryptococcosis not being considered as an early differential diagnosis) can lead to poorer case outcomes (Ecevit et al., 2006).

In koalas, a diagnosis is often obtained late in the course of disease, particularly in free-ranging animals but sometimes also in captivity (Krockenberger et al., 2003), and treatment can be difficult due to the factors mentioned in section 1.3.6.4. Thus overall, the prognosis is anecdotally poorer in koalas. Despite this, cryptococcosis can be successfully treated in captive koalas, but full resolution of cryptococcal antigenaemia and long-term

outcomes are often not reported (Woods & Blyde, 1997, Bercier et al., 2012, Wynne et al., 2012, Govendir et al., 2016).

1.3.8 Outbreaks and case clusters

Spatial and/or temporal case clusters and outbreaks of cryptococcosis can occur, presumably due to exposure of a group of animals to a common, high-level, environmental source of infection and/or a highly virulent strain. In recent times this is predominantly associated with *C. gattii* VGII, but clusters of *C. neoformans* species complex cryptococcosis have been reported in captive animals (Tell et al., 1997, Bauwens et al., 2004), and outbreaks in goats caused by the *C. gattii* VGI have been reported on several occasions across a relatively limited area in one province of western Spain (Baró et al., 1998, Torres-Rodríguez et al., 2006). Several case clusters of *C. gattii* species complex cryptococcosis where the causative organism was not identified to molecular type level have also been reported, including a group of psittacine birds housed in a Brazilian aviary (Raso et al., 2004).

In Australia, *C. gattii* VGII case clusters are occasionally documented. A flock of domestic sheep in WA exhibited numerous deaths attributed to cryptococcosis, although case details are scant and isolates could be obtained from only three sheep (all were *C. gattii* VGII) (Sorrell et al., 1996a, Malik et al., 2011). In the NT, *C. gattii* VGII case clustering has been observed amongst Aboriginal people in Arnhem Land (Fisher et al., 1993, Chen et al., 1997a). There is also some evidence suggestive of spatial and temporal case clustering in captive koalas with cryptococcosis (Krockenberger et al., 2003, Malik et al., 2011), particularly that caused by *C. gattii* VGII (Saul, 2009), but this has not been established definitively. Several cases of cryptococcosis caused by the *C. gattii* species complex were observed across multiple host species (including a parrot, macropod and rodent) at a zoological park in WA, but the molecular type was not reported (Payne et al., 2005).

The Vancouver Island *C. gattii* VGII cryptococcosis outbreak offers the prime example of the ability of this pathogen to cause spatial and temporal clustering, with cases reported in humans and across a wide range of animal species (Craig et al., 2002, Kidd et al., 2004, Lester et al., 2004). The outbreak then expanded to other areas of British Columbia in Canada and

the Pacific Northwest region of the USA (MacDougall et al., 2007, Byrnes III et al., 2009). This outbreak was caused predominantly by an emergent highly virulent strain, *C. gattii* VGIIa, and a secondary strain, VGIIb (Kidd et al., 2004). A third strain, VGIIc, was identified in the Pacific Northwest, and this may have emerged subsequent to evolutionary pressure (Byrnes III et al., 2010). Numerous contributing factors to this outbreak have already been discussed, including same-sex mating (Fraser et al., 2005) and dispersal events from South America (potentially anthropogenic) as the likely underlying origin (Hagen et al., 2013, Roe et al., 2018).

1.3.8.1 Animals as sentinels

Given that cryptococcosis is acquired from the environment, animals could reasonably be considered at the front line of exposure to this disease and thus can be used to examine the interface with the environment (Malik et al., 2011). One advantage of using animals as sentinels is that, generally-speaking and depending on the species, they have a more finite and easily defined geographical range compared to humans. The Vancouver Island outbreak provided evidence that animals could act as critical sentinels for cryptococcosis in humans, with an increase in the prevalence of animal cases noted before the disease was systematically reported in humans (Craig et al., 2002, Hoang et al., 2004, Lester et al., 2004). Domestic animal cases also provided an early indication that the outbreak was spreading beyond Vancouver Island (Lester et al., 2004, MacDougall et al., 2007). The domestic animals (Duncan et al., 2005b) and wildlife (Duncan et al., 2006) of Vancouver Island were later surveyed for cryptococcal nasal colonisation and subclinical disease to provide further insights into the distribution of *C. gattii* VGII in the area and the risks of exposure for humans. Other animals, including rodents (Singh et al., 2007) and ferrets (Morera et al., 2014), have also been proposed as useful sentinel species. Given that forested and/or rural areas are considered by some as the highest risk for exposure to members of the *C. gattii* species complex (Springer & Chaturvedi, 2010), it seems reasonable that free-ranging animals in contact with these areas offer great potential as useful sentinel species.

It is extremely difficult to identify the precise environmental source of infection in many cases of human cryptococcosis, but animals (particularly domestic and captive) provide a unique opportunity to explore this, given their known and often highly restricted range of

domicile (Malik et al., 2011). Despite this, it remains rare for definitive sources of infection to be detected. Some studies in animals have tried and failed to achieve this end (Raso et al., 2004, Torres-Rodríguez et al., 2006), while others have found some indication of a source of infection but did not support this with in-depth genotyping of isolates (Sorrell et al., 1996a, Bauwens et al., 2004, Singh et al., 2007, Morera et al., 2014).

1.4 Aims and hypothesis

Cryptococcosis is clearly a consequence of environmental exposure to members of the *C. gattii* (or *C. neoformans*) species complex, but the exact mechanism for the acquisition of naturally-occurring colonisation or disease is poorly understood. It remains unclear if this is simply a random event, or if a heavy inoculum, specific strain/s, exposure to a particular environment, and/or concurrent insults to host defence mechanisms are required to establish colonisation and disease. This thesis will therefore aim to address the following overarching hypothesis:

'Cryptococcosis is a random outcome of heavy environmental exposure'

This hypothesis will be tested by using koalas as naturally-occurring models for exposure to, and infection by, *C. gattii* VGI and VGII in Australia, with other animals (parrots and ferrets) also included.

1.4.1 Aims

This thesis will address the hypothesis through the following aims:

1. Explore the ecological associations of *C. gattii* VGI in the mycobiome of Australian tree hollows, using amplicon-based metagenomics (section 2.1).
2. Systematically document the successful diagnosis and therapy of subclinical cryptococcosis in free-ranging koalas (section 3.1 and 3.2).
3. Assess the relationship between environmental presence, nasal colonisation and disease in some free-ranging koala populations (sections 3.3 and 4.1).

4. Explore the interface between environment and disease in naturally-occurring cases of cryptococcosis in Australian animals through molecular epidemiology and phylogenetic analyses (sections 4.1 to 4.4).
5. Determine the point source of infection or exposure in naturally-occurring disease or colonisation, caused by *C. gattii* VGI, in representative cases in captive animals using molecular epidemiology (sections 4.2 and 4.3).
6. Assess clusters of cryptococcosis in koalas for their outbreak potential (sections 4.1 and 4.4).
7. Examine the ability of koalas to translocate *C. gattii* VGII into new environments (section 4.4).

Aim 1 addresses the hypothesis by determining if the microenvironment can play a role in the presence or abundance of *Cryptococcus* spp. in tree hollows, which could thus affect the likelihood of host exposure. Aims 2 to 4 further examine the potential randomness of disease through exploring the relationship between colonisation, subclinical disease, clinical disease and the environment (using captive and free-ranging koalas with naturally-occurring disease or colonisation). Aims 5 and 6 both look more specifically at interactions between animals and their environment in naturally-occurring cryptococcosis, which provides models for assessing when and how the disease is acquired. Aim 7 explores how the organism is spread by koalas in Australia, and the implications that this may have for both koalas and other host species.

2. Environmental associations

Background:

Tree hollows are an important ecological niche for *Cryptococcus* spp. (particularly *C. gattii* VGI in Australia), and therefore also present a source of infection. Little is known about the tree hollow microbiome and the role that other fungi could play in the survival, growth and proliferation of this pathogen in the environment. The ability to detect *Cryptococcus* spp. in environmental samples is a critical step in identifying potential sources of infection, yet traditional culture-based methods can be problematic. Culture-independent methods have not been explored as a method of detection for environmental *C. gattii* VGI in Australia.

This section uses amplicon-based NGS of DNA extracted directly from tree hollow debris in Australia to: characterise the mycobiome, assess the utility of this method for identifying environmental *Cryptococcus* spp., and expand the environmental associations of *C. gattii* VGI. This approach included statistical analyses to assess if *Cryptococcus* spp. was linked to other fungal taxa, thus testing for important associations within the mycobiome. The comparison between culture and NGS highlighted the limitations of both methods and provided an opportunity to discuss these in detail.

Aims:

- **Aim 1:** explore the ecological associations of *C. gattii* VGI in the mycobiome of Australian tree hollows, using amplicon-based metagenomics.

Sections:

- **2.1:** The mycobiome of Australian tree hollows in relation to the *Cryptococcus gattii* and *C. neoformans* species complexes.

Impact:

An enhanced understanding of the environmental niche of *C. gattii* VGI may help to identify risk factors for exposure to this pathogen, while improved methods for its detection could aid in the identification of sources of infection. This is applicable to the management and prevention of cryptococcosis in many species, particularly captive or domestic animals.

2.1 The mycobiome of Australian tree hollows in relation to the *Cryptococcus gattii* and *C. neoformans* species complexes

Authorship declaration:

This manuscript has been submitted to *Ecology and Evolution* and, at the time of submission of this thesis, is under review. L.J. Schmertmann is the primary author and contributed to all aspects of this study (designing and performing the research, analysing the data and preparing the manuscript). Equal authorship is shared with L. Irinyi due to his critical involvement in the NGS data analysis and assistance with the preparation of parts of the manuscript. R. Malik, W. Meyer and M.B. Krockenberger designed the research. J.R. Powell assisted in data analysis. All authors were involved in the revision of the manuscript and support its inclusion in this thesis.

Laura J. Schmertmann _____ March 2019

Mark B. Krockenberger _____ March 2019

The mycobiome of Australian tree hollows in relation to the *Cryptococcus gattii* and *C. neoformans* species complexes

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ABSTRACT

Cryptococcosis is a fungal infection caused by members of the *Cryptococcus gattii* and *C. neoformans* species complexes. The *C. gattii* species complex has a strong environmental association with eucalypt hollows (particularly *Eucalyptus camaldulensis*), which may present a source of infection. It remains unclear whether a specific mycobiome is required to support its environmental survival and growth. Conventional detection of environmental *Cryptococcus* spp. involves culture on differential media, such as *Guizotia abyssinica* seed agar. Next-generation sequencing (NGS)-based culture-independent identification aids in contextualising these species in the environmental mycobiome. Samples from 23 Australian tree hollows were subjected to both culture- and amplicon-based metagenomic analysis to characterise the mycobiome and assess relationships between *Cryptococcus* spp. and other fungal taxa. The most abundant genera detected were *Coniochaeta*, *Aspergillus* and *Penicillium*, all being commonly isolated from decaying wood. There was no correlation between the presence of *Cryptococcus* spp. in a tree hollow and the presence of any other fungal genus. Some differences in the abundance of numerous taxa were noted in a differential heat tree comparing samples with or without *Cryptococcus*-NGS reads. The study expanded the known environmental niche of the *C. gattii* and *C. neoformans* species complexes in Australia with detections from a further five tree species. Discrepancies between the detection of *Cryptococcus* spp. using culture or NGS suggest that neither is superior *per se* and that, rather, these methodologies are complementary. The inherent biases of amplicon-based metagenomics require cautious interpretation of data through consideration of its biological relevance.

INTRODUCTION

Cryptococcosis is a potentially lethal mycosis affecting both humans and animals, caused by basidiomycetous yeasts in the *C. gattii* and *Cryptococcus neoformans* species complexes (Kwon-Chung et al., 2017). The infection is acquired from the environment by the inhalation of basidiospores or desiccated yeast cells (Kwon-Chung et al., 2014). The *C. gattii* and *C. neoformans* species complexes inhabit various ecological niches and are associated with decaying organic material, such as tree hollow debris and accumulations of avian excreta (K.

Nielsen et al., 2007, Springer et al., 2014). The ecological role of *Cryptococcus* spp. is not well understood but their presence in decaying organic matter suggests that they contribute to the process of decomposition (Voříšková & Baldrian, 2013).

The *C. gattii* species complex has traditionally been regarded as tropical or subtropical, whereas the *C. neoformans* species complex is globally distributed. There is increasing evidence, however, that the *C. gattii* species complex is also prevalent in temperate climates (Kidd et al., 2004, Chowdhary et al., 2012, Colom et al., 2012). Ellis & Pfeiffer (1990) described a specific ecological association between the *C. gattii* species complex and eucalypt trees in Australia, most notably the river red gum (*Eucalyptus camaldulensis*), in a wide variety of temperate and subtropical locations. Since then, the *C. gattii* species complex has been found globally in decaying wood, particularly inside trunk hollows and in various living tree species, suggesting that trees could be its primary natural habitat (Lazera et al., 2000, Randhawa et al., 2008, Cogliati et al., 2016).

In Australia, eucalypts appear to be a key environmental niche for the *C. gattii* species complex (particularly *C. gattii* VGI), and the range of tree species from which it has been isolated continues to expand (Table 2.1.1). Koalas (*Phascolarctos cinereus*) exhibit comparatively high rates of both clinical and subclinical cryptococcosis, likely preceded by nasal colonisation by *Cryptococcus* spp., with this association presumably related to their close association with eucalypts (Krockenberger et al., 2003). In recent years, several cases of cryptococcosis have been observed in free-ranging koalas (*Phascolarctos cinereus*) inhabiting the Port Stephens (Schmertmann et al., 2018b) and Liverpool Plains (Schmertmann et al., 2019) regions of New South Wales (NSW), Australia. As the Vancouver Island *C. gattii* VGII outbreak highlighted the potential for animals to represent key sentinels for human disease (Craig et al., 2002), we were prompted to conduct environmental investigations in both areas.

The presence of a specific fungal community or critical species could support the growth of *Cryptococcus* spp. in the environment, but this remains undetermined. A study conducted in Africa attempted to characterise this notion but did not find any associations between the presence of any specific fungal taxa and *Cryptococcus* spp. (Vanhove et al., 2017). The fungal community residing in eucalypt tree hollows in association with the *C. gattii* species complex in Australia remains unexplored.

TABLE 2.1.1 Tree species from which the *Cryptococcus gattii* species complex has been isolated in Australia.

Scientific name	Common name	*Cryptococcus* spp. association	Method of first detection	Source of first detection
Angophora costata	Smooth-barked apple	*C. gattii* VGI	Culture	(Halliday et al., 1999)
Eucalyptus albens	White box	*C. gattii* VGI	Culture	(Schmertmann et al., 2019)
E. bella	Ghost gum	*C. gattii* VGII	Culture	(Sorrell et al., 1996a)
E. blakelyi	Blakely's red gum	*C. gattii* VGI	Culture	(Pfeiffer & Ellis, 1997)
E. camaldulensis	River red gum	*C. gattii* VGI	Culture	(Ellis & Pfeiffer, 1990)
E. gomphocephala	Tuart	*C. gattii* VGI	Culture	(Pfeiffer & Ellis, 1997)
E. grandis	Flooded gum	*C. gattii* VGI	Culture	(Halliday et al., 1999)
E. microcorys	Tallowwood	*C. gattii* VGI	Culture	(Krockenberger et al., 2002b)
E. rudis	Flooded gum	*C. gattii* VGI	Culture	(Pfeiffer & Ellis, 1997)
E. tereticornis	Forest red gum	*C. gattii* VGI	Culture	(Pfeiffer & Ellis, 1992)
Syncarpia glomulifera	Turpentine gum	*C. gattii* VGI	Culture	(Krockenberger et al., 2002b)

NGS = next-generation sequencing.

The detection of *Cryptococcus* spp. from the environment is important in the context of cryptococcosis in order to pinpoint potential infection sources, with culture being the primary means of achieving this. Staib's *Guizotia abyssinica* seed extract agar (bird seed agar) containing antibiotics underpins the conventional detection of *Cryptococcus* spp. from environmental samples through mycological culture (Shields & Ajello, 1966, Paliwal & Randhawa, 1978). The method is based on *Cryptococcus* spp. colonies exhibiting the brown-colour-effect, due to the production of melanin by cryptococcal laccase (a phenoloxidase enzyme) (Staib, 1962). It is therefore dependent on the visual recognition of suspect cryptococcal colonies, which can be challenging when multiple fast-growing filamentous fungi are present concurrently.

New culture-independent methods, such as next-generation sequencing (NGS), have the potential to detect any organism, including *Cryptococcus* spp., in the environment without relying on growth *in vitro*, therefore aiding in the identification of sources of infection. In addition, they can also define the microbial communities present in environmental samples (Taberlet, et al., 2012, Hamad et al., 2017, Tong et al., 2017). There has been a fundamental shift away from conventional DNA sequencing introduced by Sanger et al. (1977), considered as first-generation sequencing technology, to newer methods, such as NGS. High-throughput sequencing technologies have revolutionised

biological research and allowed for the in-depth characterisation of microbial diversity, without the need for morpho-taxonomy (Creer et al., 2016).

Fungi might represent the largest genetic diversity amongst the eukaryotes, with an estimated 5.1 million species, including taxa ranging from unicellular yeasts and microscopic moulds to large mushrooms (Blackwell, 2011). Of this enormous number of species, only a small number are known to be potential mammalian pathogens. To overcome inherent limitations of culture-based methods of characterising microbial communities, indirect molecular methods have been developed based on the total DNA content of the sample. Amplicon-based metagenomics analyses (metabarcoding) have become a widely used technology in various fields, ranging from microbial ecology studies to infectious disease surveillance (Tedersoo et al., 2014, Tonge et al., 2014, L.D.N. Nguyen et al., 2015). Currently, this is the standard tool and the most efficient method for culture-independent assessment of microbiomes, even if its broad application is still hampered by relatively high cost and the need for advanced bioinformatic analyses (Tang et al., 2015). The approach combines the methodologies of DNA barcoding (Hebert et al., 2003) with high-throughput sequencing technology. It is based on the concept that each operational taxonomic unit (OTU) can be unequivocally identified using DNA barcodes. The general strategy involves: (i) extraction of DNA from an environmental sample or organism, (ii) amplification of the species-specific DNA barcodes, (iii) sequencing of the DNA amplicons, (iv) analyses of the generated sequences using appropriate pipelines and (v) taxonomic assignment of the detected sequences. PCR-based metabarcoding has become a rapid and accurate method for species level identification from complex environmental and clinical samples without the requirement for culture, thereby providing unprecedented insights into the underlying biological diversity (Bik et al., 2012).

In this study, used amplicon-based NGS was used as a tool to assess the co-existence of *Cryptococcus* spp. with other fungi in eucalypt and other Australian native tree hollows, while simultaneously characterising the fungal community (mycobiome) of tree hollows from two areas of NSW, Australia that have recently been associated with cryptococcosis in koalas. We also compared conventional culture-based methods to NGS, to assess which was more sensitive at detecting pathogenic *Cryptococcus* spp. in environmental samples.

MATERIALS AND METHODS

Sample collection

Debris and related material from hollows were collected from 23 trees at multiple locations within the Port Stephens (9) and Liverpool Plains (14) regions of New South Wales, Australia (Table 2.1.2). Samples were collected as part of a disease investigation into cases of koala cryptococcosis. The areas for sample collection were selected based both on proximity to these cases and reported koala habitat (according to our observations and/or the recommendation of local rescue groups). Trees in these areas were then selected randomly for sampling based on the presence of one or more tree hollows that were accessible from the ground. A generous amount of material was collected from the interior of each tree hollow and placed into a clean plastic bag, which was sealed and labelled. Tree species from which samples were collected included *E. camaldulensis* (12), *E. pilularis* (2), *E. tereticornis* (2), *Eucalyptus* spp. (2), *Angophora floribunda* (1), *E. albens* (1), *E. populnea* (1), *E. robusta* (1) and *Melaleuca* spp. (1).

A mock fungal community was created (as a positive control) by obtaining a mixture of yeast and filamentous fungi isolates from the Medical Mycology Culture Collection at The Westmead Institute for Medical Research in Westmead, NSW, Australia. This comprised a total of 12 isolates and included the genera *Aspergillus*, *Candida*, *Fusarium, Kodamaea, Pichia* and *Scedosporium*, along with one *C. gattii* VGI and one *C. neoformans* VNI isolate.

Culture

Samples were inoculated onto bird seed agar (containing penicillin and gentamicin) as soon as possible after collection by introducing a sterile swab, pre-moistened with sterile saline, into the sample and then gently rolling the swab across the plate. Plates were incubated at 27°C for a minimum of seven days and monitored daily. *Cryptococcus* spp. colonies were identified by the brown-colour-effect and their yeast-like growth on bird seed agar. Once suspected cryptococcal colonies were observed, the agar plate was removed from the incubator and one or more colonies were subcultured onto Sabouraud's dextrose agar for isolation of a pure culture, which was followed by DNA extraction (see below). Samples

E2657, E2666 and E2704 had five, four and three isolates collected, respectively, from each primary isolation plate. In all other positive samples, only one isolate was collected. DNA extraction from cryptococcal isolates was performed using an established fungal DNA extraction method (Ferrer et al., 2001). Restriction fragment length polymorphism (RFLP) analysis of a *URA5* PCR product was used as described previously (Meyer et al., 2003) to determine the cryptococcal species and molecular type of each isolate.

DNA Extraction for NGS

DNA was extracted directly from the mock fungal community and approximately 20 g of each sample of tree hollow material by first grinding them with liquid nitrogen and a mortar and pestle. This homogenised the samples and aided in breaking down both the cryptococcal capsule and fungal cell walls. DNA was then extracted using the DNeasy PowerSoil kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

PCR amplification of the ITS1 region

Fragments were amplified with ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) (White et al., 1990) targeting the ITS1 region of the rRNA gene complex. PCR amplicons were generated using AmpliTaq Gold 360 Master Mix (ThermoFisher Scientific, North Ryde, NSW, Australia) for the primary PCR with the following amplification protocol: 7 min initial denaturing at 95°C, followed by 35 cycles of 30 s at 94°C, 45 s annealing at 55°C, 60 s at 72°C and 7 min final extension at 72°C. A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech Laboratories, Mountain View, California, USA). All PCRs included negative controls.

Sequencing

Sequencing of PCR amplicons was conducted with MiSeq® System of Illumina (Illumina, San Diego, California, USA) by the Australian Genome Research Facility. The Illumina bcl2fastq 2.18.0.12 pipeline was used to generate the sequence data. Pair-end reads 2 × 300bp were generated up to 0.15 GB per sample.

Bioinformatics pipeline and analysis

Reads were processed according to the protocol described in the USEARCH package (Edgar, 2010) version 10.0. The sets of OTUs were generated, then 'zero-radius OTUs' (ZOTUs) (i.e. error-corrected [denoised] sequences) were produced using the UNOISE algorithm including chimera filtering (Edgar, 2016b) to identify all correct biological sequences. The ZOTU table was normalised to the same number of reads per sample (5,000) prior to downstream analysis. Singletons were kept for downstream analysis. The taxonomy was predicted for ZOTU sequences using the SINTAX classifier (Edgar, 2016a) against both the UNITE full dataset (Kõljalg et al., 2013) (accessed on 04.03.2017) and ISHAM-ITS (Irinyi et al., 2015) database (containing all relevant ITS sequences of *Cryptococcus* spp.). All identified OTUs were retained for downstream analysis, including singletons.

Fungal OTUs from each sample were used to determine Shannon and Evenness indices as indicators of soil microbial diversity and structure, respectively. α-diversity metrics were calculated using USEARCH package (Edgar, 2010) version 10.0 (Table 2.1.3). To estimate the potential correlation between *Cryptococcus* spp. and other genera, Pearson and Spearman co-occurrence coefficients were then calculated with SparCC (Friedman & Alm, 2012) for each sample, separately. Taxon pairs with SparCC values > 0.6 were considered as exhibiting a co-occurrence relationship with a positive correlation.

MetacodeR was used to visualise the mycobiome diversity of tree hollows in heat tree format (RCore, 2013, Foster et al., 2017). A differential heat tree was created to indicate which taxa are more abundant in the presence of *Cryptococcus* spp. reads. For statistical support, one-sample Wilcoxon signed rank test was used in R (RCore, 2013) to test the hypothesis that certain taxa may be enriched for lower p value ranks than other taxa. Benjamini-Hochberg (FDR) correction (Benjamini & Hochberg, 1995) was used to adjust p values for multiple comparisons to limit the probability of even one false discovery.

RESULTS

Detection of *Cryptococcus* spp. by culture

Cryptococcal colonies were observed after culture on bird seed agar in 14/23 (61%) tree hollow samples studied (Table 2.1.2). *URA5*-RFLP analysis identified all isolates as *C. gattii* VGI. The *C. neoformans* species complex and other members of the *C. gattii* species complex were not identified in any samples. Tree species in which *C. gattii* VGI was identified include: *A. floribunda, E. albens, E. camaldulensis, E. pilularis, E. robusta, E. tereticornis* and *Melaleuca* spp. (Table 2.1.2).

TABLE 2.1.2 Detection of *Cryptococcus* spp. from 23 tree hollow samples in New South Wales, Australia using conventional culturing and next generation sequencing. Samples are ordered by positive or negative culture results and degree of growth. Bold text indicates samples with a discordance between the results of sequencing and culture.

Sample	Tree species	Region	Culture / *URA5*-RFLP results	Number of reads detected with amplicon based (ITS1) NGS		
				Total reads	*C. gattii* VGI	*C. neoformans* VNI/VNII
E2699	***Melaleuca* spp.**	**PS**	**+ / *C. gattii* VGI**	**152490**	**–**	**–**
E2771	*Eucalyptus camaldulensis*	LP	+ / *C. gattii* VGI	149970	2	–
E2666	*Angophora floribunda*	PS	++ / *C. gattii* VGI	125784	3	1
E2697	***E. robusta***	**PS**	**++ / *C. gattii* VGI**	**89683**	**–**	**–**
E2704	*E. tereticornis*	PS	++ / *C. gattii* VGI	226974	1	1
E2768	*E. camaldulensis*	LP	++ / *C. gattii* VGI	70250	1	1
E2772	*E. camaldulensis*	LP	++ / *C. gattii* VGI	206302	11	10
E2773	*E. camaldulensis*	LP	++ / *C. gattii* VGI	152861	3	7
E2774	*E. camaldulensis*	LP	++ / *C. gattii* VGI	129923	2	2
E2657	***E. pilularis***	**PS**	**+++ / *C. gattii* VGI**	**131034**	**–**	**–**
E2760	*E. albens*	LP	+++ / *C. gattii* VGI	104095	6	2
E2761	*E. camaldulensis*	LP	+++ / *C. gattii* VGI	104725	52	1
E2764	*E. camaldulensis*	LP	+++ / *C. gattii* VGI	142580	4	4
E2770	*E. camaldulensis*	LP	+++ / *C. gattii* VGI	145686	11	5
E2668	*E. tereticornis*	PS	–	158773	–	–
E2677	*Eucalyptus* spp.	PS	–	114688	–	–
E2698	*Eucalyptus* spp.	PS	–	126651	–	–
E2711	*E. pilularis*	PS	–	71419	–	–
E2757	***E. populnea***	**LP**	**–**	**105099**	**3**	**–**
E2762	***E. camaldulensis***	**LP**	**–**	**133183**	**5**	**–**
E2765	***E. camaldulensis***	**LP**	**–**	**118666**	**1**	**–**
E2766	***E. camaldulensis***	**LP**	**–**	**163204**	**1**	**1**
E2769	***E. camaldulensis***	**LP**	**–**	**108789**	**6**	**7**

LP = Liverpool Plains; PS = Port Stephens; RFLP = restriction fragment length polymorphism analysis; + = low (1-10 cryptococcal colonies), ++ = moderate (11-100 colonies), +++ = heavy (>100 colonies); – = negative.

Detection of *Cryptococcus* spp. using NGS technology

C. gattii or *C. neoformans* species complex sequence reads were identified in 16/23 (70%) samples. *C. gattii* species complex reads were detected in all these 16 samples, with *C. neoformans* species complex reads concurrently detected in 12. *C. neoformans* species complex reads were not recorded in the absence of *C. gattii* species complex reads in any sample. No *Cryptococcus* spp. reads were identified in seven samples (Figure 2.1.1 A). All *C. gattii* species complex reads were VGI, while *C. neoformans* species complex reads were VNI/VNII. *C. gattii* VGI and *C. neoformans* VNI reads were also successfully identified in the mock community.

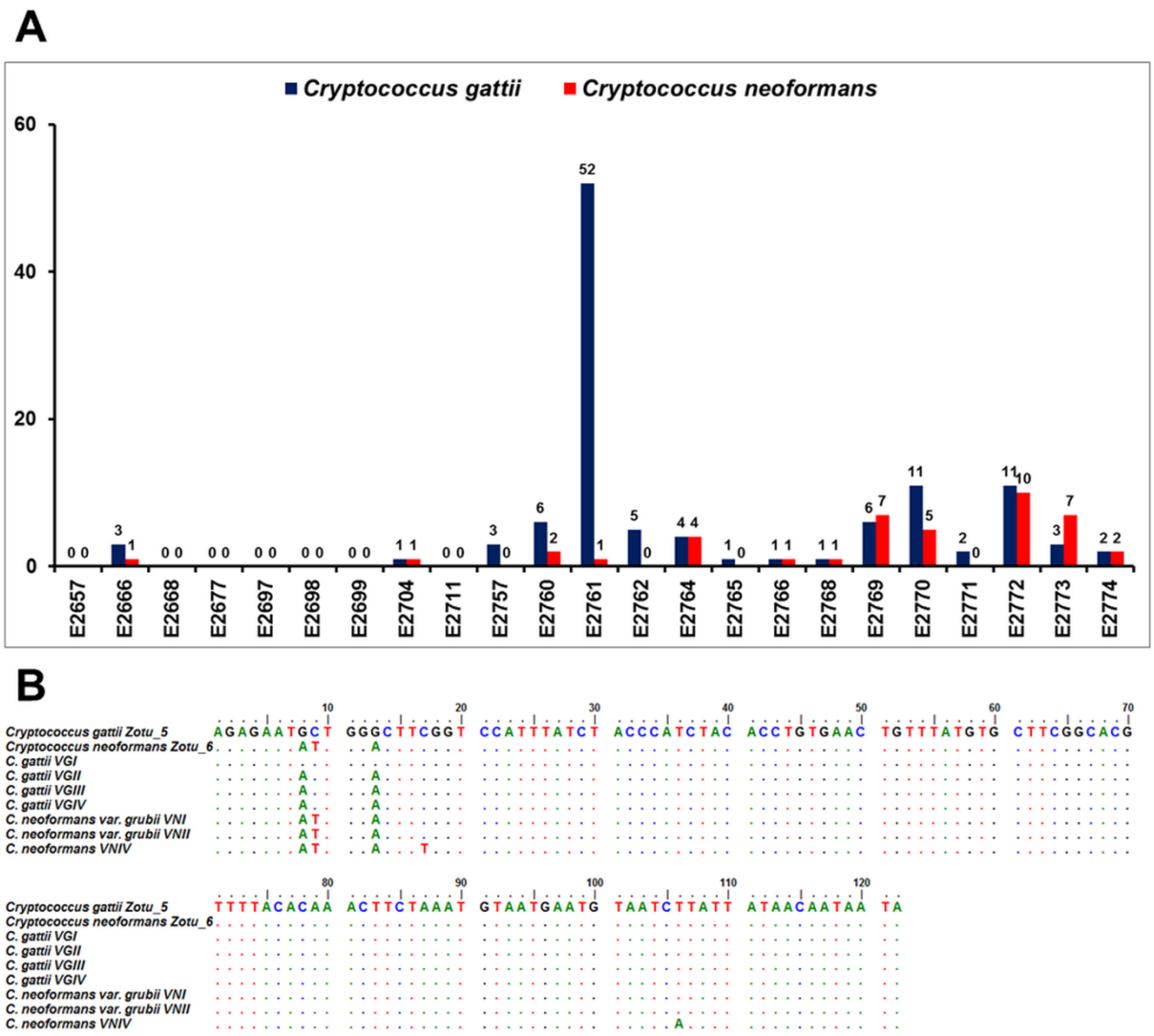

FIGURE 2.1.1 (A) Number of unique *Cryptococcus gattii*/*C. neoformans* species complex ITS1 sequence reads detected from 23 tree hollows in New South Wales, Australia. **(B)** Alignment of the *C. gattii*/*C. neoformans* species complex ITS1 region identified in next-generation sequencing (Zotu_5 and Zotu_6) and reference sequences (Katsu et al., 2004).

Concordance between NGS and culture results was observed in 15/23 (65%) samples: 11 samples were NGS- and culture-positive for the *C. gattii* species complex while four samples were NGS- and culture-negative for all *Cryptococcus* spp. In three samples, *C. gattii* VGI was identified by culture but not NGS. In another five samples, *Cryptococcus* spp. reads were identified by NGS, but culture yielded negative results (Table 2.1.2).

Mycobiome of eucalypt tree hollows

The number of generated sequence reads per sample was highly variable (mean 126,381; standard deviation 44,207) and thus the OTU table was normalised to the same number of reads (5,000) per sample. We identified a total of 2,638 OTUs which were assigned at different taxonomic levels: 199 to species (7.5%), 565 to genus (21.4%), 741 to family (28.1%), 1,185 to order (44.9%), 1,557 to class (59.0%), 1,982 to phylum (75.1%) and 2,638 to kingdom level (100%) (Figure 2.1.2). The number of OTUs differed in each sample, with a mean of 550 and standard deviation of 273.

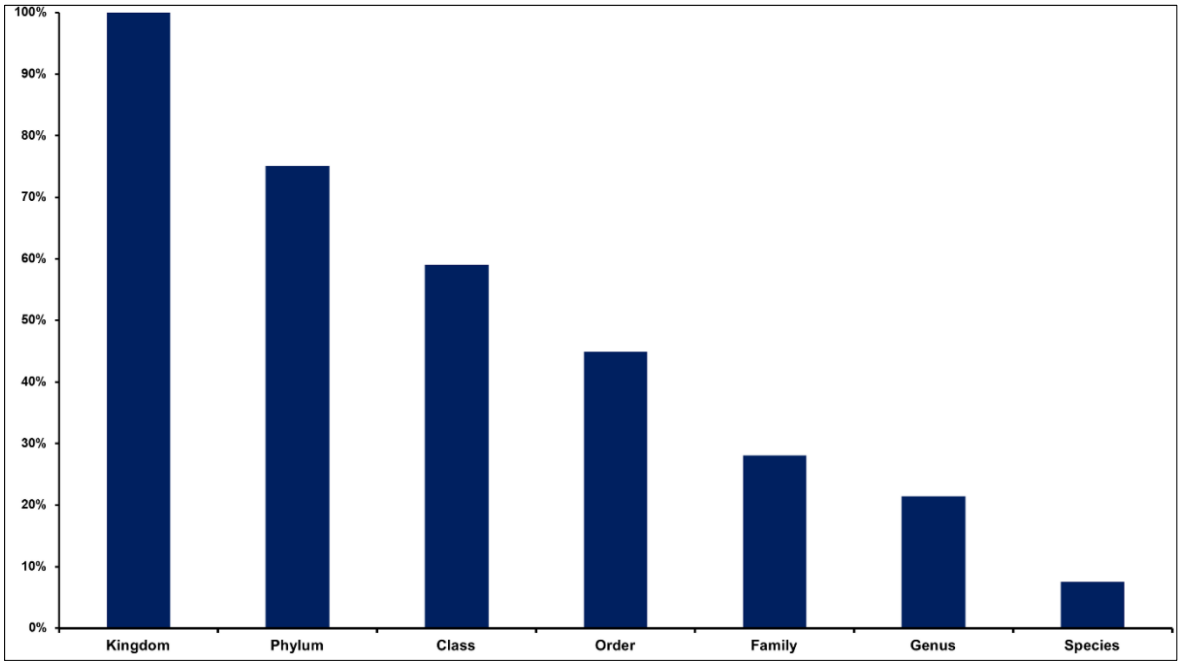

FIGURE 2.1.2 Operational taxonomic unit assignment rates at different taxonomical levels using ITS1 amplicon-based metagenomics analysis of samples from 23 tree hollows in New South Wales, Australia.

OTUs classified in the Ascomycota were observed most frequently (1,660 OTUs; 62.9% sequences), followed by the Basidiomycota (226 OTUs; 8.6% sequences)

(Supplementary figure S2.1.1). The distributions of taxa at lower taxonomical levels, such as class, order, family and genus, per sample, are shown in Supplementary figures S2.1.2 to S2.1.5. Much less abundant fungi included Mortierellomycota (38 OTUs; 1.4% sequences), Chytridiomycota (22 OTUs; 0.8% sequences), Rozellomycota (18 OTUs; 0.7% sequences) and Mucormycota (12 OTUs; 0.5% sequences). Some 25% of the sequences (662 OTUs) remained unassigned at phylum level (Figure 2.1.2).

Amongst the Ascomycota, Sordariomycetes (28.1%), Eurotiomycetes (25.9%), Dothideomycetes (12.5%) Leotiomycetes (4.4%) and Saccharomycetes (2.7%) were the most abundant classes. Amongst the Basidiomycota, Agaricomycetes (51.3% sequences), Tremellomycetes (22.6%) and Microbotryomycetes (4.9%) were most abundant.

At genus level, amongst Ascomycota, *Penicillium* (2.3%), *Aspergillus* (1.6%) and *Scytalidium* (1.5%) were the most abundant genera. In the Basidiomycota, *Trechispora* (4%), *Jaapia* (2.7%) and *Cryptococcus* (2.5%) were the most dominant. In Mortierellomycota, the most common genera were *Mortierella* (55.3%) and *Gamsiella* (2.6%). The overall ten most abundant genera in each sample are shown in Supplementary figure S2.1.6.

At species level, 901 OTUs (34.2%) were singletons, 371 (14.1%) and 205 (7.8%) OTUs were represented by only two and three reads, respectively. A total of 1,719/2,638 OTUs (65.2%) were represented by less than five reads. The most abundant genus across all samples was *Coniochaeta* (anamorph: *Lecythophora*), followed by *Aspergillus* and *Penicillium*. The mycobiome structure of the 23 tree hollows are displayed in Figure 2.1.3. In analysis of the mock community, all the known fungal species were successfully identified.

Co-occurrence analysis did not show any significant correlation between the occurrence of *Cryptococcus* spp. and other taxa or genera (Figure 2.1.4). The correlation analysis likewise did not show any statistical significance ($p < 0.05$) or correlation between *Cryptococcus* spp. and other genera (Figure 2.1.4).

The differential heat tree analysis (Figure 2.1.5) highlighted numerous taxa that differed in abundance between samples with no *Cryptococcus* spp. reads compared to those with one or more reads. In samples with no *Cryptococcus* spp. reads, Ascomycota, Eurotiomycetes, Eurotiales and *Aspergillus* were comparatively more abundant at phylum, class, order and genus level, respectively. In those with *Cryptococcus* spp. reads, numerous

taxa were identified as comparatively more abundant at phylum, class, order and family levels. At the genus level, four genera were highlighted as being of greater abundance – *Coniochaeta*, *Cryptococcus*, *Penicillium* and *Scytalidium* (Supplementary figures S2.1.5 and Figure S2.1.6)

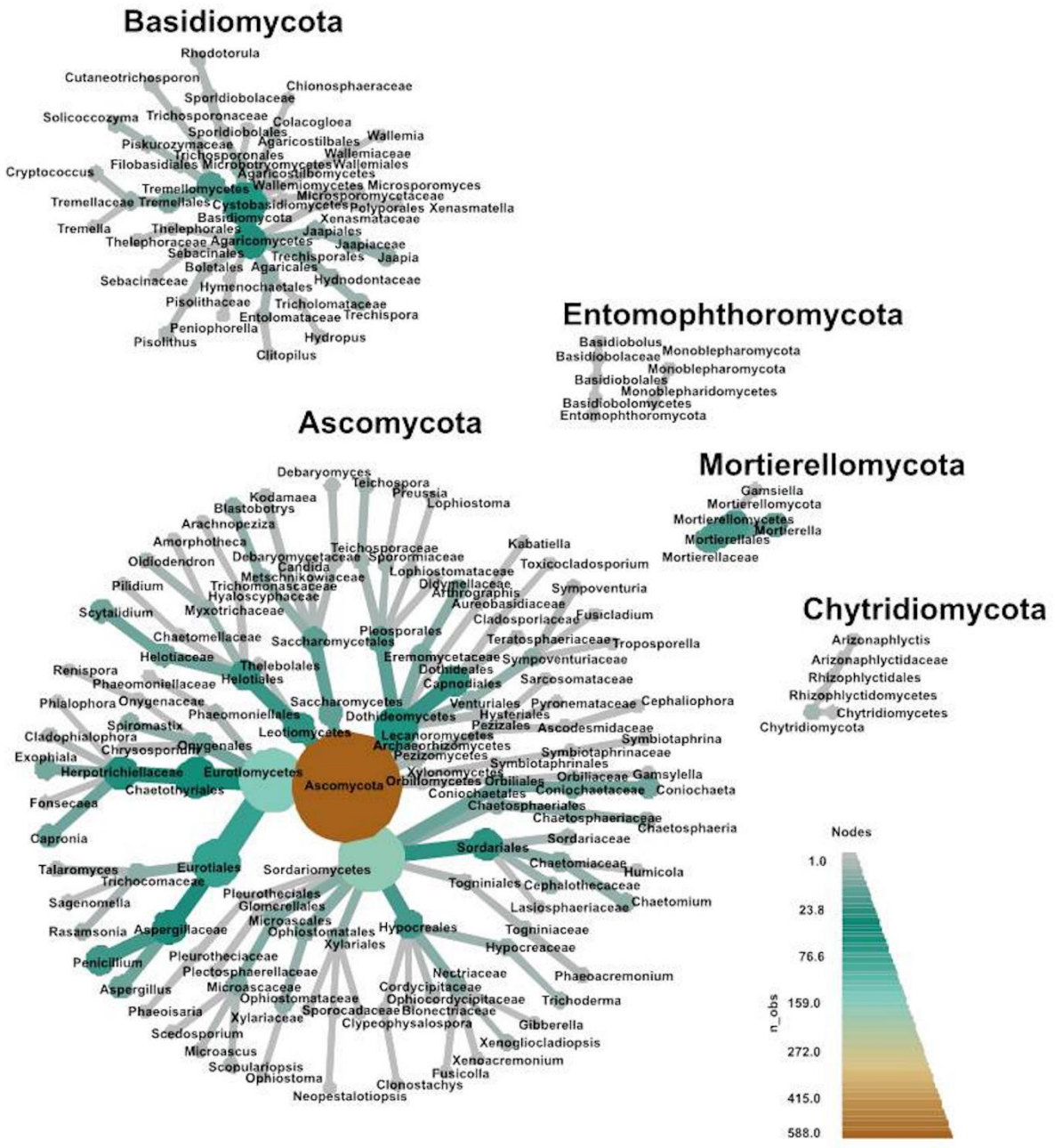

FIGURE 2.1.3 Heat tree of the mycobiome structure of the 23 tree hollows in New South Wales, Australia. The size and colour of nodes and edges are correlated with the abundance of taxa in each community.

Diversity indices did not at all highlight any specific predictors likely to explain the occurrence of *Cryptococcus* spp. in certain tree hollows. Fungal communities varied, based on OTU numbers and Shannon and Evenness indices (Table 2.1.3). The number of detected OTUs in each sample varied between 125 and 1,062, with a mean of 570. The Simpson index, which indicates whether a single large OTU dominates the sample, was also highly variable (0.022-0.952). The values closer to 1 indicated that a few OTUs dominated the sample contrary to a value closer to 0 indicating that the samples were composed of multiple OTUs, none of which dominated the sample. The Berger-Parker and Robbins frequency indexes showed that the samples with more OTUs detected had many singletons. One sample (E2697) had Simpson and Berger-Parker values indicative of a single large OTU dominating the sample. Supplementary figures S2.1.1 to S2.1.6 confirm this pattern and indicate that the single dominant OTU in this sample can be attributed to *Aspergillus*.

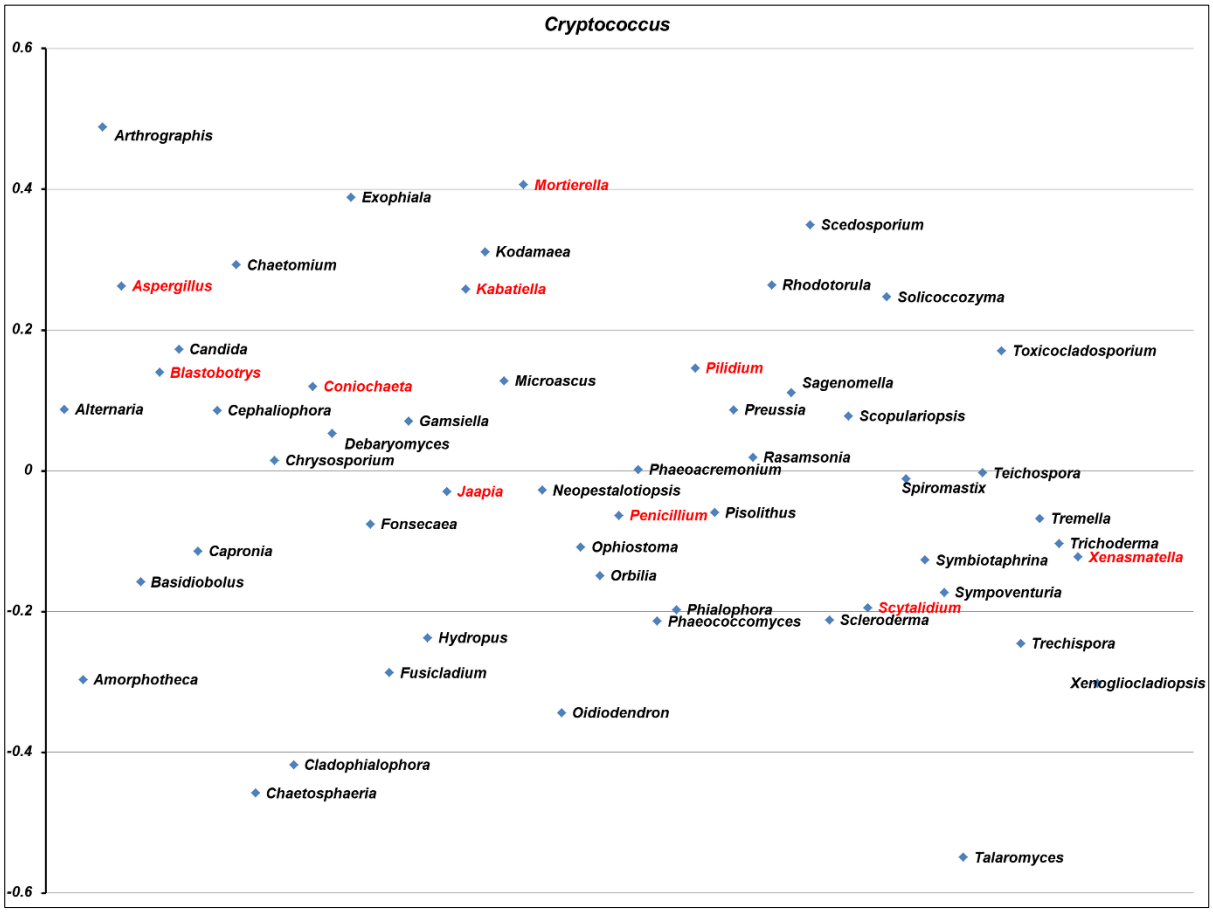

FIGURE 2.1.4 Correlation analysis using SparCC, Pearson and Spearman correlations between *Cryptococcus* spp. and other fungal genera detected in the 23 tree hollows in New South Wales, Australia. Red text denotes the 10 most common genera.

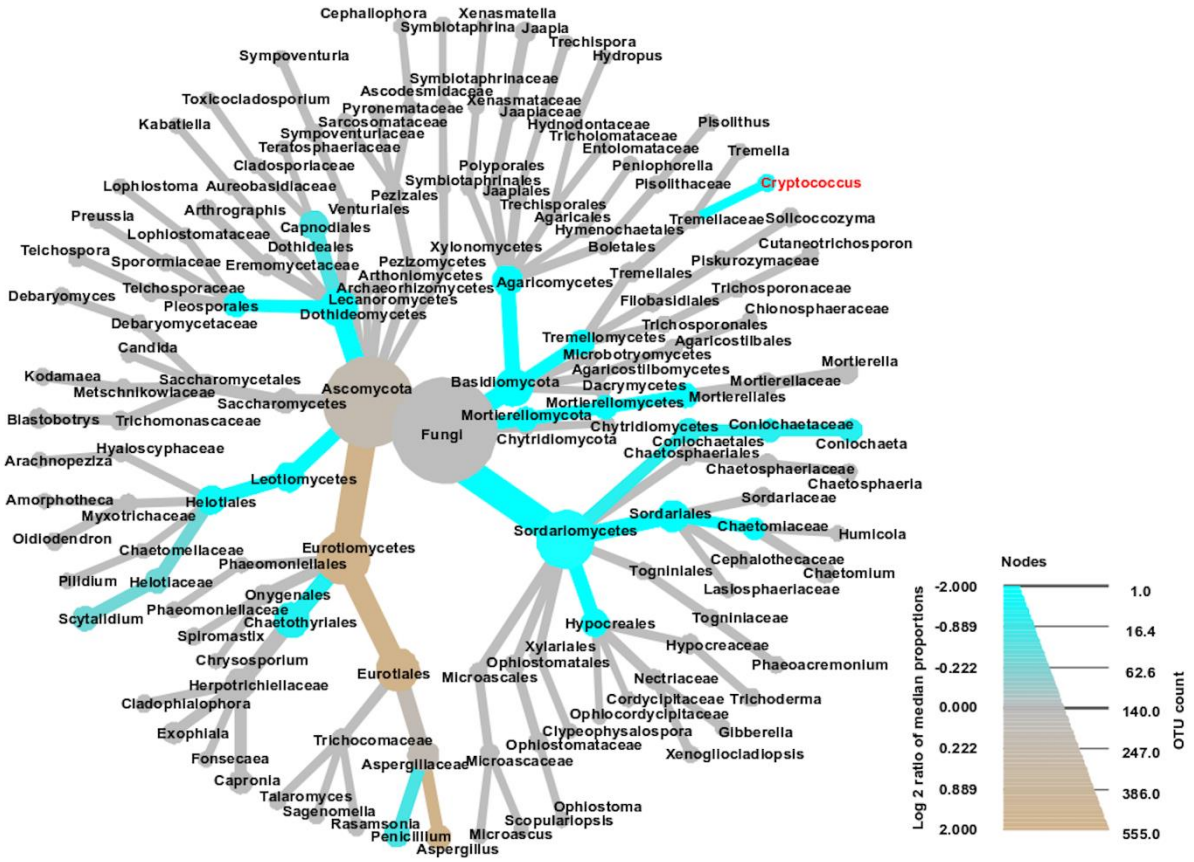

FIGURE 2.1.5 Differential heat tree showing the relative abundance of different fungal taxa in the presence of *Cryptococcus gattii/C. neoformans* species complex reads in 23 Australian tree hollows, as detected by next generation sequencing. The colour of each taxon represents the log-2 ratio of median proportions of reads observed in *C. gattii/C. neoformans* species complex negative and positive samples. Taxa coloured brown are more abundant in negative while those coloured blue are more abundant in positive samples. Only significant differences are coloured, as determined using a Wilcox rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple comparisons.

DISCUSSION

In this study, the mycobiome of Australian native tree hollows was characterised and no definitive correlations were found between the presence of *Cryptococcus* spp. and other genera. Discrepancies were established between culture-dependent and culture-independent methods used for the detection of the *C. gattii* and *C. neoformans* species complexes in environmental samples. Although these methodologies were often in agreement, discordant results were observed in 8/23 samples. These findings can likely be explained by biases in both methods, which will be explored throughout the discussion. This study has also expanded the known environmental niche of *C. gattii* VGI through its

detection in the hollows of another five tree species: three eucalypts (*E. pilularis*, *E. populnea* and *E. robusta*), a *Melaleuca* spp. and an *Angophora* spp.

TABLE 2.1.3 Estimations of fungal species richness of 23 tree hollows in New South Wales, Australia obtained with different models.

Sample	Number of reads	Diversity metrics			Evenness metrics			
		Richness	Shannon_2	Jost1	Simpson	Dominance	Berger_Parker	Robbins
E2657	131034	245	3.41	10.6	0.195	0.805	0.378	0.187
E2666	125784	535	4.1	17.1	0.158	0.842	0.342	0.185
E2668	158773	482	3.65	12.5	0.284	0.716	0.522	0.197
E2677	114688	323	3.44	10.8	0.192	0.808	0.312	0.29
E2697	89683	125	0.265	1.2	0.952	0.0478	0.976	0.413
E2698	126651	143	2.96	7.79	0.222	0.778	0.342	0.319
E2699	152490	524	4.1	17.2	0.188	0.812	0.367	0.145
E2704	226974	520	5.84	57.4	0.0381	0.962	0.0938	0.0768
E2711	71419	216	3	7.99	0.213	0.787	0.376	0.313
E2757	105099	940	6.89	118.5	0.0222	0.978	0.0726	0.119
E2760	104095	803	6.2	73.6	0.051	0.949	0.192	0.149
E2761	104725	780	5.17	36	0.0853	0.915	0.244	0.25
E2762	133183	537	3.37	10.3	0.217	0.783	0.405	0.229
E2764	142580	461	4.06	16.7	0.194	0.806	0.416	0.24
E2765	118666	560	3.7	13	0.214	0.786	0.427	0.271
E2766	163204	1062	6.41	85.1	0.0343	0.966	0.113	0.148
E2768	70250	780	5.61	48.7	0.102	0.898	0.304	0.213
E2769	108789	776	4.46	22	0.212	0.788	0.45	0.227
E2770	145686	701	5.78	55.1	0.051	0.949	0.175	0.172
E2771	149970	894	5.73	52.9	0.0665	0.933	0.163	0.169
E2772	206302	228	4.63	24.7	0.0811	0.919	0.177	0.17
E2773	152861	683	4.82	28.2	0.0997	0.9	0.246	0.161
E2774	129923	808	4.36	20.5	0.237	0.763	0.477	0.193

Richness: number of operational taxonomic units (OTUs) with at least one read for the sample.
Shannon_2: Shannon index logs to base 2. Accounts for abundance and evenness of species in the sample.
Jost1: Jost index of order 1, the effective number of species given by the Shannon index.
Simpson: probability that two randomly selected reads will belong to the same OTU. Values close to 1 indicate that a single large OTU dominates the sample; small values indicate distribution of reads over many OTUs.
Dominance: probability that two randomly selected reads will belong to different OTUs.
Berger_Parker: frequency of the most abundant OTU. Values close to 1 indicate that a single large OTU dominates the sample, small values indicate distribution of reads over many OTUs.
Robbins: Robbins index, calculated as S/(N + 1) where S is the number of singleton OTUs and N is the total number of OTUs.

Culture-based identification remains challenging due to the rapid growth of filamentous fungi which may impair the detection of *Cryptococcus* spp. in some samples. All conventional culture-based methods have limitations, including their unknown and potentially low sensitivity, cost (due to the need to frequently use multiple media) and turnaround time (often up to a week, potentially longer when the time required for

subculturing and molecular confirmation of the phenotypic identification is considered). Given the unknown sensitivity of this method, perhaps repeated cultures of each sample in this study could have yielded different results. It may also have been of use to add an inhibitor of mould growth to the bird seed agar (Pham et al., 2014, de Matos Castro e Silva et al., 2015).

It is also possible that cryptococcal DNA was detected using NGS but insufficient viable organisms were present in these samples for culture-based detection to be successful. Viable but non-culturable organisms could also be present (Hommel et al., 2019). This observation may have clinical relevance and should be considered carefully if NGS results are used as part of a disease investigation, as viable live yeast cells or basidiospores are required to initiate infection. The potential for environmental DNA to complicate NGS results is further explained later in the discussion. The culture-positive but NGS-negative results for *Cryptococcus* spp. are likely related to the multitude of biases encountered in amplicon-based metagenomics, which will be explored later in the discussion. It is also of note that the number of *Cryptococcus* spp. reads based on NGS did not appear to be consistent with the culture-based grading of low, moderate or heavy. These findings again suggest that abundance-based results are generally considered unreliable in amplicon-based metagenomics studies (N.H. Nguyen et al., 2015, Tessler et al., 2017).

Many samples were found to contain *C. neoformans* VNI/VNII sequences using NGS, as this method is unable to distinguish between VNI and VNII since their ITS1 regions are identical (Figure 2.1.1 B) (Katsu et al., 2004). These findings were not supported by the culture results, as none of the isolates collected were identified as members of the *C. neoformans* species complex. This could have occurred by chance, since only a few isolates were obtained from each positive sample for downstream molecular typing. Members of the *C. neoformans* species complex may also have been in a quiescent state (Hommel et al., 2019) or were outcompeted on the culture plates by *C. gattii* VGI and other fungi. However, this discrepancy could also be due to the potential high error rate of NGS and a very high similarity between the ITS1 regions of the *C. gattii* and *C. neoformans* species complexes. Differentiating between them relies on only three polymorphic sites in the ITS1 region (Figure 2.1.1 B) (Katsu et al., 2004), which may also explain that *C. neoformans* VNI/VNII reads were only obtained in samples that also had *C. gattii* VGI reads. All *Cryptococcus* raw

reads were extracted, visually checked and their taxonomic assignments were confirmed using BLAST. The choice of another target, more discriminatory between the *C. gattii* and *C. neoformans* species complexes, such as the entire ITS region or the *URA5* gene (Meyer et al., 2003) may have circumvented this, but the size of both regions precluded their compatibility with the technology (MiSeq® System of Illumina) used in this study. The use of long read sequencing technologies, such as MinION™ from Oxford Nanopore Technologies (Eisenstein, 2012), may improve discriminatory power by allowing for the sequencing of the entire ITS region or other targets, such as the *URA5* gene.

Although we identified 2,638 OTUs amongst the samples, only 7.5% were classified to species and 25.6% to genus level. Moreover, 34.2% of the OTUs were singletons which are largely due to the choice of the algorithm in the downstream analysis. Below order level, most OTUs remained unclassified without any taxonomic predictions. Our findings regarding the assignment success rate of OTUs agree with previous metagenomics studies carried out in a different environment (Schmidt et al., 2013, Sun et al., 2015, Soliman et al, 2017, Yuan et al., 2017).

The mycobiome of Australian tree hollows characterised in this study appears to be reasonably consistent with our expectations of a decaying, hardwood micro-environment. Three genera: *Coniochaeta*, *Aspergillus* and *Penicillium*; dominated the mycobiome. Species in the *Coniochaeta* genus (pleomorphic yeasts) are known pathogens of trees and are often isolated from necrotic wood samples but can also cause opportunistic human infections after traumatic implantation, such as keratitis, subcutaneous abscesses, peritonitis and endocarditis (de Hoog et al., 2000, Taniguchi et al., 2009, Damm et al., 2010, Khan et al., 2013). There is some speculation that yeasts may be overrepresented in NGS analyses due to their higher nucleus to cytoplasm ratio when compared to filamentous fungal species with longer cells (Lindahl et al., 2013). *Aspergillus* and *Penicillium* spp. are well-known fungal genera associated with wood degradation (soft rot fungi) in nature, since they tolerate wide ranges of temperature, humidity and pH, and attack a variety of wood substrates. Soft rot fungi are more common in hardwood, such as *Eucalyptus* spp., than in softwood which might be due to differences in the quality of the lignin (Hamed, 2013). Other genera, such as *Scytalidium, Blastobotrys, Jaapia* or *Mortierella,* are also commonly found in decaying organic matter and produce enzymes which enhance the degradation of proteins in the

wood of dead trees (Middelhoven & Kurtzman, 2007, Takahashi & Oda, 2008, Wagner et al., 2013, Telleria et al., 2015). Statistical comparisons between tree hollow mycobiomes were not considered relevant in this study, because the primary focus of the sampling was to investigate the connection between cryptococcosis cases of koalas and the environmental source of these infections, with the mycobiome analysis performed opportunistically. Since a power analysis was not performed (regarding the number of samples for mycobiome and correlation analyses) and relatively few samples were used overall, this can be considered a pilot study. A more in-depth statistical analysis, requiring additional information about trees and tree hollow characteristics and a greater sample size (justified by a power analysis), will be subject of future studies. Further work of interest to the authors will be directed towards characterising the mycobiome of the koala nasal cavity to determine how closely this reflects that of nearby tree hollows.

The lack of a relationship between the presence of *Cryptococcus* and any other fungal genera based on the correlation analysis is consistent with a prior study (Vanhove et al., 2017). The differential heat tree analysis, however, suggested some differences in the relative abundance of numerous taxa in samples with versus without *Cryptococcus* reads. At genus level, *Aspergillus* was more abundant in *Cryptococcus* NGS-negative samples and *Coniochaeta*, *Penicillium* and *Sctyalidium* were more abundant in *Cryptococcus* NGS-positive samples. However, both the correlation and heat tree analysis rely on NGS data, and as we have already demonstrated, NGS results may not always reflect the biological reality of the mycobiome. Of particular note in this study is one tree in which a heavy growth of *C. gattii* VGI was observed on culture, yet no *Cryptococcus* reads could be identified using NGS (Table 2.1.2; E2657). As previously mentioned, abundance-based results are also often considered unreliable in NGS studies (N.H. Nguyen et al., 2015, Tessler et al., 2017). Therefore, it is difficult to draw definitive conclusions from these findings, and further work, such as more systematic sampling and numerous technical replicates, would be required to determine how reliable these potential associations are. We also did not attempt to find correlations between *Cryptococcus* and bacteria or parasites in Australian tree hollows, which most likely will also influence the composition of the mycobiome but are beyond the scope of the current work.

NGS metabarcoding results should be interpreted with care, as a number of technical issues and biases inherent in amplicon-based metabarcoding have been reported, including: PCR primer selection (Pinto & Raskin, 2012, Elbrecht & Leese, 2015), tag switching (Esling, Lejzerowicz & Pawlowski, 2015, Schnell, Bohmann & Gilbert, 2015), template concentration, amplification conditions (Kennedu et al., 2014), and PCR and sequencing errors (Turnbaugh et al., 2010, Nakamura et al., 2011, Tremblay et al., 2015). Such errors are difficult to distinguish from true biological variation (Edgar, 2016b, Leray & Knowlton, 2017). The denoising algorithm used in this study aims to remove sequencing noise and preserve all biological reads in the sample, an important step in NGS data analysis. We used the UNOISE improved error correction algorithm (Edgar, 2016b). However, denoising algorithms present a challenge and another potential bias of NGS data interpretation, as defining an abundance threshold that differentiates correct sequences from random errors is difficult (Schirmer et al., 2015). The high number of singletons observed in the data set might have been attributable to the UNOISE algorithm used in the analysis. It is also well recognised that inferences from metagenomics studies are greatly influenced and varied by the fields, laboratories and analytic techniques utilised (Majaneva et al., 2015). Besides technical biases in sample preparation, DNA extraction and sequencing, there is also another level of complexity and biases in downstream analyses and databases.

Another major limitation of NGS metabarcoding is the requirement for reference databases in order to determine the phylogenetic affiliation of sequence reads. Taxonomical assignments can only be as reliable as these reference databases (Santamaria et al., 2012). This study used the UNITE full dataset (Kõljalg et al., 2013) which also includes the ISHAM-ITS (Irinyi et al., 2015) dataset containing all relevant ITS sequences of *Cryptococcus* spp. The SINTAX taxonomy classifier (Edgar, 2016a) was chosen to predict the taxonomy of the identified sequences, as it achieved comparable or better accuracy than the popular RDP Naive Bayesian Classifier (Wang et al., 2007). The SINTAX algorithm predicts taxonomy by using k-mer similarity to identify the top hit in a reference database, supported by bootstrap values for all ranks in the prediction (Edgar, 2016a).

Another bias of amplicon-based metagenomics is the uncertainty as to whether the detected DNA belongs to a live or a dead microbe. DNA is ubiquitous and stable in the environment and can account for roughly 10% of extractable phosphorus in soil (Turner &

Newman, 2005). Extracellular DNA fragments can persist over time in many environments, allowing for their detection with high-throughput sequencing technology (K.M. Nielsen, et al., 2007). Tree hollows could protect environmental DNA from some forms of degradation, including extreme heat and UV-radiation.

This study presents the first published detection of the *C. gattii* species complex in Australia from *A. floribunda, E. pilularis, E. populnea, E. robusta* and *Melaleuca* spp. to the best of our knowledge. The detection from *E. populnea*, however, was only based on NGS and could not be confirmed by culture. It is possible that this represents a sampling error when plated on culture media for the detection of fungal growth, non-viable organisms or erroneous sequence reads. Nevertheless, it is becoming increasingly clear that the ecological niche of *C. gattii* VGI in Australia extends far beyond the classic association with *E. camaldulensis* and *E. tereticornis.*

Based on our findings of discrepancies between culture and NGS-based identification of *Cryptococcus* spp. from environmental samples, neither approach can be considered definitive, and they are likely complementary. NGS may have proven unable to differentiate between the *C. gattii* and *C. neoformans* species complexes in these samples, no significant correlation between the presence of *Cryptococcus* spp. and other fungal genera or taxa could be identified, and abundance-based analyses were inconclusive. As expected, the mycobiome of Australian tree hollows reflected the microenvironment of decaying wood. Given the discrepancies between culture and NGS results and the multitude of potential biases in amplicon-based metagenomics, meaningful inferences are difficult to establish, and results must be interpreted with caution. Further improvements in NGS, such as whole genome shotgun and long-read sequencing, together with appropriate data analysis pipelines and the extension of reference databases, should significantly contribute to better characterisation and understanding of such complex microbial community structures. Further work in this area should include assessing possible correlations between *Cryptococcus* spp. and bacteria, free living amoebae and nematodes.

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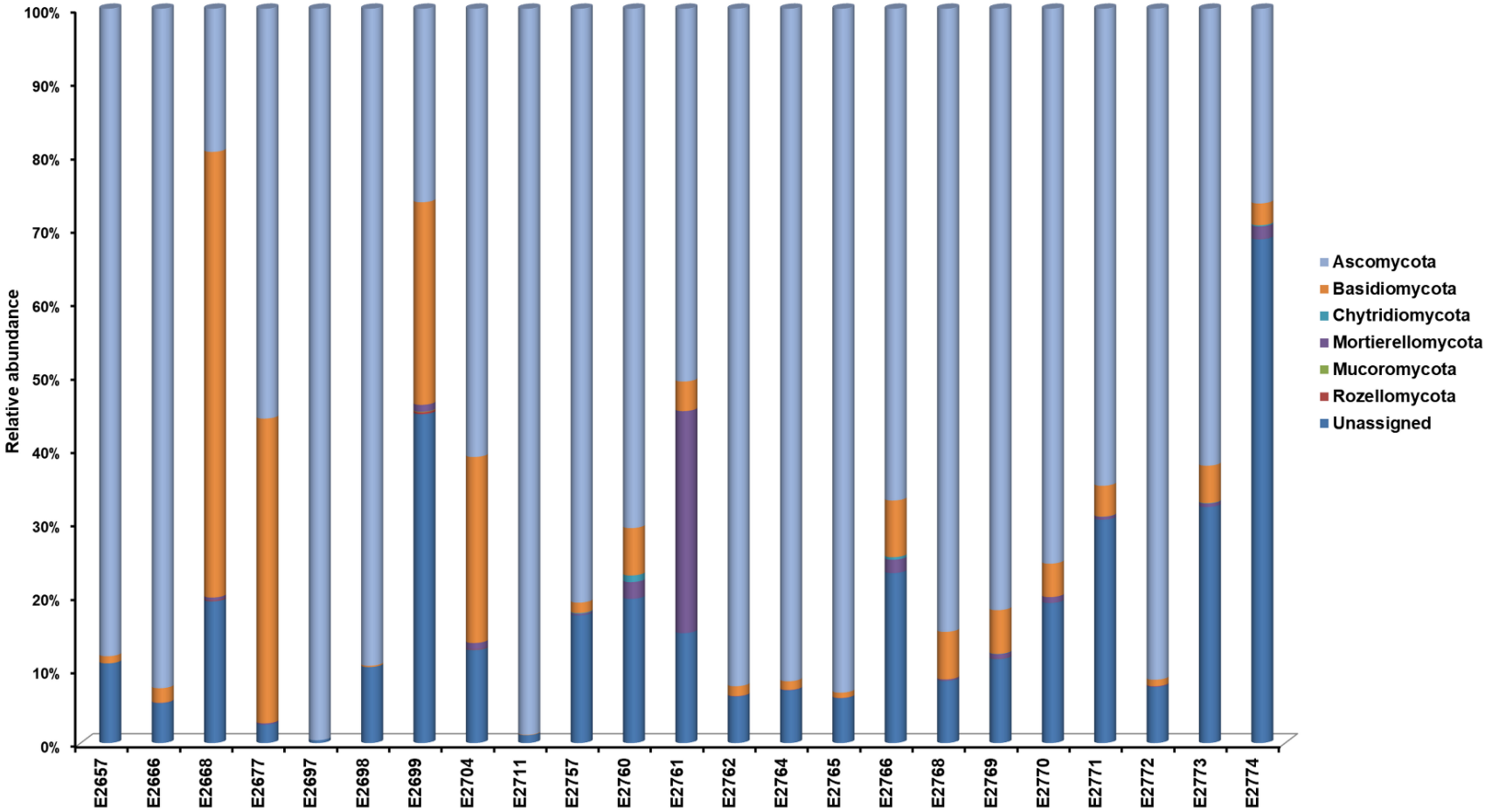
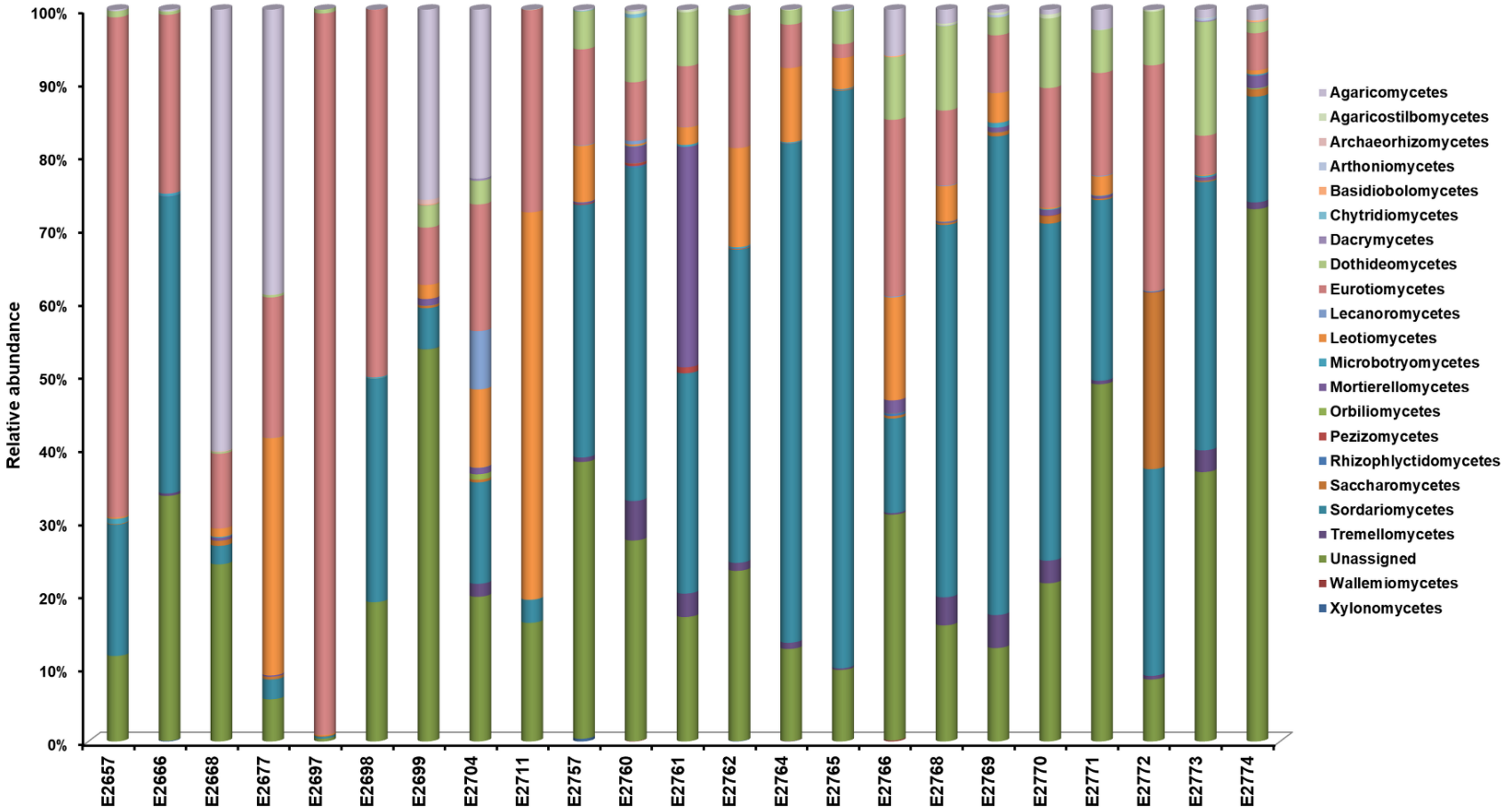
DATA ACCESSIBILITY

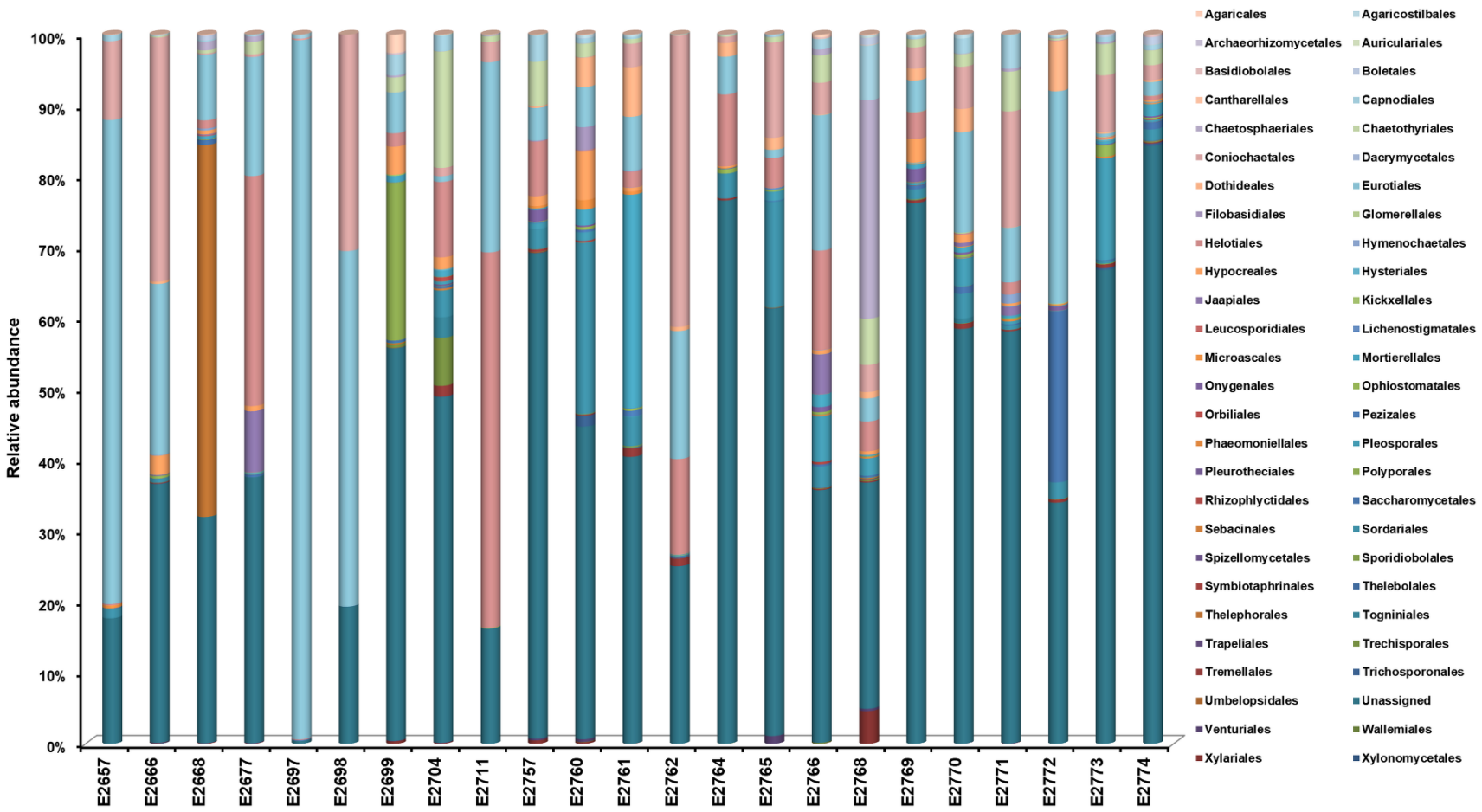
All raw reads from this study were submitted to NCBI's Sequence Read Archive under the BioProject accession PRJNA497337.



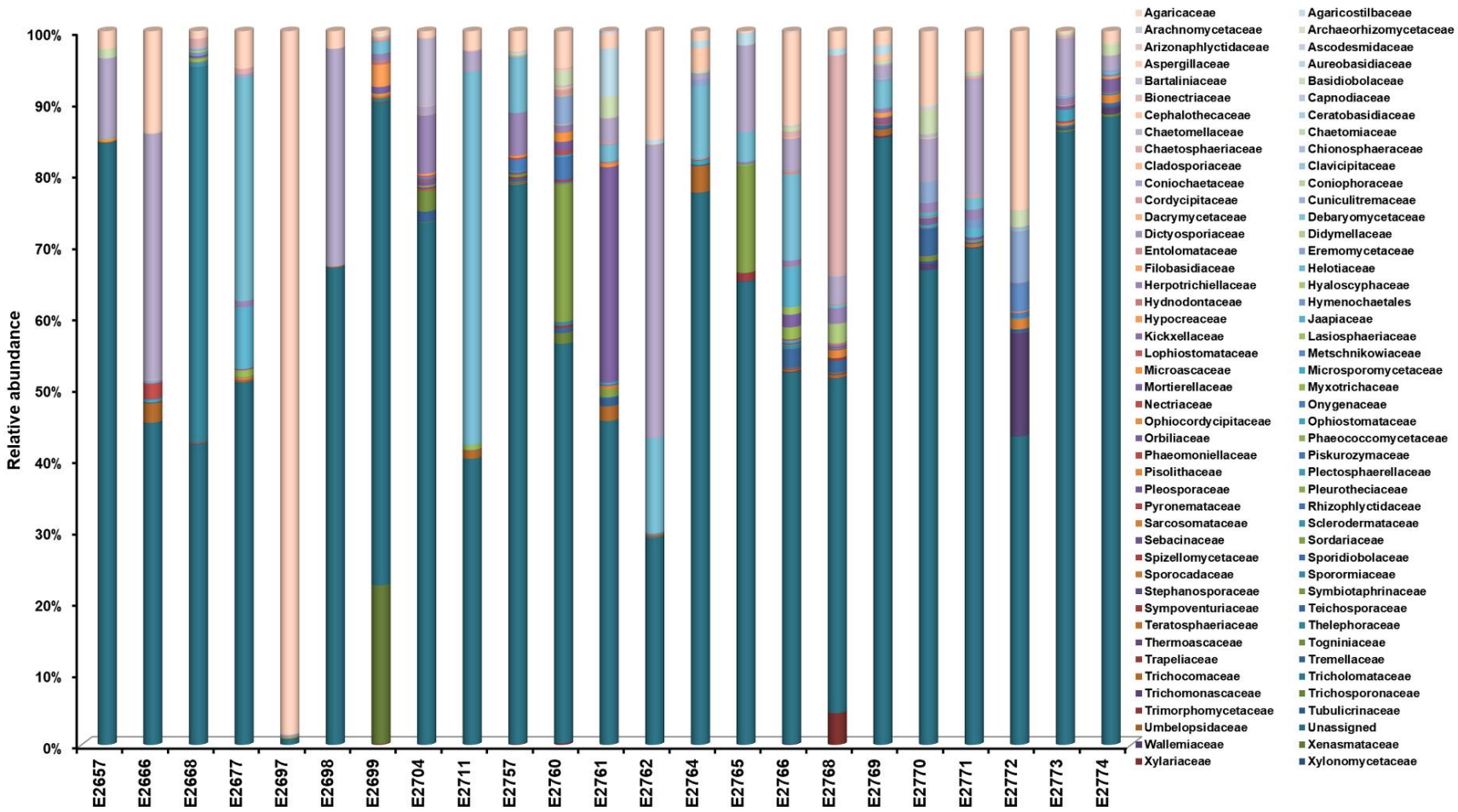
SUPPLEMENTARY FIGURE S2.1.1 The inferred taxonomic composition of fungal communities at phylum level for environmental samples collected from 23 tree hollows in New South Wales, Australia



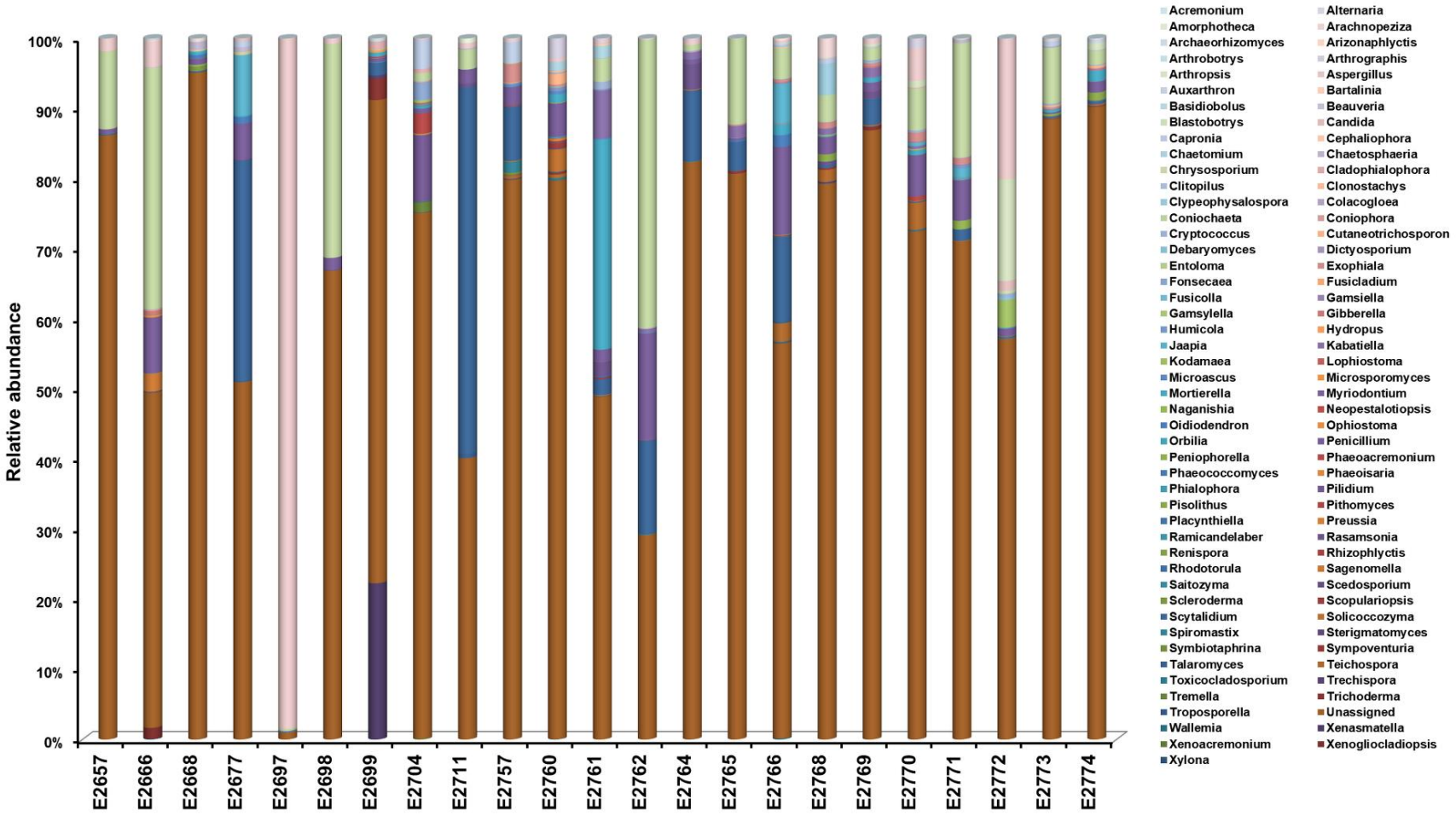
SUPPLEMENTARY FIGURE S2.1.2 The inferred taxonomic composition of fungal communities at class level for environmental samples collected from 23 tree hollows in New South Wales, Australia.



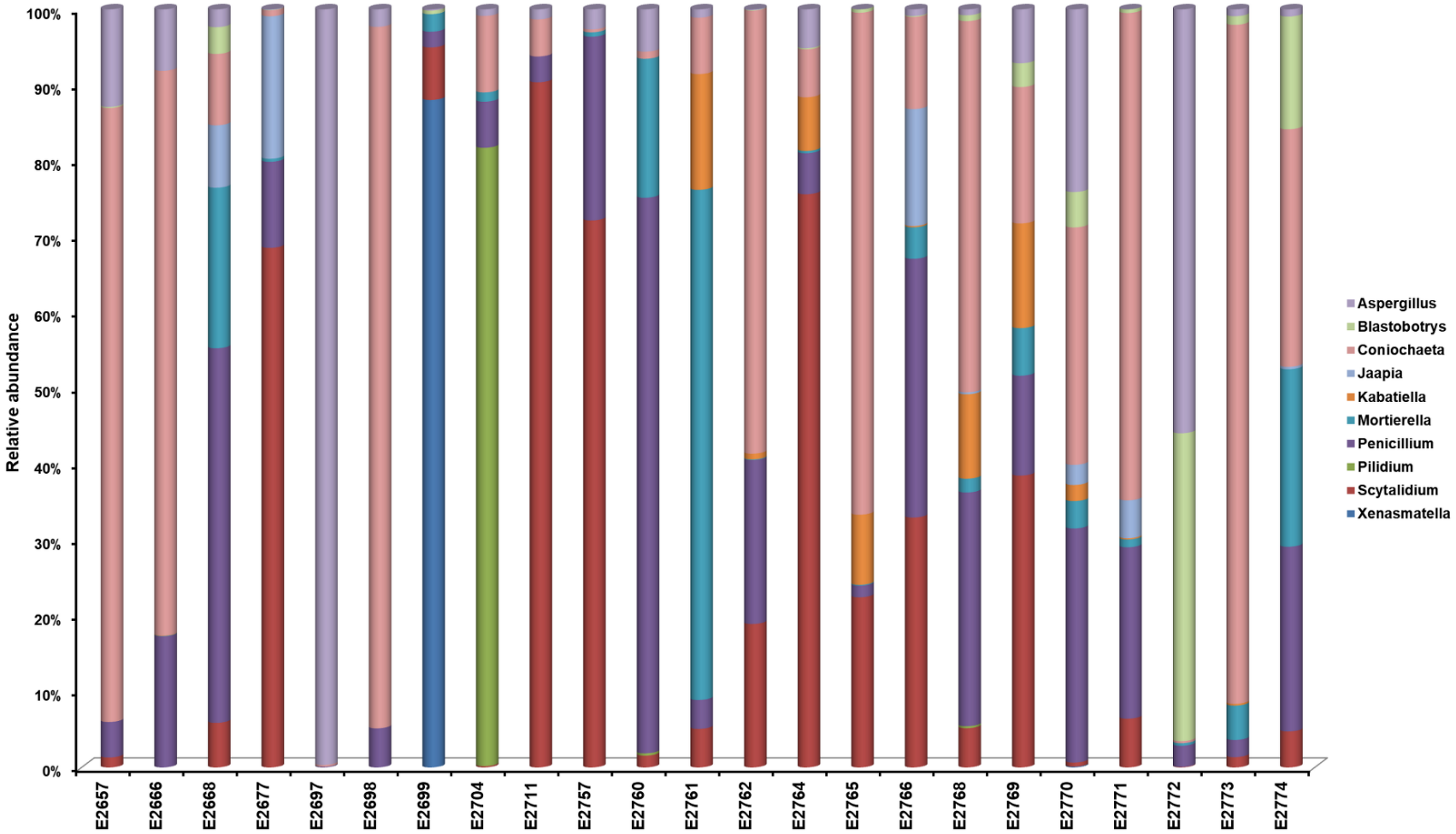
SUPPLEMENTARY FIGURE S2.1.3 The inferred taxonomic composition of fungal communities at order level for environmental samples collected from 23 tree hollows in New South Wales, Australia.



SUPPLEMENTARY FIGURE S2.1.4 The inferred taxonomic composition of fungal communities at family level for environmental samples collected from 23 tree hollows in New South Wales, Australia.



SUPPLEMENTARY FIGURE S2.1.5 The inferred taxonomic composition of fungal communities at genus level for environmental samples collected from 23 tree hollows New South Wales, Australia.



SUPPLEMENTARY FIGURE S2.1.6 The 10 most abundant genera within the mycobiome of the 23 tree hollows in New South Wales, Australia, without the unassigned operational taxonomic unit for each sample.

3. Koala cryptococcosis

Background:

Cryptococcosis has been well-characterised in captive koalas, but free-ranging populations remain largely unstudied. In particular, the acquisition and prevalence of disease (both clinical and subclinical) and nasal colonisation in these animals is unknown. The diagnosis and treatment of cryptococcosis in both captive and free-ranging animals also remain problematic: there have been no systematic studies into treatment protocols, and the most widely used serological test in this species, the LCAT, is expensive, time-consuming and inaccessible to field researchers. This has likely impeded large-scale studies in free-ranging populations. Individual and environmental risk factors for the development of colonisation or disease in free-ranging koalas remain unexplored, and it is difficult to survey animals for evidence of early exposure. In this section, three publications are presented together based on their relevance to clinical disease, diagnostics and treatment, while also providing insights into host-pathogen-environment interactions and thus addressing the central hypothesis.

Section 3.1 reports unusual cases of cryptococcosis in an adult free-ranging koala and concurrent subclinical disease in its joey (index cases for the cluster discussed in section 4.1). These cases provided an opportunity to explore the acquisition, diagnosis and treatment of clinical and subclinical cryptococcosis in the context of previously published reports. Section 3.2 compares two methodologies for serological testing in koalas (along with cats and dogs) – the LFA and the LCAT, to validate the LFA in these species. Inter-species differences in the sensitivity and specificity of this test are also discussed. Section 3.3 used a surveillance finding of cryptococcal pneumonia in an individual to prompt an investigation into the prevalence of subclinical disease and nasal colonisation in a sub-population of free-ranging koalas. The validation of the LFA in the previous section was vital to this. This explored the utility of koalas as a sentinel species while also assessing risk factors for developing cryptococcosis, an important consideration in addressing the central hypothesis.

Aims:

- **Aim 2:** systematically document the successful diagnosis and therapy of subclinical cryptococcosis in free-ranging koalas; and

- **Aim 3**: assess the relationship between environmental presence, nasal colonisation and disease in some free-ranging koala populations.

Sections:

- **3.1:** Cryptococcosis in the koala (*Phascolarctos cinereus*): pathogenesis and treatment in the context of two atypical cases;
- **3.2:** Comparing immunochromatography with latex antigen agglutination testing for the diagnosis of cryptococcosis in cats, dogs and koalas; and
- **3.3:** Prevalence of cryptococcal antigenemia and nasal colonization in a free-ranging koala population.

Impact:

An improved understanding of factors involved in the acquisition of this disease by the host, along with its effective and prompt diagnosis and treatment, will aid in the prevention and management of cryptococcosis in all species. Reports of successful treatment, and reviews of previously attempted protocols, will aid in the future treatment and management of cryptococcosis in koalas. Serological testing for cryptococcal antigen could be improved and simplified in koalas (and other animal species) through the validation of the LFA, which may allow for reliable screening, the detection of both low-grade exposure to environmental *Cryptococcus* spp. and the determination of the full resolution of disease during treatment. Critically, the LFA could also provide a means for rapid, cost-effective point-of-care serological testing, including during remote field work with free-ranging animals. Broad-scale testing of a sub-population of free-ranging koalas could also allow for risk factors involved in the acquisition of subclinical disease and/or nasal colonisation to be identified. This addresses the central hypothesis of the thesis and tests the utility of free-ranging koalas as sentinels for cryptococcosis in humans and other species in Australia.

3.1 Cryptococcosis in the koala (*Phascolarctos cinereus*): pathogenesis and treatment in the context of two atypical cases

Authorship declaration:

This manuscript was published in *Medical Mycology* in 2018 (volume 56, issue 8, pages 926-936). L.J. Schmertmann is the primary author and contributed to all aspects of this study (designing and performing the research, analysing the data and preparing the manuscript). K. Stalder, D. Hudson, P. Martin and M. Makara were involved in the acquisition of data. W. Meyer and R. Malik designed the research. M.B. Krockenberger designed the research and collected data. All authors were involved in the revision of the manuscript and support its inclusion in this thesis.

Laura J. Schmertmann _____ March 2019

Mark B. Krockenberger _____ March 2019

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Cryptococcosis in the koala (*Phascolarctos cinereus*): pathogenesis and treatment in the context of two atypical cases

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Abstract

Disseminated cryptococcosis caused by *Cryptococcus gattii* (molecular type VGI) was diagnosed in an adult free-ranging female koala (*Phascolarctos cinereus*). Subclinical cryptococcosis was later diagnosed in this koala's joey. In the adult koala, a pathological fracture of the tibia was associated with the bone lysis of marked focal cryptococcal osteomyelitis. Limb-sparing orthopedic intervention, in the setting of disseminated cryptococcosis, was judged to have a poor prognosis, and the adult koala was euthanized. The joey was removed and hand-reared. Serological testing revealed persistent and increasing cryptococcal capsular antigenemia in the absence of clinical signs of disease and it was subsequently treated with oral fluconazole for approximately 16 months, rehabilitated and released into the wild. It was sighted 3 months post-release in a good state of health and again at 18 months post-release but was not recaptured on either occasion. This is the first published report of cryptococcal appendicular osteomyelitis in a koala. It is also the first report of concurrent disease in a dependent juvenile and the successful treatment of subclinical cryptococcosis to full resolution of the cryptococcal antigenemia in a free-ranging koala. This paper provides a discussion of cryptococcal osteomyelitis in animals, host-pathogen-environment interactions and treatment and monitoring

protocols for cryptococcosis in koalas. Published reports describing the treatment of cryptococcosis in koalas are also collated and summarised.

Key words: cryptococcosis, *Cryptococcus gattii*, koala, osteomyelitis, treatment.

Introduction

Cryptococcosis, an invasive fungal disease affecting a wide variety of animals worldwide, is caused by members of the *Cryptococcus neoformans* and *C. gattii* species complexes,[1] with recent proposed division of these complexes into seven species and several hybrids remaining controversial.[2] The koala *(Phascolarctos cinereus)* exhibits a comparatively high prevalence of both clinical and subclinical cryptococcosis and of asymptomatic colonisation of the nasal mucosa by members of the *C. gattii* species complex, presumably due to its close association with eucalypts.[3–6] Up to 4% of koala mortalities have been estimated to be attributable to this disease.[7]

Inhalation is considered the primary route of infection, with koalas being no exception; thus the respiratory tract is the most common site of initial cryptococcal lesions. Dissemination, especially to the central nervous system, is a common sequel to chronic disease.[3,8] Osteomyelitis of the appendicular skeleton is a rare but well-documented manifestation of disseminated cryptococcosis in human patients.[9] While it has not previously been reported in the koala, sporadic reports are available in other animal species.[10–18]

Captive koalas have been treated for clinical and subclinical cryptococcosis with varying degrees of success using similar protocols to those for domestic small animal species, namely, combination therapy using oral triazoles and parenteral amphotericin B, in concert with surgical excision or debulking of mass lesions where possible (Table 1).[19–25] Although there are few published reports, our group frequently manages cases of clinical and subclinical cryptococcosis in captive koalas. However, there are no reports of the successful treatment of a free-ranging koala to the point of full resolution of the cryptococcal antigenemia and subsequent release into the wild.

This report presents two unique cases of koala cryptococcosis: a free-ranging adult koala with disseminated cryptococcosis including appendicular osteomyelitis and the successful treatment of subclinical cryptococcosis of the adult koala's joey. Cryptococcal osteomyelitis as an unusual clinical presentation of disease, host-pathogen-environment interactions, and protocols for the diagnosis and treatment of subclinical and clinical cryptococcosis in koalas are discussed. Published cases of attempted treatment of cryptococcosis in koalas are summarised and reviewed in brief.

Case 1

An adult female free-ranging koala with a female joey came to the attention of the public in Soldiers Point, New South Wales (NSW), Australia in September 2013. The pair were captured and taken to a local veterinary hospital due to the reported presence of a large wound on the right hind limb of the adult and its reluctance to move from a single tree over several days. On clinical examination, the adult was in good body condition and estimated to be approximately 3 to 4 years of age, based on dentition and microchip records indicating a brief capture and release event as a subadult approximately 18 months earlier. An ulcerated, fleshy, irregularly contoured, multi-lobulated mass (53 × 40 × 20 mm) protruded from the medial aspect of the distal tibia and proximal tarsus of the right hind limb (Fig. 1a) with palpable underlying bony instability. The ulcerated mass was surrounded by a 10 mm wide zone of alopecia and was subject to myiasis. The right inguinal lymph node was markedly enlarged.

Lateral and craniocaudal (Fig. 1b) radiographic projections of the right hind limb demonstrated a large soft tissue opacity corresponding to the observed mass with underlying severe osteolysis of the distal tibia and proximal tarsus. There was also diffuse soft tissue swelling of tissues distal to the right stifle and proximal to the metatarsal bones. Three-view thoracic radiographs were unremarkable.

The mass was cleaned, and the koala was treated empirically with subcutaneous enrofloxacin (Enrotril, Ilium) at a dose rate of 10 mg/kg once daily to control secondary bacterial infection and oral tramadol (Tramal Oral Drops, bioCSL Pty Ltd) at 1 mg/kg twice daily for analgesia.

Hematology revealed a moderate leukocytosis comprised of a neutrophilia and monocytosis, (Table 2) consistent with an inflammatory leukogram likely combined with a stress response. Serum biochemistries revealed a mild hyperglobulinemia, supportive of the inflammatory leukogram, but were otherwise unremarkable (Table 3). Cytological evaluation of fine needle aspirates of the mass and the right inguinal lymph node revealed large numbers of encapsulated, narrow-based budding yeast cells suggestive of *Cryptococcus* spp. Latex agglutination cryptococcal antigen testing (LCAT) was positive with a titer of 1:128. The positive cryptococcal serology results combined with cytological observations and evidence of spread to a regional lymph node were suggestive of disseminated cryptococcosis.

Table 1. A summary of published cases of the attempted treatment of cryptococcosis in koalas.

Source	Captivity	Site of lesion/s	LCAT titers Initial	LCAT titers Peak	Clinical signs	Treatment protocol	Treatment duration	Outcome
[19,20]	C	Nasal	1:512	1:2048	Nonspecific	1. FCZ 25 mg/kg BID PO then: 2. FCZ 25 mg/kg BID PO and AmpB 0.5 mg/kg SC every 72 hours then: 3. Surgical debulking and ICZ 10 mg/kg BID PO and AmpB 0.25 mg/kg intralesional every 72 hours (for seven doses) then: 4. ICZ 10 mg/kg SID PO	>1 year	Survival; antigenemia unresolved
[21]	C	Nasal	...	...	Nasal	ICZ 10 mg/kg BID PO	5 days	Death
[22]	C	Nasal	1:1024	1:2048	Nasal	1. FCZ 20 mg/kg BID PO and AmpB 0.5 mg/kg SC three times a week then: 2. KCZ 10 mg/kg BID PO and AmpB 0.5 mg/kg SC three times a week	6 months	Survival; antigenemia unresolved
[23]	C	Unknown	1:1024	1:32768	Nasal	ICZ 20–40 mg/kg/day PO and AmpB 1 mg/kg/day vaporised (pulse therapy; three pulses of 1 week, 2 weeks and 1.5 months)	6 months	Death
[24]	C	Nasal	...	...	Nasal	FCZ 15 mg/kg IV (two doses) and AmpB 0.5 mg/kg SC (one dose)	Unknown	Death
[25]	C	Unknown	1:2*	1:4*	No	FCZ 10 mg/kg BID PO	>2 months*	Survival; antigenemia resolved*
[25]	C	Unknown	1:2*	1:2*	No	FCZ 10 mg/kg BID PO	>2 months*	Survival; antigenemia resolved*
[25]	C	Unknown	1:8*	1:8*	No	FCZ 10 mg/kg BID PO	>2 months*	Survival; antigenemia resolved*
[25]	C	Unknown	1:4*	1:128*	Yes	FCZ 10 mg/kg BID PO	>2 months*	Survival; antigenemia resolved*
[25]	C	Nasal; lung*	1:256*	1:512*	Yes	FCZ 10 mg/kg BID PO	>2 months*	Death
[25]	C	Nasal*	1:32768*	1:32768*	Nasal*	FCZ 10 mg/kg BID PO and AmpB 0.7–0.8 mg/kg SC twice weekly	>18 months*	Survival; antigenemia resolved*
[25]	C	Nasal; lung; CNS*	1:512*	1:2048*	Yes	FCZ 15 mg/kg BID PO and AmpB 0.7–0.8 mg/kg SC twice weekly	≥2 weeks	Death
[25]	C	Mandibular abscess*	1:64*	...	Yes	FCZ 15 mg/kg BID PO (and AmpB 0.7–0.8 mg/kg SC twice weekly*)	>2 months*	Death
[25]	C	Eyelid*	1:4*	1:8*	Yes	FCZ 15 mg/kg BID PO and AmpB 0.7–0.8 mg/kg SC twice weekly	>2 months*	Survival
This paper	FR	Unknown	1:8	1:64	Nonspecific	1. FCZ 25 mg/kg BID PO then: 2.FCZ 50 mg/kg BID PO, gradually reduced back to 25 mg/kg BID PO	16 months	Survival; antigenemia resolved; release

AmpB, amphotericin B; BID, twice daily; C, captive; CNS, central nervous system; FCZ, fluconazole; FR, free-ranging; ICZ, itraconazole; IV, intravenous; KCZ, ketoconazole; LCAT, latex cryptococcal antigen agglutination test; PO, by mouth; SC, subcutaneous; SID, once daily. *Unpublished data provided by M.B. Krockenberger.

The koala was euthanized due to an overall poor prognosis for full functional recovery.

Post mortem computed tomography was performed at the University Veterinary Teaching Hospital Sydney. This confirmed and characterized further the extent of the marked destruction of the right distal tibia (Fig. 2). No sinonasal lesions were identified.

Necropsy was performed at the Sydney School of Veterinary Science, The University of Sydney. In addition to the ulcerated tibiotarsal lesions and inguinal lymphadenomegaly, a round subcutaneous mass (6 mm diameter) containing thick yellow to green colored fluid was detected in the ventral cervical subcutaneous tissues. Additionally, a focal cystic lesion of the dorsal abdominal wall adjacent to the aorta at the level of the adrenal glands, a pale focal lesion (4 mm diameter) in the right renal cortex, widespread multifocal streaky white pulmonary lesions and a well-circumscribed pulmonary nodule (10 mm diameter) were present. Moderate rounding of the hepatic lobar borders and widespread, uniform nodularity of the liver were also detected. No lesions were detectable grossly in the nasal cavity, upon serial sectioning of the head. Cytological evaluation of a needle aspirate from the cervical lesion revealed encapsulated yeast organisms. Histopathological examination of tissues revealed further encapsulated yeasts and associated granulomatous inflammation in the pulmonary

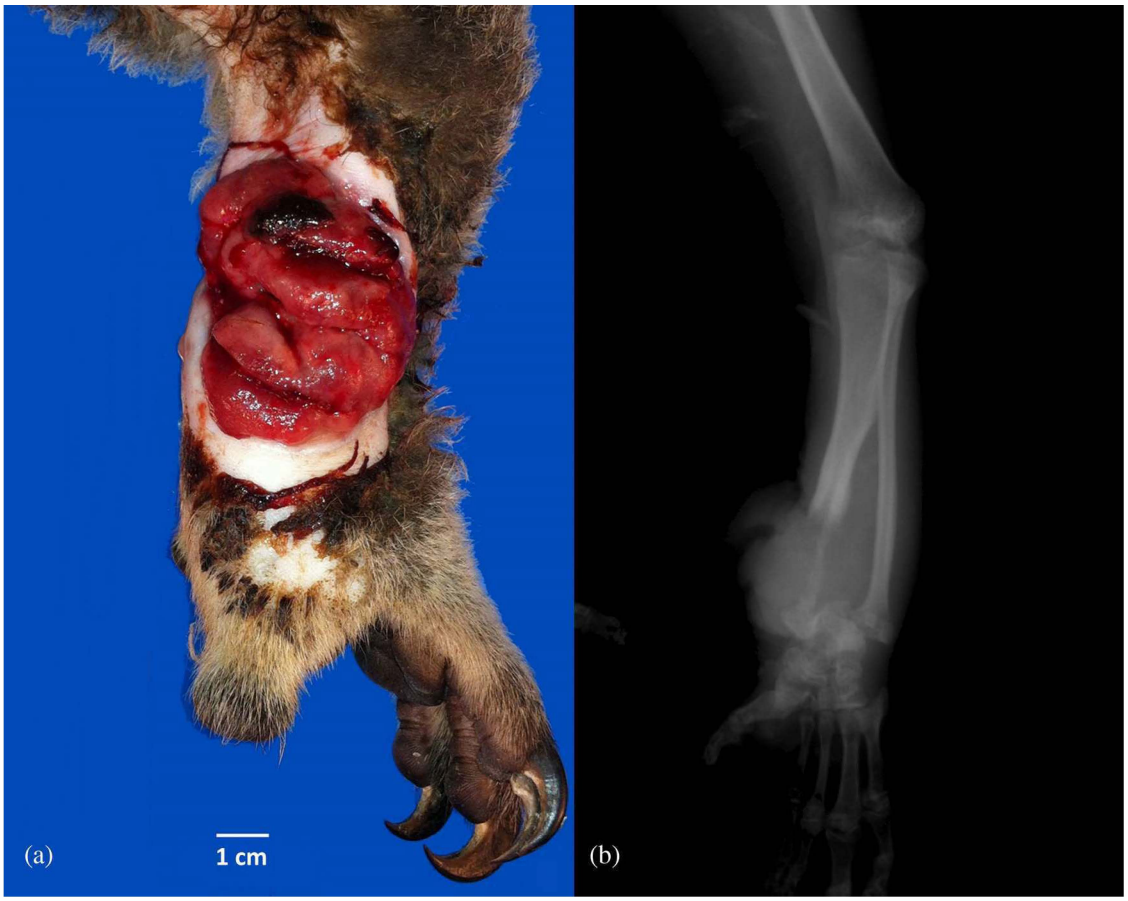

Figure 1. (a) Cryptococcal mass lesion of the distal right hind limb in an adult koala (case 1). (b) Craniocaudal radiographic projection of the distal right hind limb in case 1 showing the osteolytic changes consistent with osteomyelitis of the distal tibia and proximal tarsus and surrounding soft tissue swelling. This Figure is reproduced in color in the online version of *Medical Mycology*.

nodule, scattered throughout the pulmonary parenchyma, peri-aortic lesion, right inguinal lymph node, focal renal lesion and the mass lesion of the right hind limb. Mycotic abscessation of the right inguinal lymph node was also apparent. Small bony fragments were visible in the deeper aspects of the hind limb lesion. Mild multifocal meningitis and a focal submeningeal granuloma containing encapsulated yeasts were apparent overlying the cerebrum. The liver exhibited multifocal moderate fibrosis, occasional small aggregates of a mixed inflammatory infiltrate and increased hepatocellular turnover. Samples for culture were collected aseptically from the right inguinal lymph node and a member of the *C. gattii* species complex was isolated. Restriction fragment length polymorphism analysis of the *URA5* gene identified the isolate as *C. gattii* molecular type VGI.[26] These results confirmed the diagnosis of disseminated cryptococcosis with appendicular osteomyelitis caused by *C. gattii* molecular type VGI.

The koala tested positive for koala retrovirus (KoRV) variant KoRV-A and negative for variant KoRV-B using DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues (spleen and lymph node) and a minor adaptation of established polymerase chain reaction (PCR) protocols.[27] Other KoRV variants were not tested for.

Case 2

The female joey, a dependent backyoung weighing 0.9 kg, of case 1 was captured with its dam and presented for veterinary assessment. No abnormalities were detected on physical examination. The joey was placed in to care with Port Stephens Koalas (Port Stephens, NSW, Australia) and hand-reared after euthanasia of the adult. Approximately 1 week after the initial rescue, blood was collected for LCAT after the diagnosis of disseminated cryptococcosis in its dam was confirmed. A positive result was recorded with a titer of 1:8 (Table 4). In the absence of clinical signs, subclinical cryptococcosis was suspected. Serology was repeated one month after the initial test: the LCAT titer had

Table 2. Hematology results for case 1 with reference intervals.

Analyte	Value	Reference interval[28]
Hemoglobin(g/l)	94	88–140
Hematocrit	0.31	0.29–0.44
MCH (pg)	**29**	33.1–35.1
MCHC (g/l)	301	298–330
MCV (fl)	97	94–117
Erythrocytes ($\times 10^9$/l)	3.2	2.7–4.2
Leukocytes ($\times 10^9$/l)	**24.6**	2.8–11.2
Neutrophils ($\times 10^9$/l)	**21.2**	0.5–6.3
Lymphocytes ($\times 10^9$/l)	2.5	0.2–5.8
Monocytes ($\times 10^9$/l)	1.0	0.0–0.6
Eosinophils ($\times 10^9$/l)	0.0	0.0–1.1
Basophils ($\times 10^9$/l)	0.0	...
Reticulocytes (%)	**4.8**	1.5–3.5
nRBCs (/100 leukocytes)	1	1–20
Platelets ($\times 10^9$/l)	392	222–508

MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; nRBCs, nucleated red blood cells.

Table 3. Serum biochemistry results for case 1 with reference intervals.

Analyte	Value	Reference interval[28]
Total protein (g/l)	80	58–83
Albumin (g/l)	**26**	34–50
Globulin (g/l)	**54**	18–39
Alkaline phosphatase (U/l)	58	25–219
Alanine transaminase (U/l)	20	0–236
Amylase (U/l)	47	...
Bilirubin (μmol/l)	3.42	0–8
BUN (mmol/l)	2.14	...
Creatinine (μmol/l)	**62**	80–150
Glucose (mmol/l)	0.2–6.6	2.7–7.2
Calcium (mmol/l)	**2.07**	2.28–2.97
Inorganic phosphate (mmol/l)	**0.71**	0.79–1.96
Potassium (mmol/l)	3.7	3.5–6.8
Sodium (mmol/l)	**122**	132–145

BUN, blood urea nitrogen.

changed to 1:64 (Table 4), suggestive of expansion of the subclinical focus of infection despite the absence of any clinical signs of disease. Antifungal therapy was therefore initiated. Oral generic fluconazole (Bova Compounding©) was administered at a dose rate of approximately 25 mg/kg twice daily. Cryptococcal antigenemia was monitored every 1 to 3 months during treatment with the lateral flow assay (LFA) (CrAg® LFA, IMMY) and LCAT (CALAS®, Meridian Bioscience, Inc.). Serological testing was performed in the same laboratory in every instance (Veterinary Pathology Diagnostic Services, The University of Sydney).

During the first month of treatment, the joey became acutely unwell, exhibiting a quiet demeanour, diarrhea, and inappetence. Supportive care, including subcutaneous fluid therapy and nutritional support (Di-Vetelact®, Sharpe Laboratories and Nutrigel, Ilium), combined with an increased dose of oral fluconazole (approximately 50 mg/kg twice daily) and a single dose (10 mg) of subcutaneous dexamethasone (Dexapent, Ilium) was successful in restoring the joey to normal health after several days. The oral fluconazole dose was gradually reduced back to approximately 25 mg/kg twice daily. No further such episodes occurred, and the joey remained clinically normal until its eventual release.

Treatment continued for a total of 16 months until serology was negative for cryptococcal antigen for both the LCAT and LFA on consecutive tests at a minimum interval of 1 month (Table 4). At the time of the cessation of treatment, the koala was a subadult at over 2 years of age and ready for release, pending transfer to the Koala Hospital (Port Macquarie, NSW, Australia) for a de-humanization period. The koala remained negative for cryptococcal antigenemia when tested immediately after transfer and just prior to release, the final test being approximately 5 months after the cessation of treatment.

The koala was released approximately 21 months after capture. A microchip was implanted, and a yellow numbered ear tag was placed in the right ear prior to release to aid identification in the event of a sighting or recapture event. No other koalas with ear tags are known to be present in this region. Three months after release, a sighting of a koala with a yellow ear tag in close proximity to the release site was reported by a member of the public. Photographic evidence of the sighting demonstrates the presence of the tag in the right ear, although individual identification cannot be definitively confirmed. The koala appeared to be a young adult female. Based on the location, sex, and apparent age of the koala and presence of the ear tag, it is considered extremely likely that this sighting was of the same animal. The report and photographs suggest that the koala was behaving normally. A second sighting of a koala with an ear tag was reported in the same area approximately 18 months after release, but no photographic evidence is available.

Discussion

Cryptococcosis is predominantly acquired from the environment by inhalation. Colonization of mucosal surfaces is the initial outcome of infection, potentially followed by local disease. This may either be constrained by the normal innate and acquired immune response of the host or progress to clinical disease, depending on factors such as

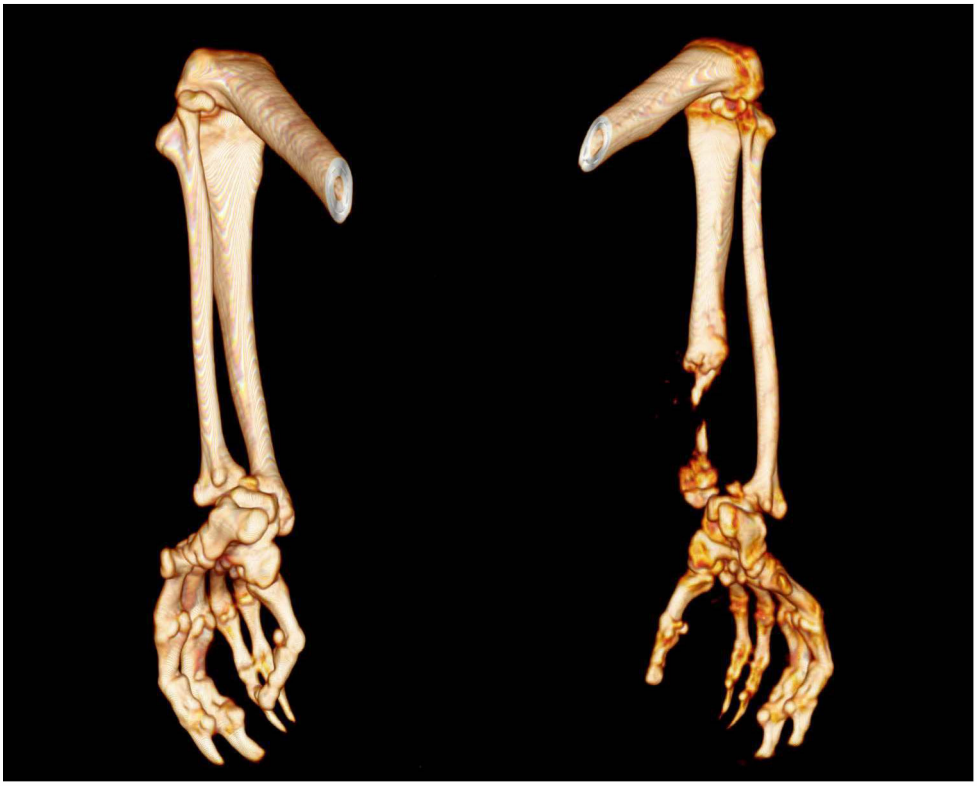

Figure 2. Computed tomographic three-dimensional reconstructions of the distal left and right hind limbs of an adult koala (case 1), comparatively highlighting the marked destruction of the right distal tibia due to cryptococcal osteomyelitis. This Figure is reproduced in color in the online version of *Medical Mycology*.

Table 4. Cryptococcal antigen serology results for case 2.

Date	LCAT	LCAT titer	LFA	Comments
October 2013	Positive	1:8	...	Initial test
November 2013	Positive	1:64	...	Treatment begins
January 2014	Positive	1:64	...	
April 2014	Positive	1:8	Positive	
June 2014	Positive	1:2	Positive	
August 2014	Negative	...	Positive	
September 2014	Negative	...	Positive	
October 2014	Negative	...	Positive	
December 2014	Negative	...	Negative	
February 2015	Negative	...	Negative	Treatment ceases
March 2015	Negative	...	Negative	After transfer
June 2015	...	...	Negative	Before release

LCAT, Latex cryptococcal antigen agglutination test; LFA, Cryptococcal antigen lateral flow assay.

the size of the inoculum and the age and overall health of the host. Following the development of a primary lesion in the upper or lower respiratory tract, the fungus has the potential to invade locally into contiguous tissues, or to disseminate hematogenously or via the lymphatics.[3,8] Solitary cutaneous lesions have been identified in numerous koalas, suggesting that direct inoculation is also a route of infection and can give rise to localized variably invasive cutaneous and subcutaneous disease.[3] The distribution of solitary cutaneous lesions is generally around the mouth and philtrum, the ears, and the base of the claws.[4] The authors speculate that cryptococcal colonization of the nails and nail beds[6] may facilitate inoculation of the skin of the head through minor grooming injuries. Appendicular osteomyelitis subsequent to disseminated cryptococcosis has been reported in canine, equine, feline and avian patients.[11,12,14,15] Several cases involving a solitary cryptococcal lesion of the appendicular skeleton have been identified

in dogs and a cat, but in all cases the possibility of dissemination from an unidentified primary lesion could not be excluded.[10,13,16–18] This report presents the first published case of cryptococcal appendicular osteomyelitis in the koala and indicates that cryptococcosis should be considered as a differential diagnosis for lameness, mass lesions, and destructive bony lesions in this species. Cryptococcal osteomyelitis and osteolysis of the axial skeleton have been identified as potential sequelae to locally invasive lesions of the upper respiratory tract in koalas as well as cats, dogs, and birds.[3,29–32] The majority of human cases of cryptococcal appendicular osteomyelitis are attributable to infection with the *C. neoformans* species complex.[9] However, in the reported animal cases, there is no apparent tendency toward any member of the *C. gattii* or *C. neoformans* species complexes as the etiological agent, with the notable exception of koalas, in which all cases have involved the *C. gattii* species complex.[3]

The prognosis for cryptococcosis in koalas is generally considered guarded to poor, depending on the severity of clinical signs and the extent of lesions at the time of diagnosis.[33] In this case, due to the degree of destruction of the distal tibia in conjunction with disseminated disease and the requirement for orthopedic intervention, the prognosis was judged to be very poor. Limb-sparing surgery with effective antifungal therapy and subsequent return to normal function for a free-ranging koala was considered unlikely to be able to be achieved in this clinical setting.

While only 77% of koalas with cryptococcosis are found to have detectable respiratory tract lesions,[3] there is a compelling case for early pathogenesis of cryptococcosis in all species to include a primary respiratory focus. Therefore, it is likely that the tibiotarsal lesion developed as a result of dissemination from the pulmonary lesions. However, it is prudent to also consider the possibility of a primary tibiotarsal lesion subsequent to direct inoculation. This may be supported by the advanced nature of the lesion, marked lymphadenopathy of a draining lymph node (in the absence of a more generalized lymphadenopathy), relatively low LCAT titer (1:128) and fairly limited dissemination. Co-infection via both inhalation and direct inoculation is also possible.

Host-pathogen-environment interactions involved in colonization of koalas by the *C. gattii* species complex and the comparatively high prevalence of subclinical and clinical cryptococcosis in this species remain relatively poorly understood. In both cases presented in this report, no obvious causes of increased host susceptibility were apparent. Retroviral testing of case 1 was positive for KoRV-A and negative for KoRV-B. These findings are consistent with the koala originating from a population likely to have 100% prevalence of endemic KoRV-A inserted in the genome.[34] KoRV-A is an endogenous variant considered unlikely to have any significant immunomodulatory effects on koalas; conversely the exogenous variant KoRV-B has been associated with altered immune function, chlamydiosis and malignant neoplasia.[27,35,36] The relationship between cryptococcosis and KoRV infection has not been studied systematically. No other evidence of concurrent disease or immunosuppression could be detected on *ante mortem* hematology or necropsy. Pathogen-related factors, including the possibility of a highly virulent strain of *C. gattii* molecular type VGI, cannot be excluded, and further epidemiological molecular analysis of the strain isolated from this koala may be pertinent.

Most reported cases of clinical cryptococcosis in koalas where the causative organism was identified to a species level have been attributed to infection with a member of the *C. gattii* species complex,[3,20] with *C. neoformans* var. *grubii* (molecular type VNI) identified as the etiologic agent in one case based on PCR and sequencing of DNA extracted from FFPE tissue.[21] This is in contrast with cats and dogs, where the *C. neoformans* species complex tends to be responsible for a greater proportion of cases, even in places like Australia where the *C. gattii* species complex is endemic.[30,31,37] This difference is likely to be attributable to the close contact between koalas and eucalypts, a common environmental niche for *C. gattii* molecular type VGI, with *Eucalyptus camaldulensis* and *E. tereticornis* both consistent environmental sources of the *C. gattii* species complex[38,39] and common feed species for koalas.[40] *E. tereticornis* trees are an important part of the bushland at Soldiers Point, NSW, Australia from which this koala originated and *C. gattii* molecular type VGI was easily recoverable from this environment in preliminary investigations, which suggests a substantial cryptococcal environmental load in this area. High environmental burdens of the *C. gattii* species complex have been associated with a higher incidence of nasal colonization and subclinical disease in koalas.[4,5] Further sampling is required to expand on this notion and the role that it may play in the host-pathogen-environment interaction in this specific environment.

The second case presents the first published report of the successful treatment of subclinical cryptococcosis in a free-ranging koala. Table 1 summarises all published cases of attempted treatment of cryptococcosis in koalas. With the exception of this publication, all involve only captive koalas. Two publications reported successful treatment to the point of survival more than one year after diagnosis, although both appear to describe the same case.[19,20] Another reported treatment success in a captive koala up to six months after diagnosis but longer term

survival is unknown.[22] In one publication focusing on the pharmacokinetics of fluconazole administered orally to koalas, treatment was attempted in nine koalas with clinical ($n = 6$) or subclinical ($n = 3$) cryptococcosis.[25] In the clinical group, full recovery was reported in two cases,[25] although a third case also achieved resolution of antigenemia and clinical signs (Krockenberger, M.B., unpublished data). Outcomes in the subclinical group were not reported but all three koalas survived and the antigenemia fully resolved (Krockenberger, M.B., unpublished data). The exact duration of treatment was unclear and cases were lost to further follow-up. Among the few published reports, common themes for successful treatment appear to include early diagnosis and aggressive, persistent antifungal therapy. However, survival and full resolution of antigenemia was achieved in one case with a LCAT titer of 1:32768 and severe clinical signs at the time of diagnosis (Krockenberger, M.B., unpublished data). Pulse therapy proved ineffective in another case.[23] In the experience of some of the current authors, numerous other captive koalas have been successfully treated to full resolution for both clinical and subclinical cryptococcosis; nevertheless, successful therapy is challenging and the majority of these cases remain unpublished. Therapeutic dose monitoring is advisable given the known pharmacokinetics of some of the azole agents. This paper is the first published report of the full resolution of cryptococcosis in a koala to the point of negative serology results and subsequent cessation of treatment.

Subclinical cryptococcosis is considered a relatively common finding in koalas, particularly in the presence of a high cryptococcal load in the environment.[5] Depending on individual circumstances, the authors generally recommend treating any koala with an antigen titer greater than 1:8, where the titer persists at this level over time, or increases further. Treatment at lower antigen titers may be considered depending on the specific case details. In case 2, the initial titer was 1:8 but changed to 1:64 after 1 month, and there was concern that the stress of adjusting to handling and captivity was activating a quiescent focus of infection in the joey, particularly knowing that it had been exposed to the same environment as its dam which had succumbed to disseminated cryptococcosis. It is possible that this progression to overt clinical cryptococcosis occurred when the joey became acutely unwell early in treatment, as the LCAT titer of 1:64 at the time is the upper limit of what we have seen in subclinical disease in this species.[5] It may have been prudent to assess the joey for the presence of any cryptococcal lesions or concurrent pathologies through fecal culture, thoracic imaging, sinonasal computed tomography, and/or hematology and serum biochemistry. Given that no causative organism was isolated, it is possible that the LCAT and LFA results represented false positives. However, this is highly unlikely given the persistence of the positive results, the response to treatment exhibited through the changing LCAT titer, the joey's exposure to the same environment from which its dam contracted cryptococcosis and the high prevalence of cryptococcosis in koalas.

The monitoring protocol used by the authors during treatment involved serum antigen testing at approximately 1-month intervals, with the intention to stop treatment after three consecutive negative tests. The first negative LCAT result was obtained approximately nine months after fluconazole treatment was initiated, and all LCAT results remained negative from this point onwards. However, the LFA remained positive for a further three months. At the time of the third consecutive negative LCAT, the LFA was still positive, consistent with it being the more sensitive of the two tests.[41–43] Therefore, it was decided to continue treatment until consecutive negative LFA results were obtained. The koala was tested again after transfer to a new facility and just prior to release into the wild, due to concern that the potential stress of transfer and dehumanization could cause recrudescence of disease. It remained antigen negative. At present, the LCAT is still the predominant serological test for cryptococcosis in animals. The LFA is not yet routinely used in veterinary diagnostics, primarily due to a lack of published evidence regarding its use in animals. However, the LFA's high sensitivity and 'point of care' accessibility make it an attractive future prospect for veterinary diagnostics.[41–44] Preliminary findings suggest that the LFA has an excellent negative predictive value but is of variable specificity for serum samples in cats, dogs, and koalas, when compared to the LCAT (Krockenberger, M.B., unpublished data). Based on this observation, the authors now commonly use the LFA as a screening test for cryptococcosis in animals in both the laboratory and the field and use the LCAT to confirm positive results and to determine antigen titers, due to the high cost of using multiple LFA test strips.

Variations of the treatment protocol for this koala have been used relatively successfully by the authors on numerous occasions for the treatment of cryptococcosis in other koalas, and are based on treatment protocols for cryptococcosis in dogs, cats and ferrets.[31,33,45,46] The dose rate used for oral fluconazole in small animal patients is typically 10 mg/kg twice daily.[31] The dose rate of 20–25 mg/kg given orally twice daily for koalas was selected at the time based on the prior experience of the authors and has been supported since by a report of overall low and highly variable bioavailability (median 0.53, range 0.20–0.97) in koalas when dosed orally at 10 mg/kg.[47] Another study showed that peak plasma fluconazole concentrations failed to reach the minimum inhibitory concentration for *C. gattii* species

complex isolates when dosed orally at 10–15 mg/kg twice daily.[25] Fluconazole levels were not monitored in this case, though the authors now routinely utilise drug concentration testing to adjust dosages and treatment protocols in koalas. More recently, the authors have begun to use oral itraconazole (10–20 mg/kg once daily with Sporanox® [Janssen-Cilag Pty Ltd]; 10 mg/kg using Lozanoc® [Mayne Pharma International Pty Ltd]) with the experience of generally shorter treatment times and a better tolerance by koalas for once daily dosing. However, the pharmacokinetic profile of oral itraconazole in the koala remains poorly understood. Although there is evidence that posaconazole has a reasonable oral bioavailability in koalas,[48] there are limited clinical data regarding its use as a standard treatment for cryptococcosis in any species, and at present it is prohibitively expensive for most veterinary applications. The use of amphotericin B in conjunction with oral triazole antifungals is recommended for most cases of clinical disease and for refractory subclinical disease, if the antigen titer remains unresponsive to oral triazole monotherapy.[25] The most recent recommendation for amphotericin B dosing in koalas is 0.7–0.8 mg/kg administered subcutaneously twice weekly in a 0.45% NaCl and 2.5% dextrose fluid bolus.[25,49] A cumulative amphotericin B dose requirement for the koala is unknown but likely should be a minimum of 20 mg/kg, based on our experience in adapting protocols developed for feline and canine cryptococcosis.[46,49] Fluconazole monotherapy was preferred in this case, largely for logistical reasons. Amphotericin B was not added to the treatment protocol based on the response to therapy seen through the changing LCAT titer. The use of corticosteroids is generally contraindicated in cases of cryptococcosis due to the risk of immunosuppression. However, glucocorticoid therapy after diagnosis has been associated with improved early survival rates in canine and feline patients with central nervous system cryptococcosis and may have a role in reducing an overactive inflammatory response.[50] In this case, dexamethasone was administered on a one-off basis in conjunction with a high dose of fluconazole due to the rapidly declining condition of the koala.

The release of this koala into the wild had the potential to cause sufficient stress to allow for a recrudescence of latent disease. The first sighting, 3 months after release, confirmed its survival, and its apparently normal behavior suggested that it was coping adequately with its new environment. The timing of this sighting was at the start of breeding season, the first for this koala as a free-ranging adult, and although this stressful period could also have resulted in recrudescence of disease, a likely repeat sighting approximately 18 months after release suggests the ongoing survival of the koala. It would be pertinent to continue to monitor this location for additional sightings to assess the health of this individual.

In conclusion, this report presents the first case of (i) disseminated cryptococcosis with appendicular osteomyelitis in a koala and (ii) the successful treatment of subclinical cryptococcosis, to the point of full resolution of the cryptococcal antigenemia and subsequent release back into the wild, in its dependent juvenile. These cases provided an opportunity to broaden the spectrum of clinical presentations of cryptococcosis and to discuss host-pathogen-environment interactions, diagnostic testing, and treatment protocols for cryptococcosis in koalas.

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Addendum

Just prior to publication, the koala was found at the base of a tree following a period of extreme hot weather. It was taken into care by Port Stephens Koalas, positively identified as the koala (ear tag and microchip) and treated for conjunctivitis and heat stress. Diagnostics were performed in our laboratory (Veterinary Pathology Diagnostic Services). Chlamydial conjunctivitis was confirmed by PCR, cryptococcal serology was negative by both the lateral flow assay (LFA) (CrAg® LFA, IMMY) and latex agglutination assay (CALAS®, Meridian Bioscience, Inc.) and nasal culture for *Cryptococcus neoformans* and *C. gattii* species complexes was negative. It is responding well to therapy for chlamydial conjunctivitis. It remains cryptococcal serology negative without any signs of cryptococcosis almost 3 years following release into the wild.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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3.2 Comparing immunochromatography with latex antigen agglutination testing for the diagnosis of cryptococcosis in cats, dogs and koalas

Authorship declaration:

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Comparing immunochromatography with latex antigen agglutination testing for the diagnosis of cryptococcosis in cats, dogs and koalas

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Abstract

Although the point-of-care cryptococcal antigen lateral flow assay (LFA) has revolutionized the diagnosis of cryptococcosis in human patients, to date there has been no large-scale examination of this test in animals. We therefore assessed the LFA in cats, dogs and koalas suspected of having cryptococcosis. In sum, 528 serum specimens (129 from cats, 108 from dogs, 291 from koalas) were tested using the LFA and one of two commercially available latex cryptococcal antigen agglutination test (LCAT) kits. The LCAT is a proven and well-accepted method in veterinary patients and therefore taken as the "gold standard" against which the LFA was compared. The LFA achieved a sensitivity of 92%, 100%, and 98% in cats, dogs, and koalas, respectively, with corresponding negative predictive values of 94%, 100%, and 98%. The specificity of the LFA was 81%, 84%, and 62% for cats, dogs, and koalas, respectively, with corresponding positive predictive values of 76%, 48%, and 69%. These findings suggest the most appropriate role for the LFA is as a screening test to rule out a diagnosis of cryptococcosis in cats, dogs, and koalas. Point-of-care accessibility makes it equally suited for use in the field and as a cage-side test in veterinary hospitals. The suboptimal specificity of the LFA makes it less suited to definitive confirmation of cryptococcosis in animals; therefore, all LFA-positive test results should be confirmed by LCAT testing. The discrepancy between these observations and the high specificity of the LFA in humans may reflect differences in the host-pathogen interactions amongst the species.

Key words: lateral flow assay, cryptococcal antigen, immunochromatography, *Cryptococcus*, veterinary.

Introduction

Cryptococcosis is a fungal infection of humans[1–3] and animals[4–8] caused by members of the *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes.[9] Rarely, other minimally encapsulated *Cryptococcus* spp. can cause disease, which is usually localized and less severe.[10] Infection typically occurs by inhalation of basidiospores, but direct inoculation can occasionally cause localized cutaneous disease.[3] In human patients, cryptococcosis classically presents as central nervous system (CNS) disease and is therefore often diagnosed using a combination

of cerebrospinal fluid (CSF) analysis (direct examination, mycological culture, and antigen testing), brain imaging studies (computed tomography [CT] or magnetic resonance imaging [MRI]) and serology.[1–3,11,12] In animals, cryptococcosis is more often associated with respiratory tract disease, classically of the nasal cavity and sinuses but also the lower respiratory tract in some species.[3–8] The propensity for dissemination to the CNS also occurs in animals, and therefore neurological changes are still commonly observed.[3–8] A definitive diagnosis in animal cases is often achieved through a combination of the direct observation of suspect organisms (using diagnostic cytology and/or histopathology), mycological culture, or serology. CT and MRI are often useful in a referral setting but are not yet accessible to most veterinary general practitioners and can be cost prohibitive.[1–8,10-12] Mycological culture remains the gold standard for diagnosis in all species, as it allows for both a definitive phenotypic and genotypic identification, and molecular or proteomic (MALDI-tof) typing of the fungal pathogen involved, and the ability to perform susceptibility testing.[3]

Measurement of circulating polysaccharide antigen (glucuronylmannan) in serum or plasma by latex cryptococcal antigen agglutination testing (LCAT) provides evidence of invasive cryptococcosis with both high specificity and sensitivity.[13] The monitoring of sequential LCAT titers has proven to be extremely useful to support therapeutic decisions during the treatment of birds, cats, dogs, ferrets and koalas with cryptococcosis.[4–7,10,14] The accuracy of the procedure is dependent on preincubation of serum with pronase (typically included as part of the test kit) and heat inactivation to remove interfering factors capable of causing false results, including rheumatoid factor, which can result in false positive agglutination, and opsonising antibodies, which can trap cryptococcal antigen.[13] The LCAT is well accepted and validated as a diagnostic test in veterinary laboratories. However, the procedure requires a skilled technician and access to laboratory equipment, including a heat block. The test is also expensive because it is time and labor intensive, which often necessitates sample batching, further delaying turn-around time.

Rapid confirmation or exclusion of the diagnosis of cryptococcosis allows clinicians to adjust diagnostic and therapeutic strategies and has the potential to hasten the diagnosis and reduce costs of investigations for many patients. A cryptococcal antigen lateral flow assay (LFA) (CrAg® LFA, IMMY, Norman, OK, USA) was developed in 2010 as a point-of-care (POC) serological test to diagnose cryptococcosis. The test detects cryptococcal polysaccharide antigen and is currently in widespread usage in medical diagnostic laboratories worldwide.[15–26] This test platform provides room temperature stability and simplicity of execution with minimal laboratory equipment and no pronase pretreatment or heat inactivation required.[17] It is therefore equally suitable as a POC test in a hospital laboratory or in the field. The test has been validated by several mycology groups around the world, with consensus from these reports and careful metanalysis[22] indicating that the LFA is highly suitable as a POC screening test, with comparable specificity and possibly superior sensitivity to the LCAT.[15–26] In Australia, the LFA has tended to replace LCAT testing in routine medical diagnostic laboratories, although the LCAT remains available at reference laboratories and is preferred when an endpoint titer is required for clinical decision making.[17]

The LFA has great potential for use in veterinary medicine as a cage-side or field screening tool to quickly establish whether cryptococcosis can be excluded as a diagnostic possibility in animal patients presenting with suspicious clinical signs, including sinonasal disease, CNS disease, or pneumonia. The test, however, remains unvalidated in animal patients. Cryptococcosis is seen in a plethora of domestic and free-ranging animals in Australia, but is most frequently diagnosed in cats, dogs[4,5] and koalas.[8] The current study was designed to determine the utility of a cryptococcal antigen LFA in animals of these three species in which cryptococcosis was a potential diagnosis.

Methods

Serum specimens included in this study were submitted for cryptococcal antigen testing to two veterinary laboratories (Veterinary Pathology Diagnostic Services, The University of Sydney, NSW, Australia, and Vetnostics, Macquarie Park, NSW, Australia) from 2011 to 2016. A total of 528 serum specimens were tested, comprising 129 from cats, 108 from dogs, and 291 from koalas. In some instances, several specimens were submitted from a single patient, as serial determinations of the cryptococcal antigen titer are typically obtained during therapy. When this occurred, each sample was considered to be a separate specimen.

Canine and feline serum was submitted from patients thought to have cryptococcosis based on signs and cytology, or where cryptococcosis was a differential diagnosis. Koala samples were generally submitted as part of routine monitoring of zoos and wildlife parks for subclinical cryptococcosis, a common condition in captive koalas. This is defined as a persistent positive LCAT in the absence of symptoms, typically with a reciprocal titer no greater than 64.[14] Samples were also included from free-living koalas in the Liverpool Plains region of New South Wales, Australia, as part of a wider study into the health of this population, approved by The University of Sydney Animal Ethics Committee (project number 2016/955). A small number of koalas with clinical signs of active (symptomatic) cryptococcosis were included also. Monitoring of koalas in facilities with high environmental levels of the *C. gattii* species complex and high rates of cryptococcal nasal colonization, subclinical and clinical cryptococcosis was undertaken with a view to commencing preemptive therapy before the development of clinical signs. This is analogous to screening high-risk human immunodeficiency virus (HIV)-positive patients for cryptococcal antigen in places such as Colombia, Uganda, and other parts of sub-Saharan Africa,

A	Feline Serum	Latex Agglutination	
		+	-
Lateral Flow Immunochromatography	+	47	15
	-	4	63

B	Canine Serum	Latex Agglutination	
		+	-
Lateral Flow Immunochromatography	+	14	15
	-	0	79

C	Koala Serum	Latex Agglutination	
		+	-
Lateral Flow Immunochromatography	+	131	60
	-	2	98

Figure 1. Latex cryptococcal antigen agglutination test and cryptococcal antigen lateral flow assay results for diagnostic serum specimens from: (A) 129 cats, (B) 108 dogs and (C) 291 koalas. This Figure is reproduced in color in the online version of *Medical Mycology*.

where early detection and preemptive therapy improves clinical outcomes and is cost effective.[18,24–29]

Paired aliquots of serum were tested with (1) an immunochromatography LFA (CrAg® LFA, IMMY, USA) according to the manufacturer's recommendations[30] and (2) a LCAT (Crypto-LA Test, Wampole Cranbury, NJ, USA, from 2011 to 2012 [when the test became unavailable], and Cryptococcal Antigen Latex Agglutination System (CALAS®), Meridian Bioscience Inc, Cincinnati, OH, USA from 2012 to 2016), according to manufacturers' instructions.[31] Pronase pretreatment was performed as a part of the standard operating protocol for all LCATs; it is incorporated as part of the CALAS® kit and was added as a preincubation step before using the Crypto-LA test. The study design considered LCAT as the gold standard for assigning cryptococcal infection status, with a positive result being defined as a reciprocal titer ≥2, although in many cases microscopy (cytological examination of smears and histological examination of tissue sections) and mycological culture were also performed to confirm the diagnosis.

Results

Cats

Of the 129 feline serum specimens tested, the findings of the LCAT and LFA (Fig. 1A) were concordant in 110 instances; 47 were both positive (LCAT reciprocal titers ranging from 2 to 131,072 for the 35 retrievable titers; median titer 128), while 63 were both negative.

Four serum specimens were LCAT-positive and LFA-negative and were considered false-negatives, as cryptococcosis was confirmed via diagnostic imaging, fine needle aspiration, and biopsy. Two of these samples, both with LCAT reciprocal titers of 16, were collected from the same cat, 2 years apart; this patient had a cutaneous lesion on the pad of one distal limb from which *Cryptococcus laurentii* was isolated. The other two serum specimens, also from a single cat, had LCAT reciprocal titers of 131,072 and 8132, but were negative with the LFA. The first specimen was collected at the time of diagnosis, and the second after several months of antifungal therapy. Disseminated intra-abdominal cryptococcosis was confirmed in this cat by abdominal ultrasonography, with *C. neoformans* var. *grubii* cultured from aspirates of the intra-abdominal lesions and identified definitively by MALDI-TOF mass spectrometry. For this patient, the LFA was repeated on heat and pronase-treated serum and still tested negative. A semi-quantitative titration was performed on both specimens using the LFA test and positive results were recorded after dilutions of 1:8 (Fig. 2) and 1:4, respectively; the endpoint reciprocal LFA titers were 1,048,576 and 524,288, respectively.

Fifteen serum specimens were LCAT-negative but LFA-positive. In these 15 cases, cryptococcosis was excluded as a possibility by further work-up and diagnostic testing. In the absence of any other evidence supporting a diagnosis of cryptococcosis, these were considered false-positives.

Dogs

Of the 108 canine serum specimens tested, the findings of LCAT and LFA (Fig. 1B) were concordant in 93 instances: 14 results were both positive (reciprocal LCAT titers ranging from 2 to 16,384 for the 13 retrievable titers; median 64) and 79 results both negative. Fifteen serum specimens were LFA-positive but

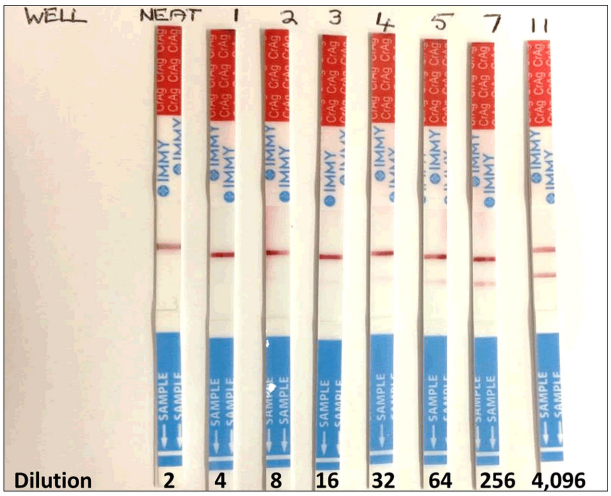

Figure 2. Prozone (or high-dose hook) effect in the cryptococcal antigen lateral flow assay (CrAg® LFA, IMMY) in a cat with a positive latex cryptococcal antigen agglutination test (reciprocal titre of 131,072) and confirmed cryptococcosis. The lateral flow assay remained negative in this specimen until a serial dilution of greater than or equal to 8. This Figure is reproduced in color in the online version of *Medical Mycology*.

LCAT-negative and considered false-positives due to a lack of any further evidence in support of a diagnosis of cryptococcosis. One dog that was LFA-positive but LCAT-negative had disseminated aspergillosis, suggesting cross-reactivity between fungal antigens. No specimens were found to be LFA-negative and LCAT-positive.

The LFA titer was compared to the LCAT titer in eight positive canine specimens, and in each instance the LFA titer was substantially higher (160 c.f. 64, 1280 c.f. 64, 80 c.f. 16, 40 c.f. 8, 80 c.f. 32, 640 c.f. 64, >2,560 c.f. 1,024, 40 c.f. 2).

Koalas

Of the 291 koala serum specimens tested, the findings of LCAT and LFA (Fig. 1C) were concordant in 229 instances; 131 tested positive with both assays (LCAT titers ranging from 2 to 131,072 for the 115 titers retrievable; median 4), while 98 tested negative using both methods. Two serum specimens from different koalas were LCAT-positive (reciprocal titers of 8 and 4) but negative with LFA, that is, false-negative results; heat and pronase treatment had no effect on these two LFA results. Follow-up sampling from both koalas 1 month later returned negative results on both the LCAT and LFA testing.

Sixty serum specimens were LFA-positive (reciprocal titers of 2 in the four specimens where the titer was determined) but negative with LCAT and were considered LFA false positives.

Sensitivity and specificity of the LFA

Using LCAT results as the gold standard, test metrics were calculated for the three different species independently (Table 1). The LFA test had excellent sensitivity for all species, 100% in canine patients, 98% in koalas (due to two specimens presumed to be LFA false-negatives), and 92% in cats (due to four specimens from two individuals that were also considered to be LFA false-negatives). Specificity was reasonable: comparable for feline and canine specimens (84% and 81%, respectively) but substantially lower in koalas (62%). The negative predictive value (NPV) was high in all species (94%, 100%, and 98% for cats, dogs, and koalas, respectively), whereas the positive predictive value (PPV) was less impressive (76%, 48% and 69% for cats, dogs and koalas, respectively) (Table 1).

A representative number of specimens were pretreated with pronase, as duplicates, to determine if this would affect the results from the LFA in certain patients, where an unexpected positive result was obtained. This preincubation step, however, had no impact on LFA test results.

A rare discordant case

A 6-year-old castrated Siamese cat was presented for progressively worsening stertor. There had been a progressive left-sided swelling over the nasal bridge associated with a left-sided mucopurulent ocular discharge. A needle aspirate was obtained from the swollen region. Nasal smears stained with Rapid-Diff® (Australian Biostain) demonstrated a small number of spherical capsulate narrow-necked budding yeasts. Cytology from the nasal bridge aspirate demonstrated inflammation featuring macrophages and lymphocytes but no yeasts. Both specimens were cultured on Sabouraud's dextrose agar at 37°C and bird seed agar. In each instance there was a light (nasal bridge) or moderate (nasal swab) heavy growth of weakly-capsulated narrow-necked budding yeast, producing brown-color-effect on bird seed agar. Molecular typing established the isolate was *C. neoformans var grubii* VNII. Interestingly, cryptococcal serology was negative, using two different Meridian CALAS® kits

Table 1. Sensitivity, specificity, positive predictive value, and negative predictive value for the IMMY CrAg® lateral flow assay in cats, dogs, and koalas when compared to latex cryptococcal antigen agglutination testing.

Species	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Cats	**92%** (47/51)	**81%** (63/78)	**76%** (47/62)	**94%** (63/67)
Dogs	**100%** (14/14)	**84%** (79/94)	**48%** (14/29)	**100%** (79/79)
Koalas	**98%** (131/133)	**62%** (98/158)	**69%** (131/191)	**98%** (98/100)

and the IMMY CrAg® LFA. The cat was treated with oral fluconazole (40 mg twice daily for 6 months) and made an uneventful recovery.

Discussion

The LFA has proven to be highly sensitive and specific in disparate cohorts of human patients.[15–26] Consequently, most human diagnostic laboratories in Australia, and likely globally, now prefer this assay to the LCAT for routine testing.[17] Our veterinary data demonstrated the LFA to be highly sensitive in all species examined, concordant with observations from human patients; however, the specificity and PPV suggest that the test metrics are less favourable in animals. The NPV derived from a large and representative number of serum specimens suggests that the LFA is an effective screening test for cryptococcosis in cats, dogs, and koalas. Thus, conducting a cage-side LFA in all animal patients with respiratory or neurological signs potentially consistent with cryptococcosis will allow veterinary clinicians to rapidly rule out this disease prior to conducting expensive and potentially risky diagnostic procedures. However, discrepant results highlight the necessity of interpreting the LFA with caution in all animal patients. The findings of this study indicate that when using the LFA as a screening test, if serum from the patient tests negative, then cryptococcosis can be excluded with confidence. If a high degree of suspicion or other compelling evidence of cryptococcal disease is evident from the diagnostic findings, the LFA should be repeated on serially diluted serum specimens and/or an LCAT should be performed to ensure that a false-negative result is not recorded. A positive LFA result in cats and dogs provides strong evidence supporting a diagnosis of cryptococcosis but requires further confirmation with the LCAT or other diagnostic tests (e.g., cytology, histology, or culture).

False-negative LFA results were noted in six specimens (four cats, two koalas). This study presents the first published reports of false-negative results using the IMMY CrAg® LFA on serum specimens in any species. In one cat from which two sequential samples were collected, it is presumed that the false-negative result occurred due to a localized infection with *C. laurentii*, a minimally encapsulated cryptococcal species that is not a member of either the *C. gattii* or *C. neoformans* species complexes. The LFA presumably failed to detect capsular antigen from this unusual cryptococcal species.

In another cat (two specimens), the false-negative LFA results were attributed to the prozone, or high-dose "hook," phenomenon. This refers to false-negative or false low-positive results in immunologic tests due to an excess of either antibodies or antigen in the diagnostic sample. It is not usually seen with immunochromatography methods, but it has been described from platforms used to measure myoglobin[32] and malarial enzyme assays.[33] A recent human study demonstrated the prozone effect was considered the probable cause of three false-negative LFA results obtained on human CSF specimens, as all three specimens tested positive at a dilution of 1:3.[34] The prozone effect has been acknowledged by the manufacturer as a potential limitation of the IMMY CrAg® LFA.[30,35] In this scenario, exceedingly high levels of cryptococcal antigen in a specimen are able to exhaust the binding sites of both the monoclonal gold-labeled antibodies and the immobilized test line antibodies, resulting in the absence of a visible test line and a false-negative result. This likely was observed because widely disseminated disease (without CNS involvement) is more often a feature of animal infections than human infections.[5] The feline patient in question was definitively confirmed to have cryptococcosis, caused by *C. neoformans* var. *grubii,* using culture, imaging, and mass spectrometry. The two false-negative LFA results in this patient had resolved by dilutions of 1:8. Therefore, in instances where there is a high suspicion of disseminated or "bulky" localized cryptococcosis but where the initial LFA result is negative, it may be prudent to repeat the LFA test using a dilution of 1:8 to rule out false-negative results, or alternately perform an LCAT.

In koalas, the two false-negative LFA results were from two patients with low LCAT titers that resolved to negative at a subsequent test and were therefore assumed to have a transient low antigenemia (subclinical cryptococcosis resolving spontaneously[14]) that may have been missed by the LFA. For logistical reasons, further diagnostic testing could not be performed on these koalas, and therefore we are unable to definitively determine the underlying cause of these discrepant results. In one discordant feline case, false-negatives were obtained from both the LCAT and LFA, despite the patient being definitively diagnosed with a sinonasal *C. neoformans* var. *grubii* VNII infection using culture and molecular typing. In this case, we speculate that the negative results were due to focal lesions resulting in an undetectable level of antigenemia and/or caused by a poorly encapsulated strain of the *C. neoformans* species complex.

False-positive results using the LFA were problematic in all three species studied. The cause of these potentially erroneous results could not be determined definitively, although laboratory error can be excluded, as all tests were performed by a competent technician and repeated. In all false-positive cases, the LFA test was also subsequently performed on pronase and heat-treated serum to ensure that this was not a factor. The inference from this finding is that the LFA should be regarded as a screening test when used on samples from cats, dogs, and koalas, with all positive results requiring confirmation by LCAT testing, and ideally also by obtaining further evidence for cryptococcal disease in the patient, such as the visualization of organisms in aspirates or biopsies and/or culture. The discordant case we present provides a salutatory lesson that no serological test is likely to be 100% reliable, and that there continues to be a requirement to further

investigate suspicious cases that test negative with either LFA or LCAT.

One false-positive LFA result (LCAT-negative) occurred in a canine patient with disseminated aspergillosis. According to the manufacturer's manual, there is some cross-reactivity between *Aspergillus* spp. capsular galactomannan and the IMMY CrAg® LFA in perhaps 10% of aspergillosis cases.[30,35] A cross-reactive capsular galactomannan from *Aspergillus* spp. was also suggested in a study using ELISA.[36] Along the same lines, colleagues in the United States have communicated that the IMMY CrAg® LFA can be positive in cats with invasive sporotrichosis (Jane Sykes, personal communication).

The situation in the koala is somewhat different and complex. Cryptococcosis is a reasonably common infectious disease in koalas, particularly in captivity, and is detected as a subclinical entity (asymptomatic cryptococcal antigenaemia) with reasonable frequency.[8,14] It is therefore recommended that koalas in Australian zoos and wildlife parks be bled on a regular basis (typically every 6 to 12 months, dependent on individual circumstances) to screen for subclinical cryptococcosis as early detection may prevent the development of clinical disease with pre-emptive azole therapy.[37] This approach has a higher likelihood of success and better welfare outcomes than waiting for clinical disease to develop, by which time the probability of successful therapy is low and the prognosis is guarded. The results of this study show that a LFA result is only straightforward to interpret when negative, as the NPV of the LFA in koalas is satisfactory (only 2/291 false negative results). A LCAT-negative/LFA-positive result might represent a false-positive LFA; on the other hand, it can be argued that the LFA is more sensitive than the LCAT in humans and capable of detecting exceedingly low concentrations of polysaccharide antigen. A LFA-positive/LCAT-negative koala serum could therefore potentially indicate very early subclinical disease or even heavy nasal colonization by *Cryptococcus* spp., without tissue invasion.[14,37–40] In one published case treated by some of the authors, a koala undergoing treatment for subclinical cryptococcosis began to test LCAT-negative but remained LFA-positive; to err on the side of caution, treatment was continued until consecutive LFA-negative results were obtained.[40] The clinical relevance of such low levels of circulating antigen is questionable and may not necessarily be consistent with a diagnosis of cryptococcosis. A comparison between the limits of detection of immunochromatography compared to latex agglutination for different *Cryptococcus* spp. isolates has shown an 8- to >200-fold higher analytical sensitivity of the IMMY CrAg® LFA compared to LCAT testing.[19,21] Supportive of this notion is the small number of canine cases in which quantitative endpoint titers were compared between LFA and LCAT methods, with the LFA titer being substantially higher (160 c.f. 64, 1280 c.f. 64, 80 c.f. 16, 40 c.f. 880 c.f. 32, 640 c.f. 64, >2,560 c.f. 1024, 40 c.f. 2). This notion is not, however, consistent with the two koalas in which the LFA was negative while the LCAT was transiently positive.

An important limitation of this study is the choice of the LCAT as the putative gold-standard serological test for cryptococcosis. Factors to consider in support of using the LCAT as a gold-standard in animals include absence of peer-reviewed publications of the IMMY test in the context of veterinary cryptococcosis and the LCAT remaining the most commonly used diagnostic test for cryptococcosis in commercial veterinary laboratories in Australia and abroad. Furthermore, in veterinary patients it is often difficult for logistical and economic reasons to obtain definitive confirmation of disease via culture and genotyping. Therefore, many animals are diagnosed with cryptococcosis based on LCAT testing combined with consistent clinical signs and sometimes cytological evaluation of aspirates, biopsies, or CSF.

Since completion of this work, another lateral flow kit (CryptoPS, Biosynex®) has come onto the market. This kit has some advantages over the IMMY CrAg® LFA in that the immunochromatography strip comes within a plastic cassette (and therefore does not require a tube or rack) (Fig. 3). It can also be purchased in smaller numbers. However, its requirement for storage at 5°C presents a distinct disadvantage when considering a test to use in the field. The kit must also be brought to room temperature before use, which would cause a further delay in results. However, in terms of sensitivity and specificity, our preliminary observations suggest that this test is comparable to the IMMY CrAg® LFA.

Our study highlights the utility of the IMMY LFA as a screening test to exclude cryptococcosis in diagnostic investigation of cats, dogs and koalas. A positive LFA result in these species should be confirmed by LCAT testing (after heat and pronase pretreatment) and/or by aspirate cytology, histopathology, or fungal culture. The format of the LFA is suitable for veterinary practice, especially in large busy hospitals and referral centers, as a rapid cage-side POC test to exclude cryptococcosis from the differential diagnosis before embarking on other expensive or invasive procedures such as cross-sectional imaging. In koalas, the test is also suitable for excluding clinical or subclinical cryptococcosis, but confirmatory testing of all positive results using the LCAT is again recommended. The propensity for low level asymptomatic cryptococcal antigenaemia in this species means that LFA-positive/LCAT-negative results should be interpreted with care. Overall, the IMMY LFA's high sensitivity, near ideal NPVs, ease of use, and rapid results make it an attractive future prospect for veterinary diagnostics.

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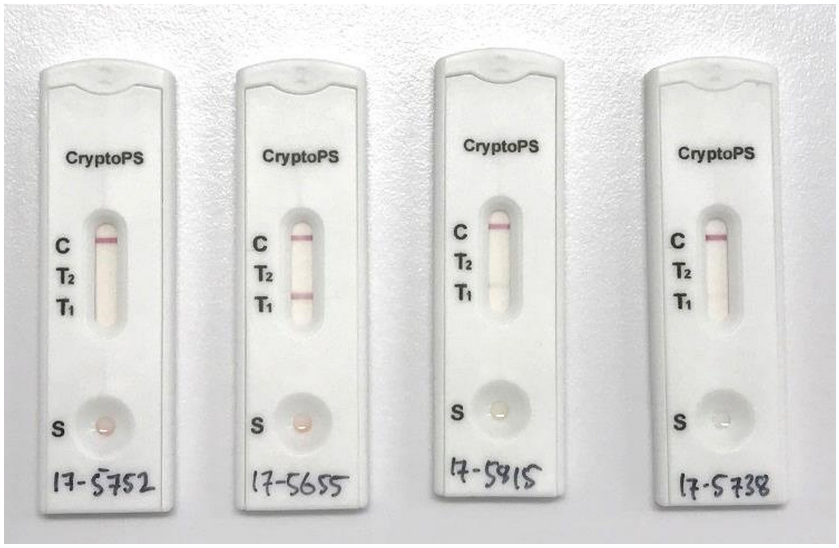

Figure 3. Alternative cryptococcal antigen lateral flow assay (CryptoPS) manufactured by BIOSYNEX®. The immunochromatography strip is provided in a plastic a cassette but must be kept refrigerated and brought to room temperature prior to use. This Figure is reproduced in color in the online version of *Medical Mycology*.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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3.3 Prevalence of cryptococcal antigenemia and nasal colonization in a free-ranging koala population

Authorship declaration:

This manuscript was published in *Medical Mycology* in 2019 (doi: 10.1093/mmy/myy144). L.J. Schmertmann is the primary author and contributed to all aspects of this study (designing and performing the research, analysing the data and preparing the manuscript). A. Kan, V.S.A. Mella and M. Crowther were involved in the collection and analysis of data. C.M. Fernandez and G. Madani collected data. R. Malik and W. Meyer designed the study and analysed the data. M.B. Krockenberger designed the study, and collected and analysed the data. All authors were involved in the revision of the manuscript and support its inclusion in this thesis.

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Prevalence of cryptococcal antigenemia and nasal colonization in a free-ranging koala population

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Abstract

Cryptococcosis, caused by environmental fungi in the *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes, affects a variety of hosts, including koalas (*Phascolarctos cinereus*). Cryptococcal antigenemia and nasal colonization are well characterized in captive koalas, but free-ranging populations have not been studied systematically. Free-ranging koalas (181) from the Liverpool Plains region of New South Wales, Australia, were tested for cryptococcal antigenemia (lateral flow immunoassay) and nasal colonization (bird seed agar culture). Results were related to environmental and individual koala characteristics. Eucalypt trees (14) were also randomly tested for the presence of *Cryptococcus* spp. by bird seed agar culture. In sum, 5.5% (10/181) and 6.6% (12/181) of koalas were positive for antigenemia and nasal colonization, respectively, on at least one occasion. And 64.3% (9/14) of eucalypts were culture-positive for *Cryptococcus* spp. *URA5* restriction fragment length polymorphism analysis identified most isolates as *C. gattii* VGI, while *C. neoformans* VNI was only found in one koala and one tree. Colonized koalas were significantly more likely to test positive for antigenemia. No associations between antigenemia or colonization, and external environmental characteristics (the relative abundance of *Eucalyptus camaldulensis* and season), or individual koala characteristics (body condition, sex, and age), could be established, suggesting that antigenemia and colonization are random outcomes of host-pathogen-environment interactions. The relationship between positive antigenemia status and a relatively high abundance of *E. camaldulensis* requires further investigation. This study characterizes cryptococcosis in a free-ranging koala population, expands the ecological niche of the *C. gattii/C. neoformans* species complexes and highlights free-ranging koalas as important sentinels for this disease.

Key words: *Cryptococcus*, *Phascolarctos cinereus*, subclinical cryptococcosis, Liverpool Plains, animal sentinel.

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Introduction

The *Cryptococcus gattii* and *Cryptococcus neoformans* species complexes comprise the etiological agents of cryptococcosis, a systemic mycosis affecting humans and a wide variety of animals that is usually acquired from the environment by inhalation.[1–3] A recent publication proposing their division into seven species[4] remains under deliberation by the cryptococcal research community. In Australia, *C. gattii* molecular type VGI has a well-established ecological niche in certain tree hollows, for example, *Eucalyptus camaldulensis* (river red gum) and *Eucalyptus tereticornis* (forest red gum),[5,6] which presents an important source of infection.[7] Our understanding of the environmental associations of *C. gattii* VGI has continued to expand to include numerous other tree species in Australia.[8–11]

Animals play an important role as sentinels for this and other environmentally acquired diseases, with reports of an increased prevalence of animal cryptococcosis cases aiding in the early characterization of the *C. gattii* VGII Vancouver Island outbreak.[12] Both captive and free-ranging animals have been considered as potential sentinels for cryptococcosis.[13,14] With forested or rural areas (particularly those with an abundance of large trees) considered by some to likely have a higher environmental abundance of the *C. gattii* species complex,[15] surveillance of free-ranging animals in particular has the potential to permit early detection of high risk areas for the acquisition of human disease.[13]

The koala (*Phascolarctos cinereus*) is one of many species known to be susceptible to cryptococcosis and is unique in its seemingly higher prevalence of disease than humans and other animal species, with approximately 3–4% of free-ranging and captive koalas found to have cryptococcal lesions at necropsy.[3,16–18] Both subclinical cryptococcosis (defined as asymptomatic cryptococcal antigenemia) and cryptococcal nasal colonization are common in seemingly healthy captive koalas.[11,19–21] The koala's tendency to develop cryptococcal nasal colonization in the presence of a high environmental burden of *Cryptococcus* spp. in captivity has led to the koala nasal cavity being considered an effective 'biological air sampler' for environmental *Cryptococcus* spp.[11] Although cryptococcal nasal colonization and antigenemia are well documented in captive koala populations, the same phenomena in free-ranging koalas are much less well characterized, and to date, no natural population has been studied systematically.[11,20] In humans, asymptomatic cryptococcal antigenemia is well described in patients infected with human immunodeficiency virus (HIV), especially in sub-Saharan Africa and other similar settings in the developing world.[22–24] It can precede clinical signs by weeks to months, with early screening tests in high-risk patients allowing preemptive oral therapy (typically using fluconazole) to prevent development of overt clinical disease.[25] A similar practice occurs in koalas, with a recent report recording successful early detection and treatment of subclinical cryptococcosis in a free-ranging individual.[26]

In 2015, a deceased adult free-ranging koala found near Breeza in the Liverpool Plains region of New South Wales, Australia, was opportunistically dissected, and multinodular cryptococcal pneumonia was identified by the authors as the probable cause of death, based on gross and histopathologic examination of tissues. No prior cases of cryptococcosis from free-ranging koalas in this region were known to the authors or recorded in the literature. This case therefore prompted an investigation into the prevalence of cryptococcosis in this population.

This study aimed to investigate exposure to environmental *Cryptococcus* spp. in the Liverpool Plains population of free-ranging koalas by determining the prevalence of both cryptococcal antigenemia and nasal colonization in relation to the presence of *Cryptococcus* spp. in the environment. Based on prior studies that identified some environmental features and individual koala characteristics as influential factors in nasal colonization and antigenemia,[11,19,20] we attempted to relate our findings to the relative abundance of *E. camaldulensis*, season, koala body condition, sex, and age.

Methods

Koala selection and field trips

A total of 181 free-ranging koalas were captured from five sites within the Liverpool Plains region of New South Wales, Australia: 73 from Dimberoy (31°08′33.4″S 150°02′16.1″E), two from Gunnedah (31°01′34.4″S 150°15′37.3″E), seven from Lake Goran (31°14′10.2″S 150°11′30.1″E), three from Nowley (31°21′19.5″S 150°04′44.6″E), and 96 from Watermark (31°13′49.7″S 150°20′17.9″E). *E. camaldulensis* trees were frequently observed at the Gunnedah, Lake Goran, Nowley, and Watermark sites but not encountered at the Dimberoy site, as identified by experienced ecologists and the use of a eucalypt identification key.[27] In the absence of systematic ecological survey data, we classified koalas from Dimberoy versus the other four sites (Gunnedah, Lake Goran, Nowley, and Watermark) as inhabiting low and high relative abundance *E. camaldulensis* areas, respectively.

Six field trips occurred at approximately six-month intervals (three in spring, two in autumn and one in winter) from September 2015 to July 2018. Some koalas were captured and tested multiple times at minimum six-monthly intervals, with 51, 17, four and two koalas captured and tested on two, three, four, or five occasions, respectively; this resulted in 286 capture events. After capture, each koala was lightly sedated with alfaxalone (Alfaxan®, Jurox; approximately 1.8 mg/kg by intramuscular injection) to minimize stress and facilitate safe sampling.

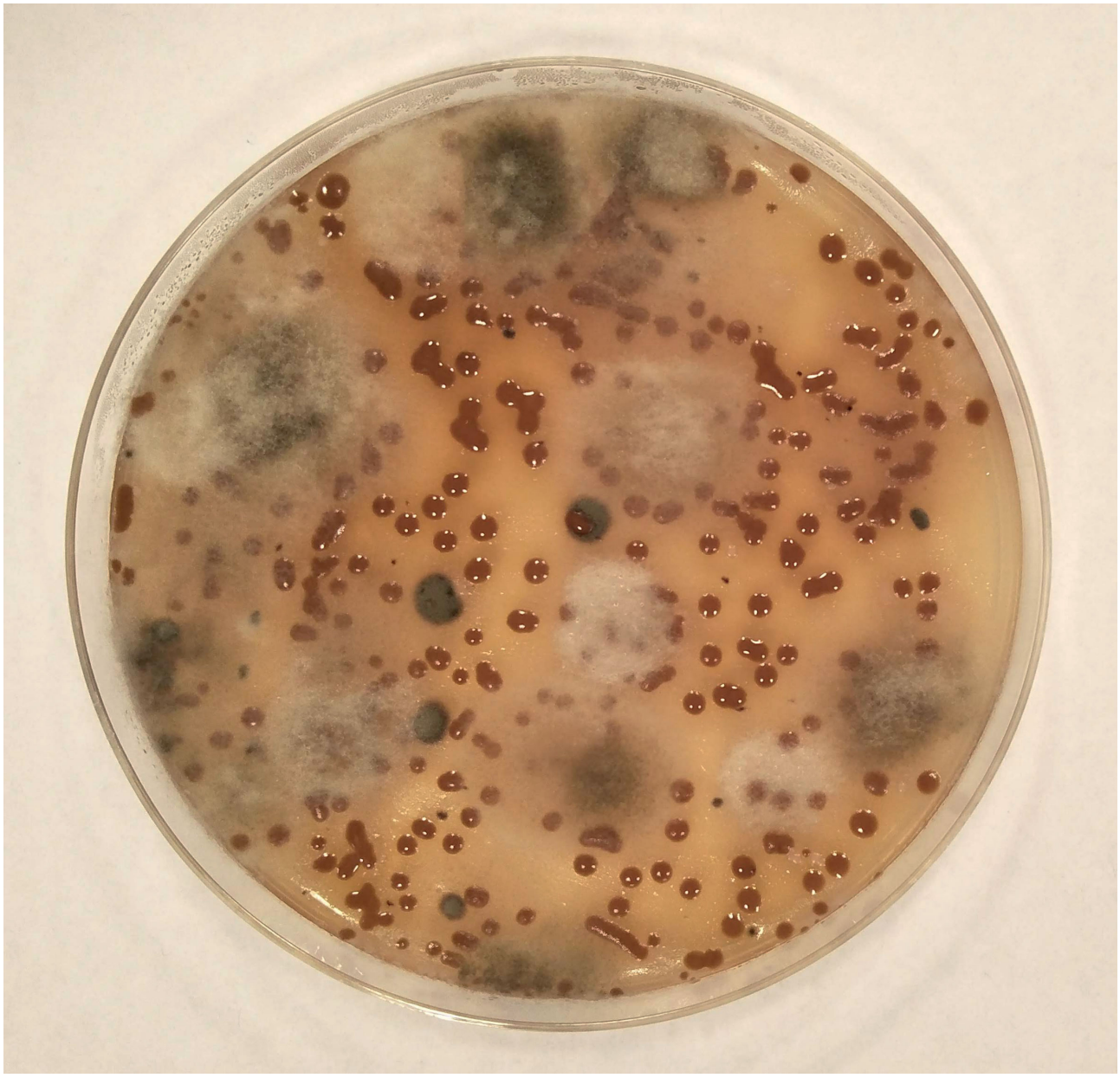

Figure 1. Bird seed (*Guizotia abyssinica*) extract agar culture of a nasal swab from a free-ranging koala in the Liverpool Plains region of New South Wales, Australia, showing a heavy growth of colony-forming units exhibiting the brown-colour-effect, later identified as *Cryptococcus gattii* VGI. This Figure is reproduced in color in the online version of *Medical Mycology*.

All koalas were examined by a veterinarian for any clinical signs consistent with cryptococcosis (including nasal discharge, facial swelling/distortion, or dyspnea); sex, body condition score (assessed on a scale from 1 to 5),[28,29] and tooth wear class (scored from 1 to 10)[30] were recorded.

Koalas were fitted with both a unique numbered ear tag and a subcutaneous microchip transponder before release for ongoing individual identification in the event of recapture or sightings. All procedures were undertaken with approval from The University of Sydney Animal Ethics Committee (project number 2016/955) and National Parks and Wildlife Service (scientific license SL101687).

Culture

Nasal swabs were collected by introducing a sterile swab moistened with saline approximately 2 cm into the nasal vestibule (one side at a time) and rotating gently, as described in previous studies.[11,19] Environmental samples, collected opportunistically from hollows in 14 eucalypts (*E. albens* [1], *E camaldulensis* [11], and *E. populnea* [2]) in the region, were obtained by running a sterile swab moistened with saline thoroughly over the interior surface of the hollow. All swabs (nasal and environmental) were inoculated on to Staib's bird seed (*Guizotia abyssinica*) extract agar containing antibiotics (penicillin and gentamicin) and

incubated at 27°C for a minimum of 7 days to identify colony-forming units (cfu) exhibiting the brown-color-effect, consistent with *Cryptococcus* spp. (Fig. 1). Positive samples were classified as having a low (1–10 cfu/plate), moderate (11–100 cfu) or heavy (>100 cfu) cryptococcal burden.[11] A single cfu exhibiting the brown-color-effect was collected from each positive sample, sub-cultured on Sabouraud's dextrose agar (SDA), and incubated at 37 °C for isolation and DNA extraction.

Molecular typing and species determination

Isolates subcultured on SDA underwent DNA extraction using the same protocol as Ferrer et al. (2001).[31] Restriction fragment length polymorphism (RFLP) analysis was performed on the product of a PCR amplification of the *URA5* gene, according to the protocol developed by Meyer et al. (2003), to determine cryptococcal species and molecular type.[32]

Cryptococcal antigen testing

Approximately 3 ml of whole blood was collected into plain tubes by cephalic venepuncture. Samples were chilled at 4°C, then centrifuged as soon as possible (on the same day) in a field laboratory for approximately 5 minutes at 5000 rpm to allow for separation of the serum. An aliquot of the serum was used to perform the cryptococcal antigen lateral flow assay (LFA) (CrAg® LFA, IMMY, Norman, OK, USA) according to the manufacturer's instructions in a field laboratory as a screening test. All remaining sera were kept frozen in liquid nitrogen until they could be transferred to a -80°C freezer. Upon return from the field, latex cryptococcal antigen agglutination testing (LCAT) (CALAS®, Meridian Bioscience, Inc., Cincinatti, OH, USA) was performed on all LFA-positive samples, according to the manufacturer's instructions. Sera from all 51 LFA-negative koalas captured during the 2016 autumn field trip also underwent LCAT testing. Reciprocal cryptococcal antigen titres were determined using the LCAT. For the purposes of this study, koalas that returned a positive LFA result were considered cryptococcal antigen seropositive, even if the LCAT was negative.

Statistical analysis

We assessed whether antigenemia or nasal colonization status had a relationship with the relative abundance of *E. camaldulensis* in a koala's habitat (low vs high), koala body condition (score of 1–2 vs score of 3–5), sex (male vs female) and an approximation of age (tooth wear class 1–5 vs tooth wear class 6–10) using 2 × 2 contingency tables and two-tailed Fisher exact tests. The same method was used to determine if a relationship existed between antigenemia and colonization status. A 2 × 3 contingency table and two-tailed Fisher exact test with Freeman-Halton extension was used to assess for dependent relationships between antigenemia or colonization status and season (autumn vs spring vs winter). P values of $< .05$ were considered statistically significant.

Results

Culture

Nasal swabs from 12/181 koalas (6.6%) were culture-positive for cryptococcal cfu on bird seed agar on at least one occasion. Eight of the 12 positive koalas were tested on more than one occasion, with 5/8 tested twice and 3/8 tested three times. Of the koalas tested twice, 2/5 were positive on both occasions, while the remaining 3/5 progressed from initially testing negative to positive on the second occasion. The koalas tested three times were either positive on all three occasions (1/3), positive only at final test (1/3) or positive twice and then negative on the final test (1/3). Among the four koalas that were culture-positive for nasal colonization on two or more occasions, the extent of colonization either increased (2/4; moderate to heavy, low to moderate), reduced (1/4; heavy to moderate) or remained stable (1/4; heavy) (Table 1). One koala (Table 1, DECC 071) that was culture-positive for nasal colonization exhibited a small amount of crusted nasal discharge at the nares and mild hemorrhage at the nasal philtrum (possibly a mild sign of sinonasal cryptococcosis) (Fig. 2). No clinical signs consistent with sinonasal, pulmonary, or neurologic cryptococcosis were observed in any other koalas with nasal colonization

Nine of the 14 (64.3%) tree hollow samples cultured positive for *Cryptococcus* spp. (Table 2), consisting of *E. albens* (1), *E. camaldulensis* (7), and *E. populnea* (1). A total of 4/9 positive trees exhibited a heavy growth (*E. albens* [1], *E. camaldulensis* [2], and *E. populnea* [1]), while 4/9 and 1/9 exhibited moderate and low growths, respectively; all were *E. camaldulensis*.

Molecular typing and species determination

URA5 RFLP analysis confirmed all isolates to be members of the *C. neoformans*/*C. gattii* species complexes. Most isolates (8/9 environmental; 16/17 koala) were identified as *C. gattii* VGI. *C. neoformans* VNI was isolated from a single koala on one occasion and from one *E. populnea* hollow (Table 2).

Serology

Serum samples from 10/181 koalas (5.5%) were positive, on at least one occasion, for cryptococcal antigen using the LFA. Five of the 10 LFA-positive koalas also tested positive using the LCAT, with reciprocal titers of 2 (DECC 092; DECC 104), 8 (DECC 198; USYD 014), and 128 (DECC 071); the remaining five koalas tested LCAT-negative (Table 1).

Of the 10 koalas that were LFA-positive, eight were tested on more than one occasion (6/8 tested twice, 2/8 tested three times).

Table 1. Free-ranging koalas (ordered by identification number), in the Liverpool Plains population of New South Wales, Australia, that tested positive for cryptococcal antigenemia and/or nasal colonization on at least one occasion in this study.

Koala ID	Sex	Location	*E. c.* abundance	Season (year)	TWC (1–10)[29]	BCS (1–5)[27,28]	LFA	LCAT (titer)	Nasal colonization*	*Cryptococcus* spp. isolated
DECC 009	M	Lake Goran	High	Autumn (2016)	4	3	-	-	-	NA
				Spring (2016)	4	3	+	-	-	NA
DECC 015	M	Dimberoy	Low	Spring (2016)	3	5	+	-	-	NA
				Autumn (2017)	5	4	+	-	-	NA
				Winter (2018)	5	4	-	ND	-	NA
DECC 017	M	Dimberoy	Low	Spring (2015)	4	4	-	ND	+	*C. gattii* VGI
DECC 051	M	Watermark	High	Autumn (2016)	4	3	-	-	++	*C. gattii* VGI
				Spring (2016)	4	4	-	ND	+++	*C. gattii* VGI
DECC 071	M	Watermark	High	Spring (2016)	4	4	-	ND	-	NA
				Autumn (2017)	4	3	+	+ (1:128)	++	*C. gattii* VGI
DECC 072	M	Watermark	High	Autumn (2016)	4	2	-	-	-	NA
				Spring (2016)	4	3	+	-	-	NA
DECC 092	M	Lake Goran	High	Spring (2016)	4	3	-	ND	-	NA
				Autumn (2017)	6	3	+	+ (1:2)	-	NA
DECC 104	F	Nowley	High	Spring (2016)	3	3	+	+ (1:2)	+	*C. gattii* VGI
DECC 130	F	Dimberoy	Low	Spring (2016)	6	2	-	ND	-	NA
				Autumn (2017)	7	2	-	ND	+	*C. gattii* VGI
DECC 135	F	Dimberoy	Low	Spring (2015)	6	2	-	ND	+++	*C. neoformans* VNI
DECC 137	F	Watermark	High	Spring (2015)	5	3	-	ND	+++	*C. gattii* VGI
				Autumn (2016)	5	4	-	-	++	*C. gattii* VGI
				Autumn (2017)	6	3	+	-	++	*C. gattii* VGI
DECC 150	F	Watermark	High	Autumn (2016)	6	2	-	-	-	NA
				Spring (2016)	6	2	-	ND	++	*C. gattii* VGI
DECC 162	F	Watermark	High	Spring (2015)	4	4	-	ND	-	NA
				Autumn (2016)	4	4	-	-	-	NA
				Spring (2017)	5	NR	-	ND	+	*C. gattii* VGI
DECC 198	F	Watermark	High	Autumn (2016)	4	3	+	+ (1:8)	+++	*C. gattii* VGI
				Spring (2016)	5	2	+	+ (1:8)	+++	*C. gattii* VGI
USYD 014	M	Watermark	High	Winter (2018)	5	3	+	+ (1:8)	+++	*C. gattii* VGI
USYD 040	M	Watermark	High	Autumn (2017)	6	3	-	ND	+	*C. gattii* VGI
				Spring (2017)	6	3	-	ND	++	*C. gattii* VGI
				Winter (2018)	6	4	-	ND	-	NA
USYD 054	F	Watermark	High	Spring (2017)	5	4	+	-	-	NA
				Winter (2018)	5	3	-	ND	-	NA

+, positive; -, negative; BCS, body condition score; *E. c., Eucalyptus camaldulensis*; F, female; LCAT, latex cryptococcal antigen agglutination test; LFA, cryptococcal antigen lateral flow assay; M, male; NA,not applicable; ND, not done; NR, not recorded; TWC, tooth wear class.

*Number of + represents the degree of cryptococcal growth on bird seed agar: + = low (1–10 colony-forming units [cfu] per plate), ++ = moderate (11–100 cfu per plate), +++ = heavy (>100 cfu per plate).

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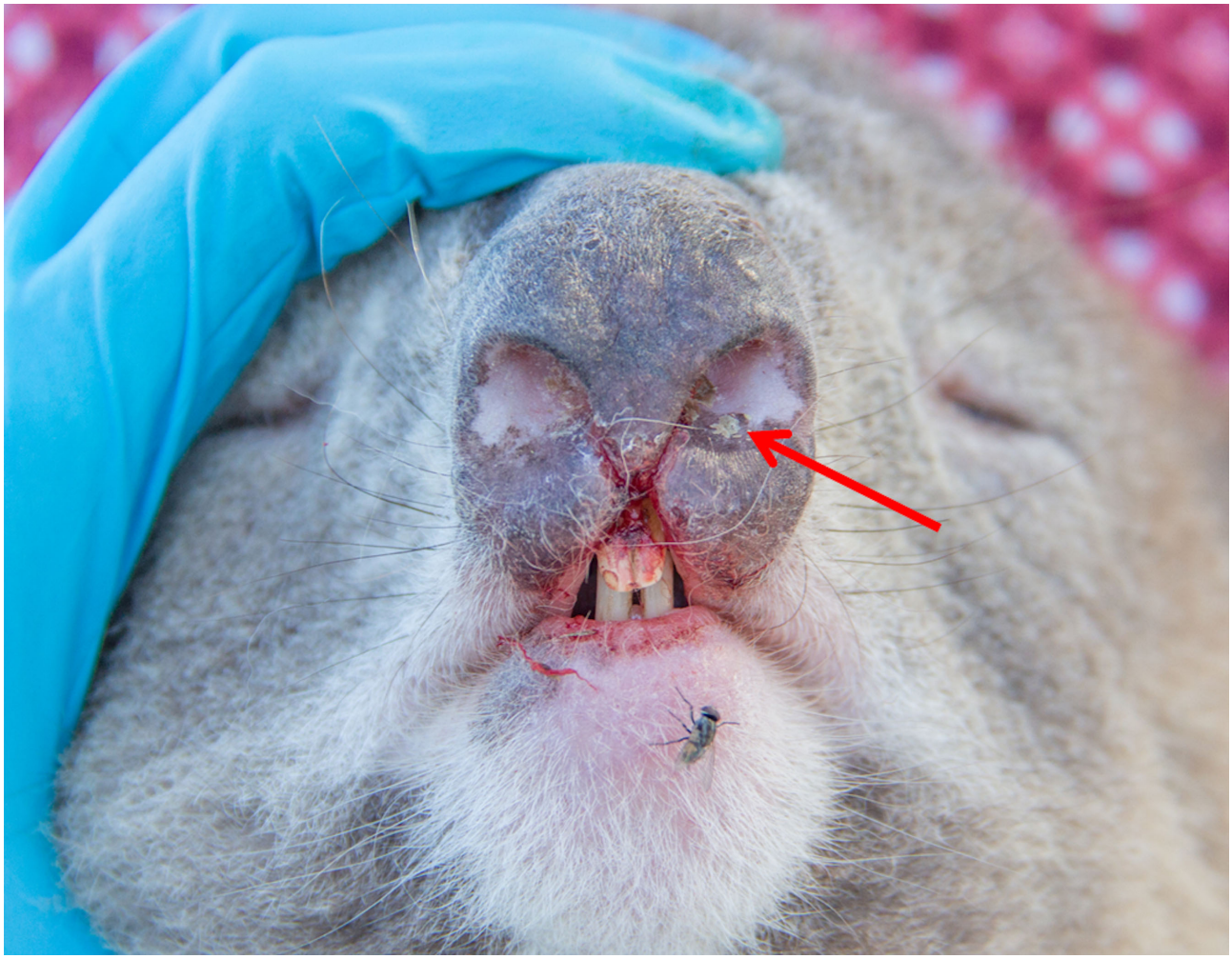

Figure 2. A free-ranging koala (ID: DECC 071) from the Liverpool Plains population of New South Wales, Australia, that tested positive for both cryptococcal antigenemia and nasal colonization, while exhibiting possible mild clinical signs of sinonasal cryptococcosis: a small amount of crusted nasal discharge (arrow) and a mild hemorrhage visible at the nasal philtrum. This Figure is reproduced in color in the online version of *Medical Mycology*.

Table 2. Culture and molecular typing results for environmental samples (ordered by sample number) collected from tree hollows in the Liverpool Plains region of New South Wales, Australia.

Sample number	Tree species	Location	Cryptococcal growth*	*Cryptococcus* spp. isolated
E2757	*Eucalyptus populnea*	Watermark	-	NA
E2760	*E. albens*	Watermark	+++	*C. gattii* VGI
E2764	*E. camaldulensis*	Watermark	+++	*C. gattii* VGI
E2765	*E. camaldulensis*	Watermark	-	NA
E2766	*E. camaldulensis*	Watermark	-	NA
E2767	*E. camaldulensis*	Watermark	-	NA
E2768	*E. camaldulensis*	Watermark	++	*C. gattii* VGI
E2769	*E. camaldulensis*	Watermark	-	NA
E2770	*E. camaldulensis*	Watermark	+++	*C. gattii* VGI
E2771	*E. camaldulensis*	Watermark	+	*C. gattii* VGI
E2772	*E. camaldulensis*	Watermark	++	*C. gattii* VGI
E2773	*E. camaldulensis*	Watermark	++	*C. gattii* VGI
E2774	*E. camaldulensis*	Watermark	++	*C. gattii* VGI
E3049	*E. populnea*	Dimberoy	+++	*C. neoformans* VNI

+, positive; -, negative; NA, not applicable.

*Number of + represents degree of cryptococcal growth on bird seed agar: + = low (1–10 colony-forming units [cfu] per plate), ++ = moderate (11–100 cfu per plate), +++ = heavy (>100 cfu per plate).

The six koalas tested twice either progressed from LFA-negative to positive (4/6), remained consistently positive (1/6), or progressed from LFA-positive to negative (1/6). One of the koalas tested on three occasions progressed from LFA-negative to positive on its final test; the same koala was consistently positive for nasal colonization across all three testings. The other koala tested on three occasions was LFA-positive twice but then became negative. Five koalas were positive for both serum antigen and nasal colonization (Table 1). As mentioned previously, one LFA-positive and LCAT-positive koala (DECC 071), also positive for nasal colonization, exhibited symptoms possibly consistent with sinonasal cryptococcosis (Fig. 2). No clinical signs consistent with sinonasal, pulmonary, or neurologic cryptococcosis were observed in any other koalas with cryptococcal antigenemia.

Serum samples from the 51 LFA-negative koalas from the 2016 autumn field trip were all LCAT-negative.

Statistical analysis

Nasal colonization was not significantly associated with the relative abundance of *E. camaldulensis* ($P = .366$), season ($P = .514$), koala body condition ($P = .764$), sex ($P = .769$), or age ($P = .787$). Antigenemia status (LFA result) was not significantly associated with season ($P = .920$), koala body condition category ($P = .305$), sex ($P = .524$), or age ($P = .354$). There was a trend for positive serum antigen status being associated with a relatively high abundance of *E. camaldulensis*, but this was not significant due to low power of the analysis ($P = .051$). Koalas positive for nasal colonization were significantly more likely to also test positive for antigenemia ($P < .001$).

Discussion

This study is the first to our knowledge to systematically establish the prevalence of cryptococcal antigenemia and nasal colonization in a population of free-ranging koalas. Our results of approximately 6% and 7% overall rates of antigenemia and colonization, respectively, should be viewed in the light of the observation that 3–4% of all koalas have detectable cryptococcal lesions at post-mortem examination.[3,17,18] Our results show substantially lower rates of nasal colonization and antigenemia compared to certain sites in studies of captive populations, where the prevalence of nasal colonization and antigenemia can reach 100% and >50%, respectively.[11,20,21] Our findings differ from preliminary studies of a small number of free-ranging koalas in Breeza, an area within the Liverpool Plains region and near the Watermark site, where all koalas were found to be negative for cryptococcal nasal colonization and antigenemia.[11,20] This discrepancy may be explained simply by the small sample size utilized in previous studies.

One koala in this study (Table 1; DECC 071) tested positive for both nasal colonization and antigenemia (LFA-positive and LCAT-positive) on one occasion, and at this time also exhibited a small amount of crusted nasal discharge and mild hemorrhage visible at the nasal philtrum, possibly consistent with sinonasal cryptococcosis. The reciprocal LCAT titer of 128 also supports the possibility of clinical disease in this koala, as such a titer falls within the range where clinical signs are expected.[20] However, the clinical signs in this koala were considered mild, and similar findings (small amounts of crusted nasal discharge) were seen occasionally in other koalas in this study that were negative for both nasal colonization and antigenemia on all occasions tested. Therefore, at the time of capture this observation was recorded but was not considered highly suspicious. The development of more prominent clinical signs in this individual may have been imminent, or other subtle symptoms may have been present but undetected due to the limited time of observation. Another koala (Table 1; DECC 198) exhibited a reciprocal LCAT titer of 8 across two field trips, 6 months apart. A persistent reciprocal titer of 8 is the point at which treatment for subclinical cryptococcosis is considered in captive koalas, depending on individual circumstances.[26] LFA and LCAT results were not available at the time of capture for any koala; therefore, in the absence of more definitive symptoms, we did not intervene with these two individuals. Once results were available, capturing these two koalas for re-assessment and possible treatment on subsequent field trips was prioritized, but neither could be relocated.

Recent observations by our group suggest that the LFA has a high sensitivity and excellent negative predictive value for the diagnosis of cryptococcosis in koalas when compared to the LCAT, making it an excellent screening test. Positive results, however, should be followed up by further testing to assess the likelihood of subclinical or clinical disease (Krockenberger, in press).[33] The LFA was also used successfully for monitoring response to treatment in a koala with cryptococcosis and remained positive for four months after the LCAT titer had become negative.[26] For these reasons, we chose to initially screen all koalas for antigenemia using the LFA and follow-up positive results with a LCAT. The internal validation performed in this study, with 51 LFA-negative samples found to also be LCAT-negative, confirmed the reliability of the LFA as a screening tool in this setting. Although 10 koalas were antigen-positive using the LFA, only 5/10 were simultaneously positive using the LCAT. This discrepancy can likely be explained by the difference in the detection limits of these two tests, with the LFA proven *in vitro* and in human patients to be sensitive to lower antigen levels compared to the LCAT.[34–36] Although such low levels of circulating antigen may not be clinically relevant in the diagnosis of overt cryptococcosis, we considered them to be pertinent to our aim of detecting evidence of exposure to *Cryptococcus* spp. We cannot definitively exclude the possibility that the discrepant results represent

false-positives, given that the LFA was associated with false-positive results due to cross-reactivity in two human patients diagnosed with *Trichosporon* spp. fungemia.[37] However, given the propensity of koalas to develop subclinical cryptococcosis[20] and the rarity of reports of cross-reactivity using the LFA, we considered LFA-positive/LCAT-negative results to be true positives for antigenemia for the purposes of this study. It is not known whether LFA-positive/LCAT-negative koalas could have very limited invasive disease, heavy nasal colonization, or both, and studies using advanced imaging (endoscopy, computerized tomography, or magnetic resonance imaging) would be required to address this question. Interestingly, we identified two koalas with heavy nasal colonization that did not test positive using either the LFA or LCAT (Table 1: DECC 051 and DECC 135).

Subclinical cryptococcosis often resolves spontaneously in captive koalas when the reciprocal LCAT titer is less than 64, particularly if there are no concurrent adverse factors (e.g., stress, poor nutrition, transport).[20] Spontaneous resolution of cryptococcal antigenemia has also been observed in cats and dogs studied on Vancouver Island and its environs.[38] Three koalas in this study (Table 1; DECC 015, DECC 198, and USYD 054) were captured again after testing seropositive for cryptococcal antigen, and the antigenemia had resolved in 2/3. We also observed the resolution of nasal colonization in one koala (Table 1; USYD 040), although this is difficult to confirm due to the unknown sensitivity of nasal swabbing as a means of determining and quantifying cryptococcal colonization status.

Our findings of a lack of correlation between nasal colonization and season or sex differ from a previous study in captive koalas, which established a potentially weak association with season and a strong relationship to sex, with more colonization apparent in spring and with males being colonized more frequently than females.[19] Seasonal variation was suggested to occur as a result of increased aerosolization of *Cryptococcus* spp. during *E. camaldulensis* flowering in spring and early summer, while sex variation was thought to be related to the greater tendency for male koalas to use smell to explore their territory.[19] Given that captive koalas appear able to amplify the amount of the agents of the *C. gattii* species complex in their proximate environment,[11] it is difficult to directly compare captive studies to the findings presented here in free-ranging koalas. In another large study, mostly of captive or hospitalized koalas but with the opportunistic inclusion of free-ranging koalas from several regions of coastal New South Wales, also no significant relationship between season and colonization status could be established.[11] However, it is difficult to assess the accuracy of any associations detected between nasal colonization and other variables, due to the previously mentioned unknown sensitivity of nasal swabbing.

Similar to our findings, sex or season had no effect on antigenemia in a study of subclinical cryptococcosis in captive koalas,[20] although a tendency toward low or negative antigen titres was observed in older koalas in one captive group.[20] In the present study, neither antigenemia nor nasal colonization was associated with tooth wear class (an approximation of age). No prior studies have compared body condition to subclinical cryptococcosis or nasal colonization status in koalas. We elected to include this variable in our analyses as we hypothesized that poor body condition could be associated with antigenemia or colonization either due to an increased susceptibility in these individuals or due to subclinical cryptococcosis causing poorer body condition. We observed, however, that neither antigenemia nor nasal colonization was associated with body condition. Given the well-established association between *E. camaldulensis* and *C. gattii* VGI,[5] we also hypothesized that nasal colonization and/or antigenemia may depend on the relative abundance of *E. camaldulensis* in an individual koala's habitat. A significant relationship could not be established, although an obvious trend was observed for antigenemia, with high relative abundance *E. camaldulensis* areas exhibiting a greater prevalence than low abundance areas (8.3% and 1.4%, respectively), suggesting greater sampling is required. This also suggests that *E. camaldulensis* is not the only environmental association of *C. gattii* VGI in this area, a point proven by its isolation from other dominant *Eucalyptus* spp. in the region. We were unable to assess for any correlation with the presence of *E. tereticornis*, another eucalypt with a strong association to the *C. gattii* species complex,[6] as trees of this species were not identified at any of the sampling sites. The study sites were likely outside of the natural range of *E. tereticornis*.[27] The relationship between nasal colonization and antigenemia status, with colonized koalas significantly more likely to also test positive for antigenemia ($P < .001$), is intuitive. Both are indicative of exposure to *Cryptococcus* spp., and it is presumed that mucosal colonization precedes tissue invasion, which in turn leads to antigenemia.[20]

C. gattii VGI was easily detected in multiple tree hollows in this study, with the isolation from *E. albens* being the first from this tree species, to the best of our knowledge. The isolation of *C. neoformans* VNI from *E. populnea* is also unique. The detections of *C. gattii* VGI from both the environment and the nasal cavities of some koalas further confirms its potential to cause disease in this region. However, systematic environmental sampling across all study sites is required to determine the environmental prevalence of *C. gattii* VGI in these areas and better characterize its habitat in this region. This would also aid in further exploring the potential relationship between the abundance of *E. camaldulensis* trees and increased risk of exposure to *C. gattii* VGI, and the potential for associations to other *Eucalyptus*

spp. The comparison of environmental and nasal colonizing isolates using a more discriminatory genotyping technique, such as multilocus sequence typing or whole genome sequencing, could also provide further evidence for environmental exposure to *C. gattii* VGI and offer insights into the virulence of different strains in this region.

The impact of host immune status and possible coinfections on cryptococcal antigenemia or nasal colonization status was not explored in this study. A study of koala major histocompatibility complex (MHC) II diversity included a small number of individuals from the Liverpool Plains region and found that these koalas belonged to a group exhibiting a comparatively high MHCII diversity.[39] Although there is evidence of the significant role of MHCII in the host response to chlamydiosis in the koala,[40] its relationship to cryptococcosis has not been examined. With the recent availability of the koala genome, there is now an improved understanding of the changes in regulation of immune genes in koalas with chlamydiosis.[41] This has the potential to offer insights into the immune response to other intracellular pathogens, such as *Cryptococcus* spp. Testing koalas for all forms of the koala retrovirus (KoRV) and *Chlamydia pecorum* would also be required to better elucidate the complex host-pathogen-environment interactions at play. KoRV in particular could play an important role, as infection with the KoRV-B variant has already been linked to malignant neoplasia,[42] chlamydiosis,[43] and changes to immune cytokine expression,[44] although the relationship with cryptococcosis has not been studied systematically. Investigating the overall pathogen burden of this free-ranging koala population and the relationship between KoRV and cryptococcosis will be the subject of future work by the authors.

Cryptococcal nasal colonization and antigenemia were found to be at a substantially lower prevalence in the Liverpool Plains population of free-ranging koalas than in certain well-studied captive populations.[11,19–21] This supports the previous assertion that captive koalas amplify *C. gattii* VGI in their immediate environment. Our findings suggest that although cryptococcosis is present in this region, and *C. gattii* VGI is detectable in both the environment and the nasal cavities of some koalas, this is unlikely to be an area of increased risk of infection for humans and other animal species. According to our analyses, cryptococcal antigenemia and nasal colonization are seemingly random outcomes of the host-pathogen-environment interactions within this region. However, further work, such as systematic environmental sampling, more discriminatory genotyping of *C. gattii* VGI strains, examining host immunity, and testing for pathogen burden is required to explore this notion. A high relative abundance of *E. camaldulensis* in a free-ranging koala's habitat may be associated with the development of cryptococcal antigenemia, but this link could not be established definitively here and requires further investigation.

Our study expands the current understanding of cryptococcosis in free-ranging koalas and the known environmental niches of the *C. gattii*/*C. neoformans* species complexes in Australia, while also highlighting the koala as an important sentinel species for this environmentally acquired disease.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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4. Fine-scale molecular epidemiology

Background:

Some aspects of the host-pathogen-environment interactions of cryptococcosis caused by the *C. gattii* species complex remain poorly understood, particularly the progression from environmental exposure to mucosal colonisation and finally, in some cases, to disease. The identification of environmental sources of infection in some animal cases of cryptococcosis, and the ability of koalas to translocate members of the *C. gattii* species complex, suggest that animals play an important role as both sentinels and a dispersal mechanism for environmental *Cryptococcus* spp. Neither phenomenon, however, has been characterised or definitively proven using high-resolution genotyping and molecular epidemiology. Captive animals offer an ideal opportunity to characterise both sources of infection and the translocation of *Cryptococcus* spp., but the artificial nature of these environments and unusually high density of animals could make this problematic for inferring some aspects of host-pathogen-environment interactions. Therefore, naturally-occurring disease in free-ranging animals presents the ideal model to study this, but cases are typically sporadic, unreported and/or logistically difficult to investigate. This section utilises in-depth environmental investigations and fine-scale molecular epidemiology to infer relationships between disease, colonisation and the environment in naturally-occurring animal cryptococcosis in captivity and the wild.

Section 4.1 investigates an unusual spatial and temporal case cluster of cryptococcosis in a sub-population of free-ranging koalas through targeted environmental sampling, and MLST and WGS of disease, colonising and environmental *C. gattii* VGI isolates. The goal was to determine if fine-scale molecular epidemiology could yield insights into the underlying cause of the cluster, while also using this as a natural model to study host-pathogen-environment interactions. In sections 4.2 and 4.3, two individual cases of cryptococcosis (one in a captive bird and another in a domestic ferret) are similarly characterised, and the possible environmental source of infection for both cases is investigated. These two sections highlight that all animal species can prove useful as naturally-occurring models and sentinels for cryptococcosis. These manuscripts also provide further indication that sources of infection can be readily identified with careful sampling in a captive context. In section 4.4, another

unusual cluster of cryptococcosis in koalas is studied, but this time its novelty was in the implication of *C. gattii* VGII as the causative agent in a geographic area (eastern Australia), where VGII is usually rare. This again provided an opportunity to use high-resolution genotyping and fine-scale molecular epidemiology to investigate the origin of this case cluster. This study also offered the chance to assess the ability of koalas to translocate *Cryptococcus* spp. and to further characterise the Australian *C. gattii* VGII population.

Aims:

- **Aim 3**: assess the relationship between environmental presence, nasal colonisation and disease in some free-ranging koala populations;
- **Aim 4**: explore the interface between environment and disease in naturally-occurring cases of cryptococcosis in Australian animals through molecular epidemiology and phylogenetic analyses;
- **Aim 5**: determine the point source of infection or exposure in naturally-occurring disease or colonisation, caused by *C. gattii* VGI, in representative cases in captive animals using molecular epidemiology;
- **Aim 6**: assess two clusters of cryptococcosis in koalas for their outbreak potential; and
- **Aim 7:** examine the ability of koalas to translocate *C. gattii* VGII into new environments.

Sections:

- **4.1**: Molecular epidemiology of cryptococcosis in koalas offers insights into host-pathogen-environment interactions;
- **4.2**: Multi-locus sequence typing as a tool to investigate environmental sources of infection for cryptococcosis in captive birds;
- **4.3**: Identification of the environmental source of infection for a domestic ferret with cryptococcosis; and
- **4.4:** Jet-setting koalas spread *Cryptococcus gattii* VGII in Australia.

Impact:

The use of high-resolution molecular epidemiology will help to explore the role that animals (particularly koalas) could play in determining sources of infection, acting as sentinels and biological samplers, and dispersing members of the *C. gattii* species complex in the environment. This section has the potential to compare naturally-occurring disease in koalas in the wild and in captivity, and show that fine-scale genotyping in both scenarios could offer insights into the underlying pathogenesis of disease (including exploring the rarity of disease isolates in the free-ranging environment). The ability of koalas to translocate *C. gattii* VGII could indicate that further strategies are required to mitigate the risk of transferred or exported koalas introducing this pathogen into new environments. This could prove vital in the management and prevention of potential outbreaks of cryptococcosis in Australia.

4.1 Molecular epidemiology of cryptococcosis in koalas offers insights into host-pathogen-environment interactions

Authorship declaration:

This manuscript is yet to be submitted for peer review. L.J. Schmertmann is the primary author and contributed to all aspects of this study (designing and performing the research, analysing the data and preparing the manuscript). R. Malik, W. Meyer and M.B. Krockenberger designed the research and analysed the data. All authors were involved in the revision of the manuscript and support its inclusion in this thesis.

Laura J. Schmertmann _____ March 2019

Mark B. Krockenberger _____ March 2019

Molecular epidemiology of cryptococcosis in koalas offers insights into host-pathogen-environment interactions

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ABSTRACT

The *Cryptococcus gattii* species complex is a group of environmental basidiomycetous yeasts that causes cryptococcosis, a severe and often fatal mycosis, in a wide variety of host species. Koalas (*Phascolarctos cinereus*) exhibit comparatively high rates of symptomatic cryptococcosis, subclinical disease and cryptococcal colonisation of the nasal passages. An unusual case cluster of cryptococcosis in koalas was investigated, with eight free-ranging koalas diagnosed in a focal region of New South Wales, Australia, over an approximately two-and-a-half-year period. Environmental sampling and molecular epidemiology (*URA5* restriction fragment length polymorphism analysis and multi-locus sequence typing [MLST]) were used to investigate this case cluster, confirming the presence of *C. gattii* VGI in 11/46 (24%) tree hollows and in the sinonasal cavities of 13/23 (57%) koalas sampled in this area. MLST analysis of *C. gattii* VGI environmental (55), koala-colonising (13) and disease-associated (8) isolates did not suggest a single emergent outbreak strain, although maximum likelihood phylogenetic analysis revealed that disease isolates mostly clustered in a separate clade to environmental isolates. Koala colonising isolates were reasonably evenly distributed amongst environmental and disease isolates in the phylogenetic tree. The apparent difference between disease and environmental isolates suggests that disease isolates could either represent a rarer subpopulation in this environment or have undergone microevolutionary changes after contact with a host. This offers new insights into host-pathogen-environment interactions of cryptococcosis and highlights the koala as an important sentinel for this disease. Although the underlying cause of this case cluster remains unclear, it is possible that co-morbidities and/or anthropogenic threats could have increased the susceptibility of this koala population to a normally sporadic disease.

INTRODUCTION

The potential for pathogenic strains of the *Cryptococcus gattii* species complex, aetiologic agents of the severe systemic mycosis cryptococcosis, to cause case clusters across numerous host species has become increasingly apparent over the past few decades (Sorrell et al., 1996b, Baró et al., 1998, Raso et al., 2004, Chen et al., 2014, Maestrale et al., 2015, Kwon-Chung et al., 2017). *C. gattii* VGII has been implicated in many of these case clusters, with the

Vancouver Island and ongoing North American Pacific Northwest outbreaks in humans and animals of particular note (Craig et al., 2002, Kidd et al., 2004, Byrnes III et al., 2009). *C. gattii* VGI has also been identified as a culprit, a notable example being clusters of caprine cryptococcosis in Spain and Italy (Baró et al., 1998, Maestrale et al., 2015). Animals are increasingly considered useful sentinels for this environmentally-acquired disease (Duncan et al., 2006, Malik et al., 2011, Morera et al., 2014). The observation of clustered cases of animal cryptococcosis was critical in the early identification of the Vancouver Island outbreak (Craig et al., 2002).

In eastern Australia, the most prevalent member of the *C. gattii* species complex to cause disease is *C. gattii* VGI (Sorrell et al., 1996a, Chen et al., 2014). This organism has a strong environmental association with eucalypt trees, particularly tree hollows of *Eucalyptus camaldulensis* and *E. tereticornis* (Ellis & Pfeiffer, 1990, Pfeiffer & Ellis, 1992). Its known ecological niche continues to expand through ongoing systematic studies, with many other trees in Australia also identified as environmental sources and thus representing potential sources of infection (Sorrell et al., 1996a, Krockenberger et al., 2002b, Vilcins et al., 2002, Saul, 2009, Schmertmann et al., 2018a). Disease caused by the *C. gattii* species complex is acquired from the environment, typically by inhalation of basidiospores or desiccated yeast cells. Occasionally, direct inoculation has been identified as a likely route of infection (Krockenberger et al., 2003, Malik et al., 2003, Chen et al., 2014). The result of host exposure is likely influenced by multifaceted interactions between the size of the inoculum, the virulence of the strain/s and the host's immune capacity (Chen et al., 2014). Microevolution within the *C. gattii* species complex is thought to be a factor in the emergence of virulent genotypes, anti-fungal resistance and relapse cases of cryptococcosis (Billmyre et al., 2014, Farrer et al., 2016, Chen et al., 2017, Cuomo et al., 2018).

Koalas (*Phascolarctos cinereus*) are in regular and close contact with eucalypts, with *E. camaldulnesis* and *E. tereticornis* being favoured feed species (Lee et al., 1988). This may in part explain the tendency for cryptococcosis to be an important cause of sporadic morbidity and mortality in free-ranging koalas (Krockenberger et al., 2003), with approximately 3-4% having detectable cryptococcal lesions at postmortem (Stalder, 2003, Krockenberger et al., 2005, Malik et al., 2011). Recent findings have also suggested rates of approximately 5-7% for both nasal colonisation and subclinical cryptococcosis (asymptomatic antigenaemia) in one

free-ranging koala population (Schmertmann et al., 2019). The situation is different in captive koalas, where the animals appear to amplify the amount of the *C. gattii* species complex in their proximate environment, resulting in rates of nasal colonisation and subclinical disease that can be as high as 100% and >50%, respectively (Connolly et al., 1999, Krockenberger et al., 2002b). Given the propensity for koalas to develop cryptococcal nasal colonisation, the sinonasal cavity of the koala could be a useful biological sampler for environmental *Cryptococcus* spp. (Krockenberger et al., 2002b) and could also present a potential site for microevolutionary change.

The Port Stephens region of New South Wales (NSW), Australia (Figure 4.1.1) supports a free-ranging koala population (Lunney et al., 1998) in which cryptococcosis was previously diagnosed sporadically (M.B. Krockenberger, pers. comm.). Over an approximately two-and-a-half-year period (September 2013 to March 2016), eight cases of cryptococcosis (five clinical; three subclinical) were identified in free-ranging koalas in this region, with 6/8 occurring in two adjacent suburbs (Salamander Bay and Soldiers Point) (Table 4.1.1). One of these six cases presented with destructive fungal osteomyelitis of the distal hind limb, while its joey had subclinical cryptococcosis (based on asymptomatic cryptococcal antigenaemia) (Schmertmann et al., 2018b). This was identified as a potential spatial and temporal case cluster, raising the possibility that an emergent highly virulent strain and/or an unusually high level of environmental infectious cryptococcal propagules were present in this area. Either of these have the potential to impact on other animal species and humans in this region.

URA5 restriction fragment length polymorphism (RFLP) analysis and multi-locus sequence (MLST) typing of the *C. gattii* and *C. neoformans* species complexes are epidemiologic tools that together offer a reasonably high discriminatory power and allow for cases to be considered in both a regional and global context (Meyer et al., 2003, Meyer et al., 2009, Hagen et al., 2010). These techniques also allow comparison of cases within a cluster and the potential identification of known highly virulent genotypes.

This study aimed to: i) determine the presence of environmental *Cryptococcus* spp. and cryptococcal nasal colonisation in koalas in two adjacent suburbs (Salamander Bay and Soldiers Point) and ii) use fine-scale molecular epidemiology and phylogenetic analyses to both characterise disease strains and draw comparisons with environmental and nasal

colonising isolates. This will provide insights into the underlying cause of this case cluster and the host-pathogen-environment interactions underpinning cryptococcosis in koalas.

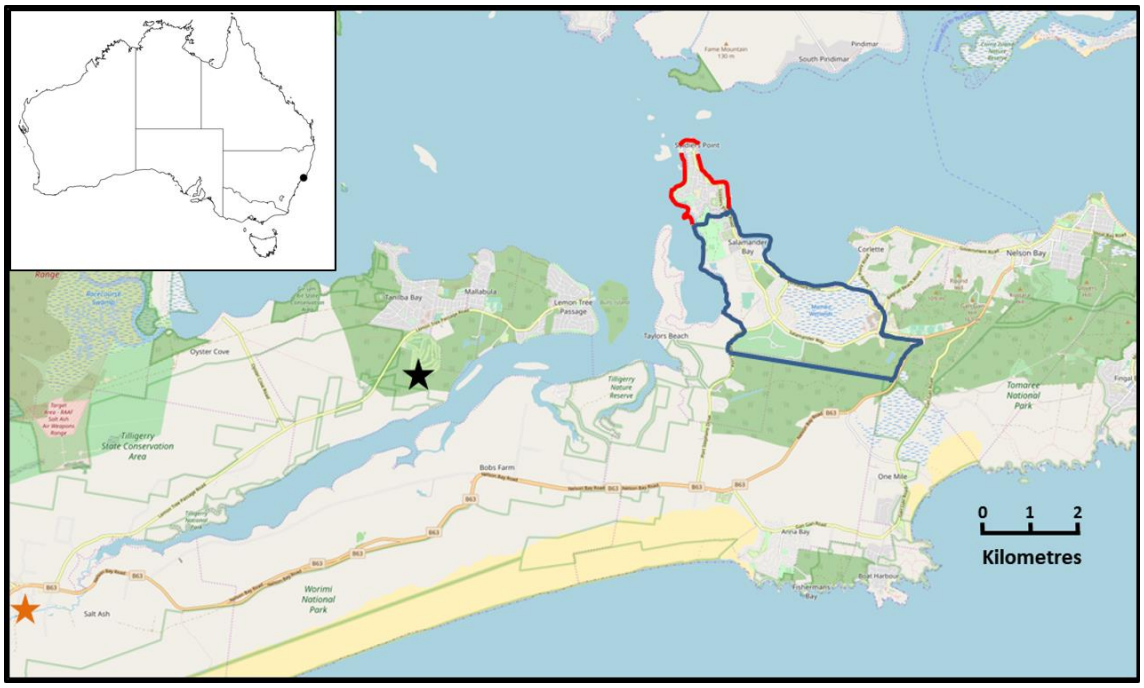

FIGURE 4.1.1 Map of the Tomaree and Tilligerry peninsulas within the Port Stephens region of New South Wales, Australia with the suburbs of Salamander Bay and Soldiers Point outlined in blue and red, respectively. Six koalas were diagnosed with cryptococcosis within these two adjacent suburbs from 2013 to 2017. Stars indicate the approximate locations of two other koalas diagnosed with cryptococcosis nearby, with the orange and black stars indicating the suburbs of Williamtown and Tanilba Bay, respectively. **Inset**: map of Australia with the approximate location of the Port Stephens region identified.

TABLE 4.1.1 Clinical details of eight koalas diagnosed with cryptococcosis in the Port Stephens region of New South Wales, Australia.

Koala	Disease	Serology		Nasal colonisation	KoRV (A/B)	Location	Source
		LFA	LCAT (titre)				
1*	C	NR	+ (1:512)	NR	+/-	Soldiers Point	[1]
2	SC	+	+ (1:64)	NR	NR	Soldiers Point	[1]
3*	C	NR	NR	NR	NR	Tanilba Bay	This study
4	C	NR	NR	NR	NR	Salamander Bay	This study
5*	C	NR	NR	+	NR	Williamtown	This study
6*	C	+	+ (1:131,072)	+	+/-	Salamander Bay	This study
7	SC	+	+ (1:8)	+	NR	Salamander Bay	This study
8	SC	+	+ (1:2)	+	NR	Soldiers Point	This study

*Disease isolate available. C = clinical; KoRV = koala retrovirus; LCAT = latex cryptococcal antigen agglutination test; LFA = cryptococcal antigen lateral flow assay; NR = not recorded; SC = subclinical; + = positive; - = negative. [1] Schmertmann et al., 2018b.

MATERIALS AND METHODS

Environmental Sampling

Areas for sampling were identified in the Salamander Bay and Soldiers Point areas of NSW, Australia, based on known koala habitats (advised by local koala rescue groups), the capture location of diseased koalas, viewing satellite images to identify bushland corridors and opportunistically. Trees were examined for the presence of one or more accessible hollows.

All accessible hollows were sampled, resulting in a total of 46 trees, 19 from Salamander Bay and 27 from Soldiers Point (Table 4.1.2), across three sampling trips in April 2015, September 2015 and November 2017. The interior surface of each tree hollow was thoroughly swabbed until the swab tip was discoloured but still intact. Sterile cotton-tipped swabs, pre-moistened with sterile saline, were used for this and were then refrigerated as soon as possible after collection. If possible, a generous amount (up to several hundred grams) of debris inside each hollow was also collected into clean plastic bags. These were labelled, sealed and stored at room temperature in the dark. Identification of each tree species was performed using a taxonomic key (Brooker & Kleinig, 2006).

Koala nasal swabs

Nasal swabs (collected by inserting a sterile moistened swab into the nasal vestibule on both sides and rotating gently (Connolly et al., 1999, Krockenberger et al., 2002b) from 23 free-ranging koalas in the Port Stephens region (Table 4.1.3) were submitted to Veterinary Pathology Diagnostic Services (VPDS) at The University of Sydney. The swabs were obtained from rescued koalas (captured by a local organisation, typically due to suspected injury or disease, or being in unsuitable habitat [e.g. adjacent to major roads]) as part of health checks performed by a local veterinary hospital prior to release. This hospital began to routinely collect nasal swabs for disease surveillance after the first cases of cryptococcosis were identified in 2013, and nasal swabbing was considered simpler and less invasive than blood collection from conscious free-ranging individuals. Koalas in Salamander Bay and Soldiers Point accounted for 9/23 nasal swabs. Koalas with clinical or subclinical cryptococcosis accounted for 4/23 nasal swabs.

Disease isolates

Disease isolates were available from four koalas with clinical cryptococcosis (Table 4.1.1), with isolates from three of these koalas provided by VPDS. The fourth koala (Table 4.1.1; Koala 6) was diagnosed with severe systemic cryptococcosis and euthanased during the time of this study. In this case, disease isolates were collected at postmortem as part of this study. This koala had widely disseminated disease and cryptococcal lesions were observed on gross, cytological and histopathological examination in the lung, brain, kidney, urine, lymph nodes, vertebrae and skin. Swabs and samples of some representative lesions were collected for culture. Unfortunately, no isolate was available from the fifth koala, as this diagnosis was made based on cytological and histopathological findings from samples collected at postmortem by local attending veterinarians.

Culture

All swabs (environmental, nasal and disease) were inoculated onto Staib's bird seed agar (containing penicillin and gentamicin) as soon as possible, by gently rolling the swabs across the entire agar plate. Debris samples were inoculated onto bird seed agar by agitating a small amount of debris (approximately 5g) in 100 ml of sterile saline, allowing the debris to settle for 5 minutes then spreading 1 ml of the supernatant across the plate. In some cases, a sterile moistened swab was also introduced into the bagged samples to mimic the process of swabbing the interior of a tree hollow; this swab was then rolled across the agar plate.

Plates were incubated at 27°C for 7-10 days and monitored a minimum of once daily for cryptococcal growth (yeast colonies exhibiting the brown-colour-effect). Samples were considered negative if no such colonies were observed after 10 days of observation. If suspect cryptococcal colonies were observed, the sample was classified as positive and then characterised as having a low (1-10 colonies), moderate (11-100 colonies) or heavy (>100 colonies) degree of cryptococcal growth. One to eight cryptococcal colonies from each positive plate were then sub-cultured on Sabouraud's agar at 37°C for isolation and DNA extraction.

Typing of isolates

DNA was extracted from all isolates using a pure heavy culture on Sabouraud dextrose agar and an established protocol for fungi (Ferrer et al., 2001). The *URA5* gene was amplified and underwent RFLP analysis as previously described (Meyer et al., 2003). Species and molecular types were assigned through comparisons with known *C. gattii* VGI-IV and *C. neoformans* VNI-IV standards (VGI: WM 179; VGII: WM 178; VGIII: WM 175; VGIV: VM 779; VNI: WM 148; VNII: WM 626; VNIII: WM 628; VNIV: WM 629) (Meyer et al., 2003).

The mating type specific genes *MF***a** and *MF*α were amplified by PCR together with known mating type **a** and α standards (WM 06.38 and WM 179, respectively) using established primers (Halliday et al., 1999, Fraser et al., 2003). The mating type was determined by visualising the PCR products on a 1.5% agarose gel run at 80V for 20 minutes and comparing the results to the known standards.

PCR amplification of the seven MLST loci (*CAP59, GPD1,* IGS1, *LAC1, PLB1, SOD1* and *URA5*) was performed using the primers and conditions specified by the consensus MLST scheme for the *C. neoformans* and *C. gattii* species complexes (Meyer et al., 2009). The *C. gattii* MLST database (http://mlst.mycologylab.org) was then used to assign allele and sequence type numbers.

Phylogenetic analysis

MLST sequences were concatenated then aligned by MUSCLE (Edgar, 2004), with subsequent alignments undergoing a maximum likelihood phylogenetic analysis to construct a neighbour joining tree (using the Jukes-Cantor model with gamma distribution). An Australian *C. gattii* VGIIb ST 7 strain (WM 13.373) was included for outgrouping. This strain was selected as it appears to be the most common *C. gattii* VGII ST in Australia (Carriconde et al., 2011). Bootstrap values were obtained from 1,000 replicates using a maximum likelihood algorithm. All alignments and analyses were performed using the MEGA7 program (Kumar et al., 2016).

Koala retrovirus testing

One koala (Table 4.1.1; Koala 6) was tested for koala retrovirus (KoRV) using genomic DNA extracted from a blood sample. An established PCR protocol was then used to test DNA for variants KoRV-A and KoRV-B (Xu et al., 2013). KoRV results were already available for another koala (Table 4.1.1; Koala 1) from a previous publication (Schmertmann et al., 2018b).

Statistical analysis

The possibility of dependent relationships between environmental sample culture results and location (Salamander Bay versus Soldiers Point) or tree species (*E. tereticornis* versus others) and between nasal swab culture results and koala location (Salamander Bay and Soldiers Point versus others) were assessed using 2x2 contingency tables and Fisher's exact test (two-tailed). P values < 0.05 were considered significant.

RESULTS

Culture

Samples from 14/46 (30%) trees (4 in Salamander Bay; 10 in Soldiers Point) cultured positive for *Cryptococcus* spp. on bird seed agar (Table 4.1.2). A total of 55 environmental isolates were successfully subcultured from 11 of these trees: *Angophora floribunda* (1), *E. pilularis* (1), *E. robusta* (1), *E. tereticornis* (5), *Melaleuca* spp. (1) and *Eucalyptus* spp. (2).

Nasal swabs from 13/23 (57%) koalas were positive for *Cryptococcus* spp., including all koalas from Salamander Bay and Soldiers Point (Table 4.1.3). A single strain from each positive koala was subcultured, resulting in 13 koala colonising isolates.

A total of eight disease isolates were available from four clinical cases, with 5/8 collected from one individual (Table 4.1.1; Koala 6). In this case, *Cryptococcus* spp. was cultured from vertebral and skin lesions, and the bronchi, urine and meninges, with one isolate collected from each of these sites. Three other disease isolates (from Koalas 1, 3 and 5 in Table 4.1.1) were also available.

TABLE 4.1.2 Bird seed agar culture, molecular typing and multi-locus sequence typing results for environmental samples from 46 trees in New South Wales, Australia.

Sample	Tree species	Location	Culture	*Cryptococcus* spp.	MT	ST
E2650	*Eucalyptus haemastoma*	SP	++	NT	NT	NT
E2652	*E. tereticornis*	SP	+++	*C. gattii* VGI	α	51
E2657	*E. pilularis*	SP	+++	*C. gattii* VGI	α, **a**	51, 154
E2660	*E. tereticornis*	SP	++	*C. gattii* VGI	α	51
E2661	*E. tereticornis*	SP	-			
E2662	*E. tereticornis*	SP	-			
E2663	*Eucalyptus* spp.	SP	+	NT	NT	NT
E2666	*Angophora floribunda*	SP	+++	*C. gattii* VGI	α	51, 188, 365, 366
E2667	*Melaleuca* spp.	SP	-			
E2669	*E. tereticornis*	SP	++	*C. gattii* VGI	α	366
E2670	*E. tereticornis*	SP	-			
E2672	*E. tereticornis*	SP	++	NT	NT	NT
E2675	*E. haemastoma*	SP	-			
E2688	*E. pilularis*	SB	-			
E2690	*Eucalyptus* spp.	SB	-			
E2692	*Melaleuca* spp.	SB	-			
E2695	*E. robusta*	SB	-			
E2697	*E. robusta*	SB	++	*C. gattii* VGI	α	366
E2698	*Eucalyptus* spp.	SB	-			
E2699	*Melaleuca* spp.	SB	+	*C. gattii* VGI	α	51
E2700	*E. robusta*	SB	-			
E2701	*E. tereticornis*	SB	-			
E2702	*A. costata*	SB	-			
E2703	*E. tereticornis*	SB	-			
E2704	*E. tereticornis*	SB	++	*C. gattii* VGI	α	51
E2705	*E. tereticornis*	SB	+	*C. gattii* VGI	α	51
E2706	*E. pilularis*	SB	-			
E2707	*Eucalyptus* spp.	SB	-			
E2708	*A. costata*	SB	-			
E2709	*E. pilularis*	SB	-			
E2710	*Eucalyptus* spp.	SB	-			
E2711	*E. pilularis*	SB	-			
E3035	*E. longifolia*	SP	-			
E3036	*Eucalyptus* spp.	SP	+++	*C. gattii* VGI	α	51
E3037	*E. tereticornis*	SP	-			
E3038	*E. tereticornis*	SP	-			
E3039	*Eucalyptus* spp.	SP	++	*C. gattii* VGI	α	51
E3040	*E. longifolia*	SP	-			
E3041	*E. longifolia*	SP	-			
E3042	*Eucalypt* spp.	SP	-			
E3043	*E. resinifera*	SP	-			
E3044	*Eucalypt* spp.	SP	-			
E3045	*E. longifolia*	SP	-			
E3046	*Eucalypt* spp.	SP	-			
E3047	*E. tereticornis*	SP	-			
E3048	*E. tereticornis*	SP	-			

+ = low degree of cryptococcal growth (1-10 colonies), ++ = moderate (11-100 colonies), +++ = heavy (>100 colonies); - = negative; MT = mating type; NT = not typed; SB = Salamander Bay; SP = Soldiers Point; ST = sequence type.

Typing

In total, 76 *Cryptococcus* spp. strains underwent molecular typing, with all subsequently being identified as *C. gattii* VGI using *URA5* RFLP analysis. Mating type analysis identified 75/76 as α; one environmental isolate (from an *E. pilularis* tree containing two other mating type α strains) was mating type **a** (WM 15.436).

TABLE 4.1.3 Bird seed agar culture, molecular typing and multi-locus sequence typing results for nasal swabs from 23 koalas in the Port Stephens region of New South Wales, Australia.

Koala	Location	Disease	Culture	*Cryptococcus* spp. (molecular type)	MT	ST
LS1	Salamander Bay		+	*C. gattii* (VGI)	α	154
LS3	Nelson Bay		-			
LS36	Corlette		-			
LS37	Corlette		-			
LS78	Bobs Farm		+++	*C. gattii* (VGI)	α	188
LS79	Corlette		+	*C. gattii* (VGI)	α	51
LS81	Soldiers Point		+++	*C. gattii* (VGI)	α	395
LS82	One Mile		++	*C. gattii* (VGI)	α	366
LS83	Williamtown		-			
LS86	Corlette		-			
LS87	Salamander Bay	SC	++	*C. gattii* (VGI)	α	396
LS90	Salamander Bay	C	+++	*C. gattii* (VGI)	α	57
LS91	Nelson Bay		-			
LS92	Salamander Bay		+++	*C. gattii* (VGI)	α	188
LS94	Bobs Farm		-			
LS95	Boat Harbour		-			
LS96	Corlette		-			
LS98	Soldiers Point		++	*C. gattii* (VGI)	α	51
LS152	Anna Bay		-			
LS176	Soldiers Point		++	*C. gattii* (VGI)	α	51
LS233	Soldiers Point	SC	+++	*C. gattii* (VGI)	α	57
14-7190	Salamander Bay		+	*C. gattii* (VGI)	α	51
14-8139	Williamtown	C	+	*C. gattii* (VGI)	α	51

+ = low degree of growth (1-10 colonies), ++ = moderate (11-100 colonies), +++ = heavy (>100 colonies), - = negative, C = clinical, MT = mating type, SC = subclinical, ST = sequence type.

All 76 isolates progressed to MLST analysis. Nine unique sequence types (STs) were identified: ST 51 (45/76), ST 366 (12/76), ST 57 (8/76), ST 188 (4/76), ST 154 (2/76), ST 365 (2/76), ST 364 (1/76), ST 395 (1/76) and ST 396 (1/76). Five of these STs were novel to the

MLST database (ST 364, ST 365, ST 366, ST 395 and ST 396). A total of 15 different allele types (AT) were identified in this study for the seven MLST loci. Three loci were highly conserved across all samples, with only one AT identified (*CAP59, GPD1* and *PLB1*). Two different ATs were observed at *LAC1* and *URA5* loci. The IGS1 and *SOD1* loci were the most variable, each with four unique ATs.

Five STs were identified amongst the 55 environmental isolates, with ST 51 and ST 366 accounting for most of the isolates (39/55 and 11/55, respectively). The remaining five isolates were ST 188 (2/55), ST 365 (2/55) and ST 154 (1/55). Of the 11 trees from which multiple *C. gattii* VGI strains were successfully isolated, 9/11 exhibited a clonal population (all isolates had the same ST). In one *E. pilularis* tree, two STs (ST 51 and ST 154) were identified, and both mating types (**a** and α) were also identified in this tree. In a single *A. floribunda* tree, four unique STs were detected (ST 51, ST 188, ST 365 and ST 366).

Amongst the 13 nasal colonising isolates, seven STs were identified, comprising of ST 51 (5), ST 57 (2), ST 188 (2), ST 154 (1), ST 366 (1), ST 395 (1) and ST 396 (1). All five disease isolates collected from the same koala (Table 4.1.1; Koala 6) were ST 57. The remaining three disease isolates were ST 51, ST 57 and ST 364. Four STs identified in this study were found only in koalas as colonising or disease isolates (ST 57, ST 364, ST 395 and ST 396) and were not detected in the environment.

Nasal colonising and disease isolates were available from the same individual in two cases. In one case, colonising and disease isolates were clonal according to MLST analysis (both ST 57). In the other koala, the colonising isolate was disparate to the disease isolate (ST 51 versus ST 57, respectively).

Phylogenetic analysis

A total of 72 isolates progressed to maximum likelihood phylogenetic analysis (55 environmental, 13 colonising and 4 disease isolates). Four disease isolates were excluded as all were of an identical ST and originated from the same individual (as mentioned above). The isolates separated in to two main clades with high bootstrap support (Figure 4.1.2). Clade 1 contained 65/72 isolates. The seven isolates in clade 2 were environmental (1), colonising (3)

and disease (3) isolates. Clade 1 was predominantly environmental (54) and colonising (10) isolates, with a single disease isolate.

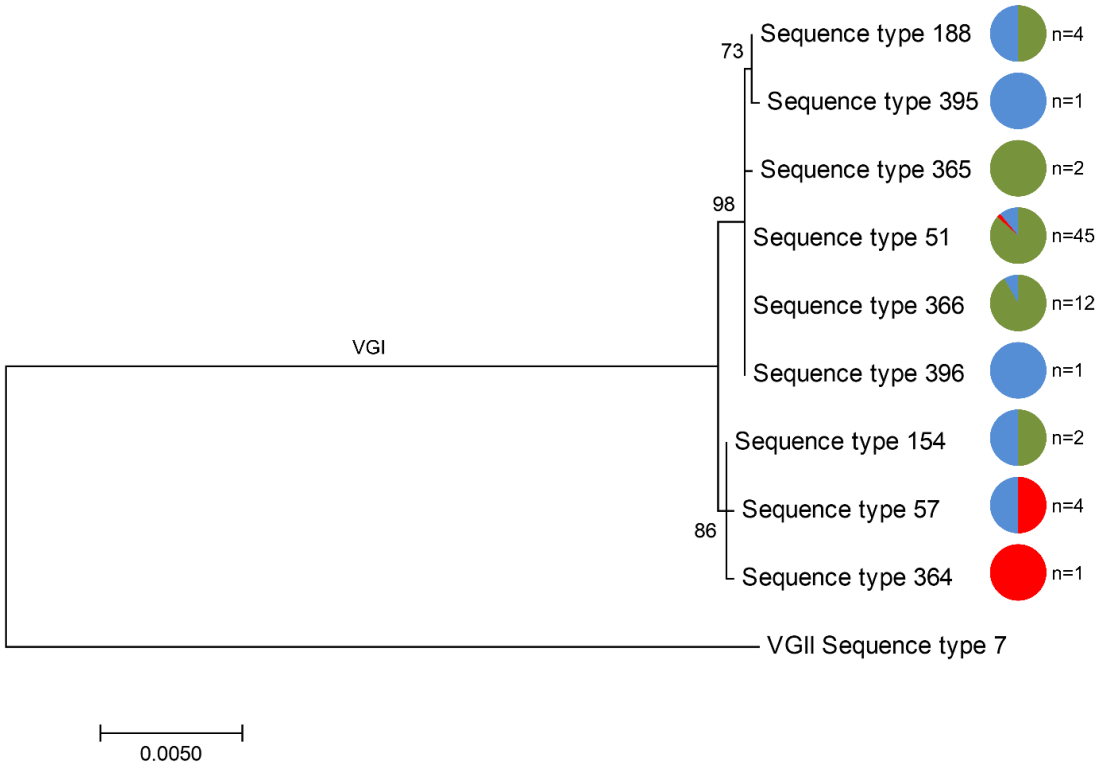

FIGURE 4.1.2 Maximum likelihood phylogenetic tree, using the Jukes-Cantor model with gamma distribution, of the nine *Cryptococcus gattii* VGI sequence types (STs) identified amongst koala disease, nasal colonising and environmental isolates from the Port Stephens region of New South Wales, Australia. *C. gattii* VGIIb ST 7 is included as an outgroup. Bootstrap values were obtained from 1,000 replicates using a maximum likelihood model, with values above 70 shown. Pie charts indicate the distribution of environmental (green), colonising (blue) and disease (red) isolates across each ST. Scale bar indicates the number of nucleotide substitutions per site.

Koala retrovirus testing

The two koalas tested were both positive for variant KoRV-A and negative for variant KoRV-B (Table 4.1.1; Koalas 1 and 6).

Statistical analysis

No significant relationships were established between environmental sample culture results and location ($p = 0.34$) or tree species ($p = 0.46$). Nasal colonisation was dependent on

location (p = < 0.01), with koalas from Salamander Bay and Soldiers Point significantly more likely to be colonised than koalas from other areas within the Port Stephens region.

DISCUSSION

The key finding of this study is the separation of most environmental and disease isolates into separate phylogenetic clades. This has significant implications for our understanding of the host-pathogen-environment interactions involved in koala cryptococcosis. In addition, we identified that colonising isolates were largely representative of the *C. gattii* VGI population in this area. We were unable to definitively ascertain the underlying cause of this case cluster, but an unusually high environmental prevalence of *C. gattii* VGI and/or single emergent outbreak strain appear to be unlikely. Increased host susceptibility in this area remains a possible contributing factor.

We identified the phylogenetic separation of most disease and environmental isolates (Figure 4.1.2) into two clades, well-supported by bootstrap values, and that four of the nine STs characterised in this study were found only in koalas (as colonising or disease isolates). These findings support a previous fine-scale epidemiological study into cryptococcosis of animals, where minor sequence differences differentiated disease strains from common environmental strains to which the animal was exposed (Schmertmann et al., 2018a). We also identified that colonising isolates were evenly distributed throughout the phylogenetic tree, which suggests that koalas are exposed to a wide range of *C. gattii* VGI strains in their environment. Through this broad exposure, it is possible that koalas 'select' for the most pathogenic strains from the environment, while these strains exist as rare environmental subpopulations. We performed a follow up Fisher's exact test (two-tailed) to compare the distribution of disease and non-disease isolates across the two clades, and we found that disease isolates were significantly more likely to be grouped in the lower clade (p = < 0.01).

Our findings could also be considered supportive of microevolution as a mechanism for the observed difference. Multiple isolates collected from relapse cases of central nervous system cryptococcosis in one study exhibited phenotypic and genetic changes over time, including adaptations to virulence mechanisms and drug resistance, indicative of microevolutionary change (Chen et al., 2017). While this phenomenon was not observed in

the single *C. gattii* VGI isolate included in that study (Chen et al., 2017), these findings still suggest that the sustained contact between koala nasal colonising isolates and the host mucosa (and likely the mammalian innate immune system) could also result in microevolutionary changes.

The environmental associations of *C. gattii* VGI continue to expand in Australia beyond the historical link with *E. camaldulensis* and *E. tereticornis* (Ellis & Pfeiffer, 1990, Pfeiffer & Ellis, 1992). Our findings highlight the importance of considering seemingly uncommon associations for this pathogen when performing environmental sampling, as the greatest ST and mating type diversity found in this study occurred in *A. floribunda* and *E. pilularis* tree hollows, respectively. *Cryptococcus* spp. colonies were observed in approximately 30% of the tree hollows sampled in this study, but *C. gattii* VGI could only be isolated and definitively identified in approximately 24%. This was typically due to the rapid overgrowth of filamentous fungi impeding the isolation of *Cryptococcus* spp. from the primary plate. In the experience of the authors and according to the literature, for a targeted environmental sampling study (focusing on the tree hollow as a known ecological niche) in eastern Australia, the herein obtained result is not indicative of an unusually heavy environmental burden of *C. gattii* VGI. For example, one study found that in two other coastal areas of NSW supporting large free-ranging koala populations, 19-28% of environmental samples were positive (Krockenberger et al., 2002b). Similar studies have also been conducted in several areas with no koalas, or very low population densities: in the northern Sydney and Blue Mountains regions of NSW, 11% and 9% of samples were positive, respectively (Krockenberger et al., 2002b, Vilcins et al., 2002), while in the Murray-Darling and Murrumbidgee River basins of NSW, up to 45% and 64% of samples were positive, respectively (Krockenberger et al., 2002b, Saul, 2009). It must be considered that these regions display differences in the tree population, with *E. camaldulensis* common in the inland river basins and Liverpool Plains and *E. tereticornis* more common in coastal areas (Brooker & Kleinig, 2006), as this could also affect the environmental prevalence of *C. gattii* VGI. It is, however, reasonable to conclude that our findings are still not supportive of an atypically heavy environmental burden of *C. gattii* VGI as the cause of this case cluster.

This study found that koala nasal colonising isolates were reasonably evenly distributed across the entire phylogenetic tree and exhibited a substantial ST diversity.

Colonising isolates reflected most environmental and disease STs, suggesting that they represent an excellent cross-section of the local *C. gattii* VGI community. This highlights the utility of the koala sinonasal cavity as an effective biological air sampler. Indeed, our findings suggest that koala nasal swabs may even be superior to environmental sampling to explore cryptococcal diversity in any given region. A large systematic study of cryptococcal nasal colonisation in the Liverpool Plains population (located in north-western NSW) of free-ranging koalas found that approximately 7% of koalas were colonised (Schmertmann et al., 2019). In another study, many hospitalised free-ranging koalas from a different region of coastal NSW were tested for nasal colonisation, with 35% found to be positive (Krockenberger et al., 2002b). The rates of nasal colonisation found in the present study, 57% amongst all koalas tested and 100% for those from Salamander Bay and Soldiers Point, are considerably higher. Indeed, rates of close to 60% and 100% are more representative of koalas residing in captive environments with high environmental cryptococcal burdens (Krockenberger et al., 2002b). The statistical relationship established between nasal colonisation and location suggests that koalas in the two hotspot suburbs are significantly more likely to be colonised than those from other areas of the Port Stephens region. The koala population density of these two suburbs is unknown, but it is unlikely to vary substantially from other nearby areas with suitable koala habitats (Lunney et al., 1998). When interpreting the results of this study, it is important to consider both the small sample size of koalas and the fact that some colonised koalas had presented to a veterinarian for illness or injury. Four of the colonised koalas (three from Soldiers Point and Salamander Bay; one from elsewhere in the Port Stephens region) were also considered to have clinical or subclinical cryptococcosis (Table 4.1.1; Koalas 5-8). To determine if colonised koalas with confirmed cryptococcosis were biasing the statistical analysis, we performed another Fisher's exact test (two-tailed) excluding these four koalas and found that the dependent relationship between colonisation status and location persisted ($p = < 0.01$).

Of the nine *C. gattii* VGI STs identified in this study, only four had been previously characterised, with ST 51 and ST 154 detected globally in environmental, veterinary and human isolates and ST 57 and ST 188 detected in Europe and North America as veterinary and human isolates, according to the *C. gattii* MLST Database. In the present study, ST 57 was detected amongst koala colonising and disease isolates, and ST 188 amongst environmental

and colonising isolates. The presence of five previously uncharacterised STs (ST 364, ST 365, ST 366, ST 395 and ST 396), one of which accounted in 16% (ST 366) of the isolates, might be indicative of a high diversity in this region, although the nucleotide substitution scale of the dendrogram (Figure 4.1.2) is not particularly suggestive of this. The finding may simply reflect a paucity of molecular epidemiology studies of *C. gattii* VGI from this area.

MLST results provided no evidence of an emergent outbreak strain, but the possibility of changes to virulence genes outside of the sequenced MLST loci cannot be excluded. If highly virulent strains of *C. gattii* VGI were implicated, a concurrent increase in cases in other wildlife species, domestic animals and humans could also reasonably be expected. The same could be true if the cause was an extremely high environmental presence. In the opinion of an experienced local veterinarian, the case load of cryptococcosis in companion animals in this area is not unusual (D. Hudson, pers. comm.). We opportunistically investigated one local companion animal case (a cat residing in Soldiers Point with sinonasal cryptococcosis) but identified *C. neoformans* VNII as the aetiological agent, thus it was considered unrelated to the koala case cluster. Any increase in the prevalence of human cases could not be ascertained. A comprehensive search through the records of the Medical Mycology Culture Collection at The Westmead Institute for Medical Research for other *C. gattii* species complex isolates from the Port Stephens region yielded no results. This collection contains thousands of environmental, veterinary and human clinical isolates from Australia and worldwide spanning several decades. Given the seemingly comparatively high rates of cryptococcosis reported in koalas (Krockenberger et al., 2003, Stalder, 2003) and the potential for long incubation periods and dormancy in humans and animals with *C. gattii* species complex cryptococcosis (Chen et al., 2014), it is possible that an increased prevalence of cases in other species will emerge in time. Therefore, it is pertinent to consider the koala as an important sentinel in this region and to monitor for further cases.

Host immune status and pathogen burden are other important considerations as potential contributors to this case cluster. KoRV-B, one of the exogenous KoRV variants, has the potential to cause immunomodulatory effects, with links between KoRV-B infection and chlamydiosis, malignant neoplasia and variations in immune cytokine expression well-established (Xu et al., 2013, Maher & Higgins, 2016, Waugh et al., 2017). Both koalas tested in this study were KoRV-B negative and KoRV-A positive. KoRV-A is an endogenous variant

considered unlikely to cause significant alterations to host immunity, and the KoRV-A status of these two individuals is consistent with prior findings of 100% prevalence in koalas from other areas of NSW (Simmons et al., 2012). Therefore, it appears unlikely that KoRV infection is implicated in this case cluster. It was not possible to ascertain the KoRV status of other koalas in this study, as samples appropriate for this type of testing (sufficiently intact koala genomic DNA) were unavailable. The potential relationship between cryptococcosis and KoRV has not been systematically studied.

Numerous other threats to this koala population are prevalent, including habitat fragmentation, motor vehicle strikes, dog attacks and bush fires, with the interaction of multiple threats known to contribute to a high risk for population decline (Lunney et al., 2007, Rhodes et al., 2011). Particularly in Soldiers Point, the koala habitat is highly fragmented by private property and roads. It is possible that the increasing number of stressors and threats to koalas in this area is contributing to a higher incidence of this normally sporadic, environmentally-acquired disease.

Further work could include environmental sampling throughout other areas of the Port Stephens region and the application of whole genome sequencing (WGS) as a higher resolution molecular epidemiology tool. WGS could shed further light on the relationship between these strains and could highlight if microevolutionary changes are occurring. The authors also aim to explore the impact of pathogen burden on koala populations, particularly co-infection with *C. gattii* VGI and exogenous KoRV variants.

Underlying causes of this case cluster remain elusive in the absence of the identification of an emergent outbreak strain or unusually high environmental prevalence of *C. gattii* VGI. Our findings indicate a genotypic distinction between disease and environmental isolates, offering further insights into the host-pathogen-environment interactions of cryptococcosis. This study cemented the utility of free-ranging koalas as both sentinels for this disease and biological samplers to assess the diversity of environmental *C. gattii* VGI.

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DATA ACCESSIBILITY

A representative sequence for each allele type identified in this study was submitted to GenBank, with accession numbers MK092949-MK092963. All isolates are stored in the Medical Mycology Culture Collection at The Westmead Institute for Medical Research in Westmead, NSW, Australia. Culture collection accession numbers are available in Supplementary Table S4.1.1.

SUPPLEMENTARY TABLE S4.1.1 List of cryptococcal isolates from the Port Stephens region of New South Wales, Australia included in this study. MLST data, strain information, allele types and STs are listed. Strains are ordered by ST number.

						Allele and sequence types							
WM number	**Species**	**Date**	**Category**	**Specific source**	**MT**	***CAP59***	***GPD1***	***IGS1***	***LAC1***	***PLB1***	***SOD1***	***URA5***	**ST**
14.340	*C. gattii* VGI	Nov 2014	Colonising	Koala - nasal	α	16	5	3	5	5	32	12	51
14.341	*C. gattii* VGI	Nov 2014	Disease	Koala - lung	α	16	5	3	5	5	32	12	51
15.169	*C. gattii* VGI	Dec 2014	Colonising	Koala - nasal	α	16	5	3	5	5	32	12	51
15.422	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
15.423	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.424	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.425	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.426	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.427	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.428	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.429	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.430	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.431	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.432	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.433	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.434	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.435	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.437	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
15.438	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
15.439	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
15.440	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
15.441	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
16.01	*C. gattii* VGI	Apr 2015	Enviro	*Melaleuca spp.*	α	16	5	3	5	5	32	12	51
16.02	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
16.04	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
16.27	*C. gattii* VGI	Oct 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
16.28	*C. gattii* VGI	Oct 2015	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
16.29	*C. gattii* VGI	Oct 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
16.30	*C. gattii* VGI	Oct 2015	Enviro	*E. pilularis*	α	16	5	3	5	5	32	12	51
16.53	*C. gattii* VGI	Dec 2015	Colonising	Koala - nasal	α	16	5	3	5	5	32	12	51
17.112	*C. gattii* VGI	Apr 2016	Colonising	Koala - nasal	α	16	5	3	5	5	32	12	51
17.113	*C. gattii* VGI	Jul 2016	Colonising	Koala - nasal	α	16	5	3	5	5	32	12	51
17.114	*C. gattii* VGI	Jul 2016	Enviro	*E. tereticornis*	α	16	5	3	5	5	32	12	51
18.140	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.141	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.142	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.143	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.144	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.145	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.146	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.147	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.148	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.149	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.150	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
18.151	*C. gattii* VGI	Nov 2017	Enviro	*Eucalyptus* spp.	α	16	5	3	5	5	32	12	51
15.170	*C. gattii* VGI	Dec 2014	Disease	Koala - hind limb	α	16	5	3	5	5	65	12	57
16.57	*C. gattii* VGI	Feb 2016	Colonising	Koala - nasal	α	16	5	3	5	5	65	12	57
16.62	*C. gattii* VGI	Feb 2016	Disease	Koala - meninges	α	16	5	3	5	5	65	12	57
18.131	*C. gattii* VGI	Mar 2016	Colonising	Koala - nasal	α	16	5	3	5	5	65	12	57
15.436	*C. gattii* VGI	Apr 2015	Enviro	*E. pilularis*	a	16	5	3	5	5	45	12	154
16.32	*C. gattii* VGI	Oct 2015	Colonising	Koala - nasal	α	16	5	3	5	5	45	12	154
16.05	*C. gattii* VGI	Apr 2015	Enviro	*Angophora floribunda*	α	16	5	12	5	5	32	12	188
16.08	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	12	5	5	32	12	188
16.52	*C. gattii* VGI	Dec 2015	Colonising	Koala - nasal	α	16	5	12	5	5	32	12	188
16.63	*C. gattii* VGI	Mar 2016	Colonising	Koala - nasal	α	16	5	12	5	5	32	12	188
13.375	*C. gattii* VGI	Sep 2013	Disease	Koala - lymph node	α	16	5	3	5	5	45	48	364
16.03	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	20	5	5	111	12	365

16.07	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	20	5	5	111	12	365
16.06	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
16.09	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
16.10	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
16.11	*C. gattii* VGI	Apr 2015	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
16.12	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	20	5	5	32	12	366
16.13	*C. gattii* VGI	Apr 2015	Enviro	*E. tereticornis*	α	16	5	20	5	5	32	12	366
16.26	*C. gattii* VGI	Oct 2015	Enviro	*E. robusta*	α	16	5	20	5	5	32	12	366
16.55	*C. gattii* VGI	Dec 2015	Colonising	Koala - nasal	α	16	5	20	5	5	32	12	366
18.136	*C. gattii* VGI	Sep 2017	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
18.137	*C. gattii* VGI	Sep 2017	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
18.138	*C. gattii* VGI	Sep 2017	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
18.139	*C. gattii* VGI	Sep 2017	Enviro	*A. floribunda*	α	16	5	20	5	5	32	12	366
16.54	*C. gattii* VGI	Dec 2015	Colonising	Koala - nasal	α	16	5	12	57	5	32	12	395
16.56	*C. gattii* VGI	Jan 2016	Colonising	Koala - nasal	α	16	5	97	5	5	32	12	396

Apr = April; Dec = December; Enviro = environmental; Feb = February; Jan = January; Jul = July; Mar = March; MT = mating type; Nov = November; Oct = October; Sep = September; ST = sequence type; WM = Westmead Mycology.

4.2 Multi-locus sequence typing as a tool to investigate environmental sources of infection for cryptococcosis in captive birds

Authorship declaration:

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Brief Report

Brief Report

Multi-locus sequence typing as a tool to investigate environmental sources of infection for cryptococcosis in captive birds

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Abstract

A systematic investigation into environmental sources of infection was conducted at an Australian zoological park after cryptococcosis, caused by *Cryptococcus gattii* VGI, was diagnosed in a red-tailed black cockatoo (*Calyptorhynchus banksii*) residing in a large aviary with a diverse range of other avian species. A single tree with an extensive hollow was identified as the likely source of infection based on heavy culture of *C. gattii* VGI, multi-locus sequence typing and phylogenetic analysis of environmental and disease-related isolates. This led to the careful removal of the tree to reduce the risk of future cases of cryptococcosis in this aviary.

Key words: cryptococcosis, *Cryptococcus gattii* VGI, avian, molecular epidemiology, multi-locus sequence typing.

Brief Report

Cryptococcosis is a severe mycosis of birds, mammals, and reptiles, which is acquired from the environment (typically by inhalation) and caused by members of the *Cryptococcus neoformans* and *C. gattii* species complexes.[1,2] The taxonomy of these species complexes is under ongoing debate, with the proposed division into seven species[3] not yet universally accepted. In Australia, avian cryptococcosis is most commonly caused by members of the *C. gattii* species complex and manifests as localised invasive upper respiratory disease.[4] The prevalence of avian cryptococcosis in both captive and free-ranging birds is unknown; while typically considered to be a sporadic disease, outbreaks caused by the *C. gattii* species complex can occur, as reported in a Brazilian aviary.[5] Avian cryptococcosis is often seemingly refractory to treatment and mortality is reported in many cases.[4,6,7] Tree hollows of many species provide an environmental niche for the *C. gattii* species complex, with *Eucalyptus camaldulensis* and *E. tereticornis* in Australia exhibiting particularly strong associations with *C. gattii* VGI and therefore present a potential source of infection.[8,9] Many native Australian birds, including red-tailed black cockatoos (*Calyptorhynchus banksii*), nest and shelter in eucalypt hollows.[10]

A red-tailed black cockatoo at a zoological park in Australia was diagnosed with cryptococcosis after it was noticed to be sneezing and have a subcutaneous swelling adjacent to the eye (Fig. 1, inset), later identified as a cryptococcal lesion by histopathological examination of a biopsy and subsequent

Figure 1. A dead eucalypt in the free flight aviary at a zoological park in Australia found to be consistently heavily contaminated by *Cryptococcus gattii* VGI. Inset: cryptococcal lesion (featherless area indicated by white arrow) in a red-tailed black cockatoo (*Calyptorhynchus banksii*). This Figure is reproduced in color in the online version of *Medical Mycology*.

mycological culture (Gribbles Veterinary Pathology, Clayton, Victoria, Australia). Serum cryptococcal antigen latex agglutination testing (CALAS®, Meridian Bioscience, Inc.) was positive (titer of 1:2048). The cockatoo was captive bred and raised, privately in Australia. Three months prior to presentation, the cockatoo had been transferred to a large free flight aviary with numerous other avian inhabitants, from a small education aviary on the same site in which it had been held for 8 years. The diagnosis prompted concern that other inhabitants of both aviaries may be at risk of developing cryptococcosis. An investigation aimed to identify environmental sources of infection, using culture, multi-locus sequence typing, and phylogenetic analysis, to guide management practices and prevent further cases of cryptococcosis.

Environmental samples were collected in May 2015 (Table 1; samples 1 to 10) from both aviaries. Sampling sites were selected randomly and through consideration of likely environmental niches for *Cryptococcus* spp. and areas favored by the cockatoo and other birds. Additional samples were collected from one site in August 2015 and February 2017 (Table 1; samples 11 to 20).

Samples were inoculated onto birdseed agar as previously described.[11] Two or three mucoid colony forming units exhibiting the brown-color-effect were isolated from all

Table 1. Birdseed agar culture and molecular typing results for environmental samples collected from two aviaries at a zoological park in Victoria, Australia.

Sample	Date	Aviary	Sample site	Culture result[a] (degree of growth[b])	*Cryptococcus* spp. (sequence types)	Culture collection accession numbers
1	May 2015	FF	Large stag hollow	Positive (H)	*C. gattii* VGI (ST57 [$n = 2$], ST154 [$n = 1$])	WM16.14, WM16.15, WM16.16
2	May 2015	FF	Large stag hollow	Positive (L)	*C. gattii* VGI (ST57 [$n = 2$]), *C. neoformans* VNI*	WM16.17, WM16.18, WM16.19
3	May 2015	FF	Large stag hollow	Negative		
4	May 2015	FF	Pooled substrate	Negative		
5	May 2015	FF	Feeding perch	Negative		
6	May 2015	FF	Feeding perch	Negative		
7	May 2015	Ed	Pooled substrate	Negative		
8	May 2015	Ed	Perch surface	Negative		
9	May 2015	Ed	Main perch	Negative		
10	May 2015	Ed	Secondary perch	Negative		
11	August 2015	FF	Large stag hollow	Positive (M)	*C. gattii* VGI (ST57 [$n = 1$], ST154 [$n = 1$])	WM16.22, WM16.23
12	August 2015	FF	Large stag hollow	Negative		
13	August 2015	FF	Large stag hollow	Positive (H)	*C. gattii* VGI (ST57 [$n = 1$], ST51 [$n = 1$])	WM16.24, WM16.25
14	February 2017	FF	Large stag hollow	Positive (M)	⋀	
15	February 2017	FF	Large stag hollow	Negative		
16	February 2017	FF	Large stag hollow	Positive (H)	⋀	
17	February 2017	FF	Large stag hollow	Negative		
18	February 2017	FF	Large stag hollow	Negative		
19	February 2017	FF	Large stag hollow	Negative		
20	February 2017	FF	Large stag hollow	Positive (L)	⋀	

[a]Presence (positive) or absence (negative) of mucoid colony forming units (CFUs) exhibiting the brown-colour-effect (BCE) on birdseed agar.
[b]Number of CFUs exhibiting the BCE; L = low (1-10 CFUs); M = moderate (11-100 CFUs); H = heavy (>100 CFUs). Ed = Education, FF = Free flight, ST = sequence type.
*Sequence type not determined.
⋀Molecular type and sequence type not determined.

positive samples collected in 2015. In addition, a disease-related isolate was provided by Gribbles Veterinary Pathology (Clayton, Victoria, Australia). All isolates are stored in the Medical Mycology Culture Collection at The Westmead Institute for Medical Research in Westmead, New South Wales, Australia. *URA5* gene restriction fragment length polymorphism (RFLP) analysis determined the species and molecular type.[12] Multilocus sequence typing (MLST) was performed using the consensus scheme for the *C. neoformans* and *C. gattii* species complexes,[13] with allele types (AT) and sequence types (ST) assigned using the Fungal MLST Database (http://mlst.mycologylab.org). Each AT sequence was submitted to Genbank (Supplementary table 1). Concatenated MLST sequences were aligned and maximum-likelihood phylogenetic analysis was performed using the program MEGA7.[14] Polymerase chain reaction (PCR) amplification of the *MF***a** and *MFα* genes using published primers determined the mating type.[15,16]

The 'large stag' (Fig. 1), a dead eucalypt of unknown species in the free flight aviary, was the only culture-positive site. Its extensive hollow was heavily culture-positive across multiple samplings (Table 1). The disease-related isolate and nine environmental isolates were identified as *C. gattii* VGI. One environmental isolate was identified as *C. neoformans* VNI. MLST analysis of *C. gattii* VGI isolates revealed four STs: ST57 ($n = 6$), ST154 ($n = 2$), ST51 ($n = 1$), and ST159 ($n = 1$) (Supplementary table 2). AT differences were observed at the *GPD1* and *SOD1* loci (two and three unique ATs, respectively). The disease-related isolate (ST159) differed from two environmental isolates (ST154) by only a single base pair insertion in the *GPD1* allele, which was confirmed by repeated PCR and sequencing. Phylogenetic analysis revealed a low genetic diversity amongst the *C. gattii* VGI isolates, but the identified clades had no bootstrap support as indicated by low bootstrap values (not shown). All isolates were mating type α.

This report presents a systematic investigation of environmental sources of avian cryptococcosis and associated molecular epidemiology. A similar investigation was previously attempted in a small avian outbreak but the *C. gattii* species complex could not be detected in the proximate environment.[5] We found a single tree hollow, used extensively by the aviary's inhabitants

(particularly the large parrots), to be consistently heavily contaminated by *C. gattii* VGI. Yeasts of the *C. gattii* species complex were not detected at any other site. Several trees in the free flight aviary could not be excluded as infection sources as the tree heights did not permit sampling, although no hollows were visible.

Most of isolates in this report were identified as *C. gattii* VGI (AFLP4[17]) by RFLP analysis. A 2014 publication describing a similar environmental investigation, prompted by several cases of cryptococcosis in ferrets, identified likely sources of infection based on matching disease and environmental isolates by amplified fragment length polymorphism (AFLP) analysis.[18] MLST analysis, a more discriminatory tool than RFLP or AFLP analysis, was used in the present study and revealed a subtle difference (single base pair insertion at the *GPD1* allele) between the disease-related strain and one of the identified environmental STs. If RFLP analysis alone was used, the strains would have appeared to be identical. The lack of an exact MLST match could be attributed to the disease-related strain representing a rare subset of environmental strains at a lower prevalence in the environment; in this case further sampling may eventually reveal an exact match. However, the insertion could also reflect a micro-evolutionary change.[19] The Fungal MLST Database (http://mlst.mycologylab.org) records for the STs identified in this study suggest that all are well-characterised, with STs 51 and 154 found worldwide in all types of samples (environmental, veterinary, and human). STs 57 and 159 appear to be primarily disease-associated isolates, with a few detections in human and veterinary samples from North America, Europe, Asia, and Africa. This could represent further evidence that ST159 is a rare environmental isolate and could also suggest that our detection of ST57 in the environment is unusual, but this speculation requires further investigation. The use of an even more discriminatory tool, such as whole genome sequencing, may aid in clarifying the relationship between these strains and could reveal a greater genetic diversity than detectable with MLST alone. The incubation period of *C. gattii* species complex cryptococcosis ranged from 2 to 11 months in a systematic study of *C. gattii* VGII disease in humans[20] but likely varies beyond this range. Possible incubation periods as short as 2–4 weeks have been reported in humans.[21] Substantially longer incubation periods can also occur; for example a presumptive seven year incubation period was recently reported in another avian patient.[6] In the present study, the cockatoo's residence at this site for over 8 years suggests that infection was acquired locally, although a longer incubation period is theoretically possible.

Since upper respiratory disease is considered the most common manifestation of avian cryptococcosis, the cockatoo's lesion likely reflected invasive paranasal sinus disease. Localised cutaneous disease can occur, often subsequent to pecking injuries in heavily contaminated environments.[4] Osteomyelitis of the distal humerus was the presenting lesion in another case.[22] The tendency for avian cryptococcosis to manifest as superficial disease is widely thought to be a consequence of the higher core body temperatures of birds precluding the growth of *Cryptococcus* spp. at more central anatomical sites. However, a recent study of thermotolerance in the *C. neoformans* species complex found that it was able to grow at avian core body temperature but that avian macrophages may be able to suppress its growth,[23] which could explain why disseminated disease appears to be less common, but can occur.[4,6,7] In the present study, no other cases of cryptococcosis were identified in either aviary. The cockatoo's transfer to the free flight aviary—a large, unfamiliar enclosure with significantly reduced human contact—may have been a sufficient stressor to increase its susceptibility to disease.

The identification of a single tree as a potential source of infection has implications for the ongoing management of this aviary. In captive koala enclosures, the 'furniture' is known to become heavily contaminated by *Cryptococcus* spp.[11] Therefore, precautionary regular replacement, decontamination and/or screening of incoming 'furniture' are logical recommendations. In this case, the size of the hollow and its multiple openings made decontamination or blocking access to it difficult. As such, removing the tree was considered the best option but proved logistically challenging, with its size necessitating the use of a chainsaw. The potential risk to personnel from the aerosolisation of cryptococcal infectious propagules was mitigated by the use of appropriate personal protective equipment.[24] Ideally the aviary's inhabitants should be monitored with regular examinations, culture of nares swabs, and cryptococcal antigen testing. However, this presents a significant stressor and logistical challenge in such a large aviary with many birds unaccustomed to handling and is therefore unfeasible.

Captive animal cryptococcosis offers a unique opportunity to systematically investigate environmental sources of infection. MLST has further elucidated the complexities of host-pathogen-environment interactions involved in avian cryptococcosis, although additional sampling and finer-scale molecular epidemiology is required to better understand these intricacies. Our findings also reinforce the importance of careful consideration of enclosure 'furniture' and the ongoing monitoring of enclosures and animals in captivity, particularly when known environmental niches of the *C. gattii* species complex are present.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors have no conflicts of interest to declare. The authors alone are responsible for the content and writing of the paper.

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SUPPLEMENTARY TABLE 1 Allele sequences deposited to GenBank – allele types and corresponding accession numbers for *Cryptococcus gattii* VGI isolates used in this study.

Locus	Allele type number	GenBank accession number
CAP59	16	MH286888
GPD1	5	MH286889
	14	MH286890
IGS1	3	MH286891
LAC1	5	MH286892
PLB1	5	MH286893
SOD1	32	MH286894
	45	MH286895
	65	MH286896
URA5	12	MH286897

SUPPLEMENTARY TABLE 2 List of *Cryptococcus gattii* VGI isolates collected for this study, including strain information, allele types and sequence types. Strains are ordered by sequence type number. Colours indicate allele type differences.

							Allele and sequence type numbers							
WM number	Other number	Date	Country	Category	Specific source	MT	*CAP59*	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	ST
16.25	E2693-B	August 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	32	12	51
16.14	E2678-A	May 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	65	12	57
16.15	E2678-B	May 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	65	12	57
16.17	E2679-A	May 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	65	12	57
16.18	E2679-B	May 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	65	12	57
16.22	E2689-A	August 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	65	12	57
16.24	E2693-A	August 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	65	12	57
16.16	E2678-C	May 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	45	12	154
16.23	E2689-B	August 2015	Australia	Enviro	MZ FFA	α	16	5	3	5	5	45	12	154
15.164	-	March 2015	Australia	Vet	RTBC	α	16	14	3	5	5	45	12	159

Enviro = environmental; MT = Mating type; MZ FFA = Melbourne Zoo free flight aviary; RTBC = red-tailed black cockatoo; ST = sequence type; Vet = veterinary.

4.3 Identification of the environmental source of infection for a domestic ferret with cryptococcosis

Authorship declaration:

This manuscript was submitted to the *Journal of Veterinary Diagnostic Investigation* and, at the time of submission of this thesis, is under review. L.J. Schmertmann is the primary author and contributed to all aspects of this study (designing and performing the research, analysing the data and preparing the manuscript). A. Wardman performed the research and analysed the data. L. Setyo and A. Kan performed the research. W. Meyer, R. Malik and M.B. Krockenberger designed the research and analysed the data. All authors were involved in the revision of the manuscript and support its inclusion in this thesis.

Laura J. Schmertmann _____ March 2019

Mark B. Krockenberger _____ March 2019

Identification of the environmental source of infection for a domestic ferret with cryptococcosis

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ABSTRACT

Cryptococcosis, caused by yeasts of the *Cryptococcus gattii*/*C. neoformans* species complexes, is an environmentally acquired disease affecting a broad range of host species. Amongst nine communally housed ferrets, a 5-year-old castrated ferret domiciled in an outdoor enclosure in Sydney, Australia was diagnosed with sinonasal cryptococcosis. All clinical signs resolved during 18 months of itraconazole therapy, but it was eventually euthanased due to splenic haemangiosarcoma. At postmortem, microscopic foci of persistent cryptococcosis were detected in brain and nasal tissues. The diagnosis raised concerns that the owners and other ferrets were exposed to a common environmental source of infection, thus prompting an investigation. Soil samples, swabs of a hollow eucalypt log (used for behavioural enrichment) and nasal swabs from eight asymptomatic ferrets were collected. Nasal exudate (obtained at diagnosis) and tissues (collected at postmortem) were available from the clinical case. Bird seed agar culture identified a heavy growth of *Cryptococcus* spp. from one environmental site (the log), one nasal swab and nasal exudate, brain and nasal tissues from the clinical case. All other samples were culture-negative. Sub-cultured isolates (17; 14 environmental, 1 nasal and 2 disease) were *Cryptococcus gattii* VGI (11/17, including both disease isolates) and *C. neoformans* VNI (6/17, including the colonising nasal isolate). Multi-locus sequence typing revealed both disease and colonising isolates as identical to environmental isolates collected from the log, confirming it as the source of clinical disease and nasal colonisation. The log was removed to prevent further exposure to a high environmental load of *Cryptococcus* spp.

INTRODUCTION

Cryptococcosis, an uncommon but important fungal disease of a wide variety of human and animal hosts, is caused by yeasts in the *Cryptococcus neoformans* and *C. gattii* species complexes (Kwon-Chung et al., 2017). Infection typically occurs subsequent to the inhalation of either desiccated yeast cells or reproductive basidiospores from the environment, meaning that respiratory tract disease is a common manifestation. There are well-established environmental niches recorded for these organisms, including accumulations of avian excreta (*C. neoformans* species complex) and tree hollows in a wide range of tree species (both

species complexes) (Malik et al., 2011, Chen et al., 2014). In Australia, eucalypt trees exhibit a particularly strong association with *C. gattii* VGI (Ellis & Pfeiffer, 1990, Pfeiffer & Ellis, 1992).

Ferrets (*Mustela putorius furo*) are one of many vertebrates in which cryptococcosis has been documented. Their behavioural tendencies [digging, soil sniffing, exploring confined spaces (Boyce et al., 2001)] and high prevalence of intercurrent nasal cavity disease [snuffles (Lewington, 2007)] and nonspecific upper respiratory conditions (Johnson-Delaney & Orosz, 2011) may predispose them to exposure, colonisation and disease caused by yeasts of the *C. neoformans* and *C. gattii* species complexes (Malik et al., 2011, Richardson & Perpiñán, 2017). This has led to ferrets being considered a sentinel species for human cryptococcosis (Malik et al., 2002, Wyre et al., 2013, Morera et al., 2014). In reviews of the ferret cryptococcosis literature (Wyre et al., 2013) and subsequent case reports (Morera et al., 2014), ferrets can be affected by both the *C. neoformans* and *C. gattii* species complexes, with no clear link between cryptococcosis and immunosuppression in this host species. They present with a range of clinical signs and lesions affecting a variety of body sites, including both the upper and lower respiratory tracts (the usual primary site of infection), central nervous system (CNS), gastrointestinal tract and cutaneous disease (Malik et al., 2002, Wyre et al., 2013). Direct inoculation from penetrating injury may also be a mechanism by which localised subcutaneous disease develops (Malik et al., 2002).

A 5-year-old, albino, castrated male (gib) domestic ferret residing in western Sydney, Australia, was diagnosed with sinonasal cryptococcosis. The affected animal was communally housed with eight other asymptomatic ferrets. It had been presented with a four-week history of violent sneezing, with a bilateral bloody purulent nasal discharge. The ferret had access to a confined outdoor area, which included some soil and a large hollow eucalypt log provided for environmental enrichment and as a place of refuge. On physical examination, marked facial asymmetry was noted with swelling of the nasal bridge on the right-hand side (Figure 4.3.1). Cytology was performed on the nasal discharge, revealing numerous encapsulated yeasts displaying narrow-necked budding and degenerate inflammatory cells (largely macrophages and some neutrophils). In addition, there were some raised circular (0.5 cm diameter) alopecic skin nodules over the neck, face and body. Cytological examination of smears from fine-needle aspirates of the skin lesions also revealed encapsulated yeasts with an inflammatory infiltrate dominated by macrophages (Figure 4.3.2). Based on the

characteristic cytology, a diagnosis of cryptococcosis was made. The infection presumably started in the sinonasal cavity, with subsequent invasion of the adjacent bone and extension to subcutaneous tissues of the nasal bridge, and haematogenous dissemination giving rise to the multifocal skin nodules.

The ferret was anaesthetised using isoflurane in 100% oxygen to facilitate the collection of blood by jugular venipuncture. After centrifugation to harvest serum, cryptococcal antigen testing was performed using both the lateral flow assay (LFA; CrAg® LFA, IMMY, Norman, Oklahoma, USA) and latex agglutination test (LCAT, CALAS®, Meridian Bioscience, Inc., Cincinnati, Ohio, USA). Serological testing was positive using both LFA and LCAT, the latter recording a reciprocal titre of 1,024. Medical therapy was commenced with itraconazole (Sporanox® 100 mg capsules, Janssen, North Ryde, New South Wales, Australia) monotherapy at a dose of 10 mg/kg once daily orally mixed in with the ferret's normal food. No possible causes of immunosuppression [e.g. lymphoma (Erdman et al., 1992)] were indicated by the history or physical examination, although further investigations were precluded due to financial constraints.

Four weeks after commencing therapy, nasal discharge and sneezing had resolved but nasal bridge swelling was still evident. The multifocal skin lesions resolved slowly over the next few months. The medication was generally well tolerated, but some intermittent periods of anorexia occurred which resolved when itraconazole therapy was interrupted for 2-3 days. By 12 months after commencement of therapy, facial asymmetry had disappeared. The ferret was maintained on itraconazole therapy, because complete resolution of the cryptococcal antigenaemia was considered unlikely at this stage and further serological testing was not possible due to financial constraints.

The ferret had been on itraconazole for 18 months when it developed generalised weakness, inappetence, polydipsia, an unkempt coat and became acutely dyspnoeic. On physical examination, cutaneous bruising and subcutaneous ecchymoses on the ventrum were evident, accompanied by marked dyspnoea and tachypnoea. The ferret was in poor body condition, with dull mentation and pale mucous membranes. At the owner's request, the ferret was euthanased with pentobarbitone without further investigations and the body was submitted for postmortem examination, during which haemabdomen and a ruptured splenic haemangiosarcoma with hepatic metastases were identified. A serum sample was

unavailable, but opportunistic cryptococcal antigen testing of serosanguinous peritoneal fluid was positive using both the LFA and LCAT (reciprocal titre of 32), and histopathological examination of tissues revealed microscopic cryptococcal lesions in the cerebrum (Figure 4.3.3) and nasal turbinates. Other age-related abnormalities observed incidentally on gross and microscopic examination included chronic renal disease and dilated cardiomyopathy.

This case prompted concerns regarding whether the eight companion ferrets and their owners could be at risk of developing cryptococcosis from common exposure to a localised identifiable source of heavy environmental contamination. Our aim was therefore to: i) determine if exposure of the other ferrets to *Cryptococcus* spp. had occurred via culture of nasal swabs (to assess for colonisation) and serological testing (for subclinical disease) where possible; ii) conduct environmental sampling and mycological culture to determine if a source of infection was present in the proximate environment and iii) use fine-scale molecular genotyping to explore the epidemiology and characterise *Cryptococcus* spp. isolates.

MATERIALS AND METHODS

Sample collection

Three pooled soil samples (from pot plants and garden beds to which ferrets had access) and three samples from a log within the enclosure (obtained by running pre-moistened sterile cotton swabs over the surfaces of the log) were collected for culture.

Nasal swabs from the eight asymptomatic ferrets were collected by inserting a sterile, pre-moistened, thin-tipped cotton urethral swab into the nares and rotating gently while under manual restraint. Samples were obtained over several weeks after the diagnosis of the clinical case. A blood sample from one asymptomatic ferret was also opportunistically collected by jugular venepuncture while it was under general anaesthesia to investigate an intra-abdominal mass (later determined to be intestinal adenocarcinoma).

A swab of the nasal exudate from the clinical case, collected at the time of diagnosis using a similar method to that described above, was available for mycological culture. Several tissue samples (nasal turbinate, lung and brain tissue) collected at postmortem examination after 18 months of antifungal therapy were also available for culture.

Serology

Serum antigenaemia was determined using the LFA (CrAg® LFA, IMMY) and LCAT (CALAS®, Meridian Bioscience, Inc.) kits according to the manufacturers' instructions on samples from one asymptomatic ferret and the ferret with invasive cryptococcosis. Serosanguinous abdominal fluid from the clinical case was obtained at postmortem for LFA and LCAT testing.

Culture

All environmental and nasal swabs were inoculated onto Staib's bird seed agar (containing penicillin and gentamicin) by gently rolling them across the entire surface of an agar plate. Soil samples were inoculated by agitating approximately 5-10 g of the sample in 50 mL of sterile saline, allowing the mixture to settle for 10 minutes and then spreading 1 mL of supernatant across the surface of a bird seed agar plate (Krockenberger et al., 2002b). Tissue samples were inoculated directly onto the bird seed agar plates. All plates were incubated at 27°C and checked daily. Plates were considered positive for *Cryptococcus* spp. if yeast colonies exhibiting the brown-colour-effect were visually identified and were classified as either exhibiting a low (1-10 colonies), moderate (11-100 colonies) or heavy (>100 colonies) degree of cryptococcal growth. One or more of these colonies were randomly sub-cultured from positive plates onto Sabouraud's dextrose agar for isolation and DNA extraction. If more than one colony morphology exhibiting the brown-colour-effect was observed (as in Figure 4.3.4), then several colonies of each type were randomly selected for sub-culture. Samples were considered negative if no cryptococcal colonies were visible after 10 days of observation.

Molecular and mating type determination

DNA was extracted from all isolates using a previously established protocol for fungi (Ferrer et al., 2001). Restriction fragment length polymorphism (RFLP) analysis of a PCR product of the *URA5* gene was then performed and compared to known standard strains in the *C. gattii* and *C. neoformans* species complexes (*C. gattii* VGI: WM 179; VGII: WM 178; VGIII: WM 175; VGIV: WM 779; *C. neoformans* VNI: WM 148; VNII: WM 626; VNIII: WM 628; VNIV: WM 629) to determine species and molecular type (Meyer et al., 2003).

PCR amplification of the MF**a** and MFα genes was performed for all isolates using established primers (Halliday et al., 1999, Fraser et al., 2003). PCR products were compared to known standards (mating type **a**: WM 06.38; mating type α: WM 179) using gel electrophoresis to determine mating type.

Multi-locus sequence typing and phylogenetic analysis

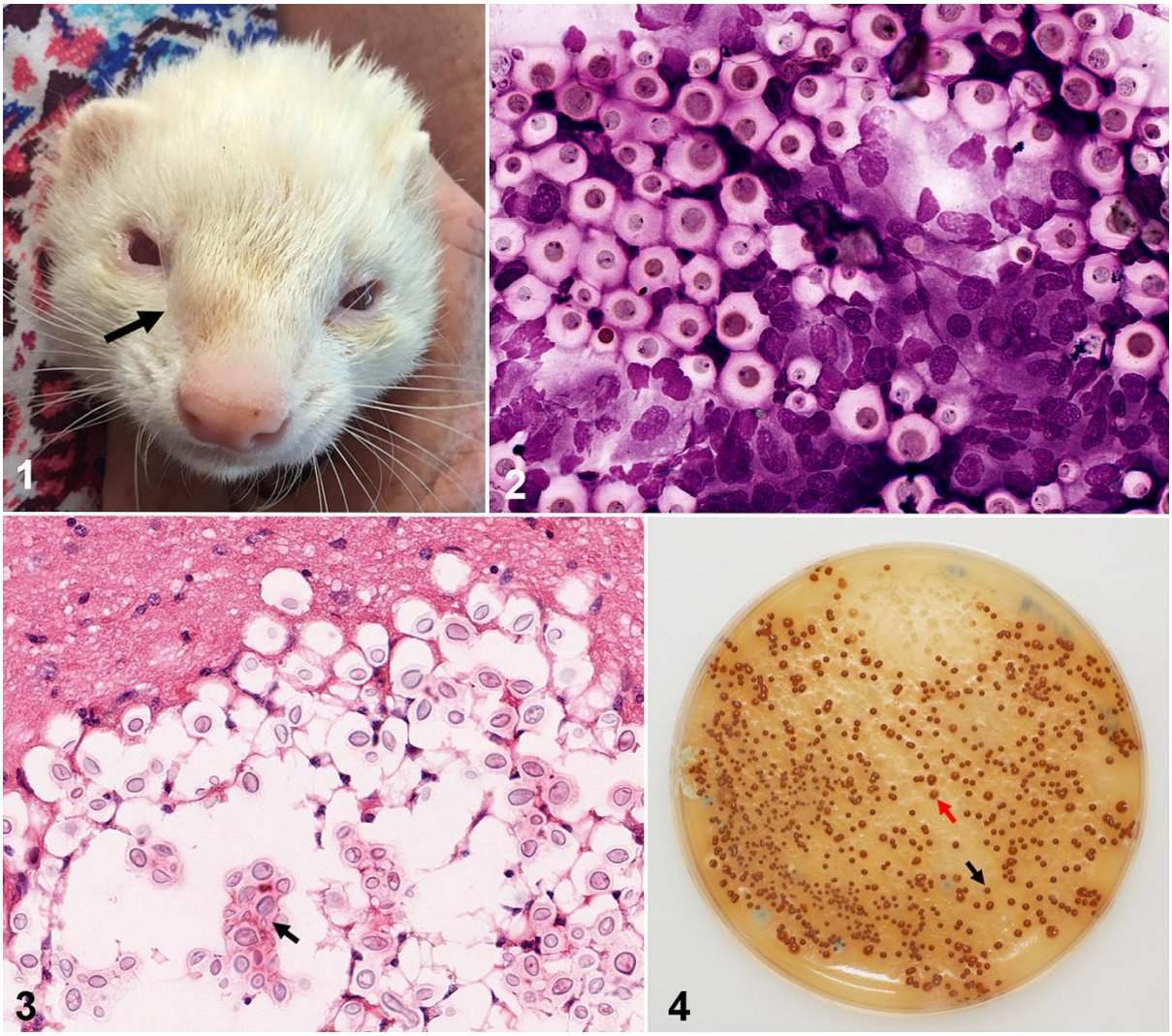
All isolates underwent multi-locus sequence typing (MLST) according to the consensus scheme for the *C. gattii* and *C. neoformans* species complexes (Meyer et al., 2009). This involved PCR amplification and sequencing of seven loci (*CAP59, GPD1,* IGS1, *LAC1, PLB1, SOD1* and *URA5*) followed by assignment of allele and sequence types using the Fungal MLST Database (http://mlst.mycologylab.org).

Concatenated MLST sequences were aligned by MUSCLE (Edgar, 2004) and underwent maximum likelihood phylogenetic analysis, using the Kimura 2-parameter model (Kimura, 1980) with gamma distribution, to construct a phylogenetic tree. Four standard strains were included for comparison and outgrouping (*C. gattii* VGI: WM 179; VGIV: WM 779; *C. neoformans* VNI: WM 148; VNII: WM 626). Bootstrap values were calculated using the maximum likelihood algorithm and 1,000 replicates. All analyses were performed using the MEGA7 program (Kumar et al., 2016).

RESULTS

Serology

A serum sample from one asymptomatic ferret was negative for cryptococcal antigen using both the LFA and LCAT. The clinical case tested positive on both the LFA and LCAT (reciprocal titre of 1,024). The serosanguinous abdominal fluid, collected at postmortem from the clinical case, returned positive LFA and LCAT (with a reciprocal titre of 32) results.



FIGURES 4.3.1 - 4.3.4 Cryptococcosis in a ferret. **FIGURE 4.3.1** Facial asymmetry in a ferret later confirmed to be sinonasal cryptococcosis caused by *Cryptococcus gattii* VGI; note the swelling (arrow) in the right naso-ocular subcutaneous tissues. **FIGURE 4.3.2** Fine-needle aspirate of a cutaneous nodule in a ferret - abundant large yeast cells with rare narrow-necked budding (later determined to be *C. gattii* VGI) are present; note the prominent negatively-stained capsules with surrounding inflammatory cells (predominantly macrophages). Diff-Quik. **FIGURE 4.3.3** Cryptococcosis in the cerebrum of a ferret; note the numerous heavily encapsulated yeasts, some exhibiting narrow-based budding (arrow), consistent with *Cryptococcus* spp. (later identified as *C. gattii* VGI); also note the minimal inflammatory response. Haematoxylin & Eosin. **FIGURE 4.3.4** Bird seed agar culture of a swab collected from a hollow eucalypt log used for environmental enrichment in a ferret enclosure in western Sydney, Australia; note the heavy growth of yeast colonies exhibiting the brown-colour-effect (consistent with *Cryptococcus* spp.) and presence of 2 distinct colony morphologies: paler brown and more mucoid (red arrow, later identified as *C. gattii* VGI), or darker brown and drier (black arrow, later identified as *C. neoformans* VNI).

Culture

All three swabs collected from the hollow log were heavily positive (>100 colonies) for *Cryptococcus* spp. on primary culture (Figure 4.3.4). All soil samples were negative. Of the eight asymptomatic ferrets from which nasal swabs were collected, one was heavily culture-positive for *Cryptococcus* spp. The nasal exudate swab from the clinical case was also heavily culture-positive for *Cryptococcus* spp. Of the tissue samples collected postmortem, the nasal turbinates and brain were moderately (11-100 colonies) culture-positive for *Cryptococcus* spp., while the lung was negative.

Molecular and mating type determination

A total of 17 isolates proceeded to DNA extraction and molecular typing; environmental isolates collected from the positive log (14/17), an asymptomatic ferret nasal swab isolate (1/17) and disease isolates from the clinical case (2/17: one from the nasal exudate at the time of diagnosis; one from the brain at postmortem). *URA5* RFLP analysis determined that *C. gattii* VGI accounted for 11/17 isolates, comprising nine environmental and the two disease isolates. The remaining six isolates were *C. neoformans* VNI, comprising the nasal colonising isolate from the asymptomatic ferret and five environmental isolates.

A total of 15/17 isolates were identified as mating type α. Two isolates (both environmental *C. gattii* VGI from the hollow log) were mating type **a** (Supplementary tables S4.3.1 and S4.3.2).

Multi-locus sequence typing and phylogenetic analysis

Amongst the 11 *C. gattii* VGI isolates, two sequence types (ST) were identified: ST 154 (10 isolates) and ST 541 (1 environmental isolate). Allele type (AT) variation between these two STs was seen only at the IGS1 locus, with two unique ATs identified, differing by just a single nucleotide polymorphism (SNP) (Supplementary table S4.31). Of the six *C. neoformans* VNI isolates, 5/6 were ST 13 while the remaining isolate was ST 23. Variation between ST 13 and 23 occurred at the *CAP59*, *GPD1*, *LAC1*, *PLB1* and *URA5* loci, with two unique ATs identified for each (Supplementary table S4.3.2).

The phylogenetic tree (Figure 4.3.5) confirmed the *URA5* RFLP results by its separation of isolates into clades corresponding with their species and molecular type standards, with high bootstrap support.

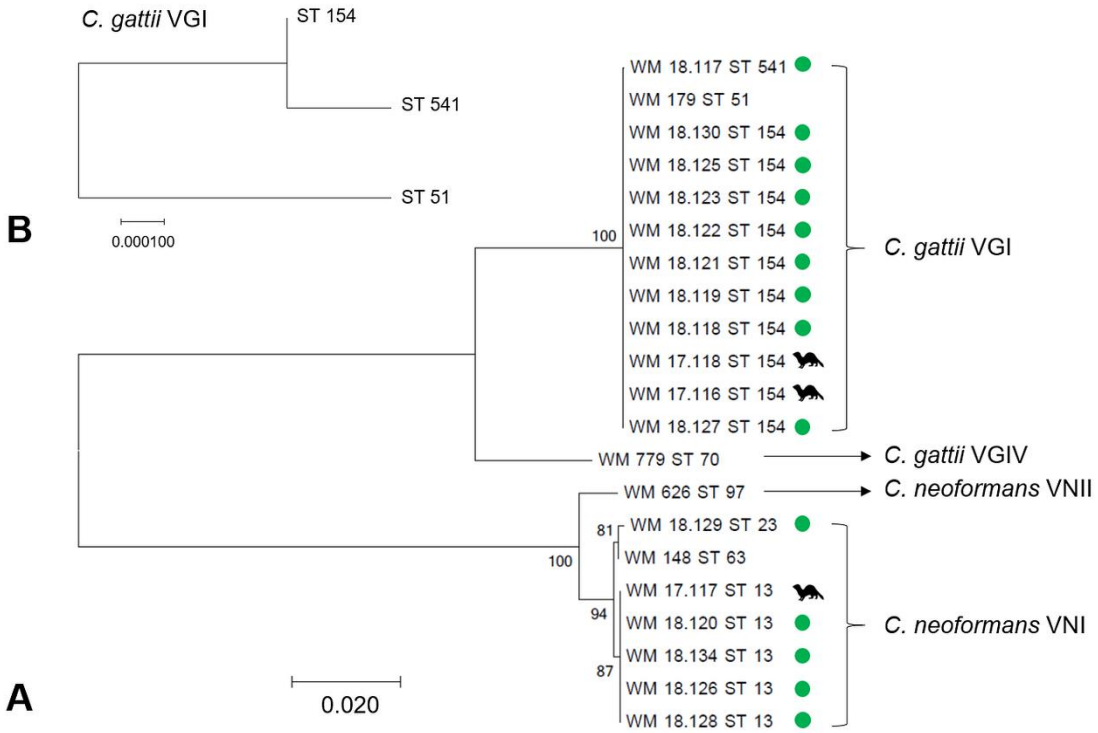


FIGURE 4.3.5 (A) Maximum likelihood phylogenetic tree based on concatenated MLST sequences (from *Cryptococcus gattii* VGI and *C. neoformans* VNI isolates collected from a group of ferrets and their environment in western Sydney, Australia, and 4 standard strains [WM 148, WM 179, WM 626 and WM 779]), using the MEGA7 program and the Kimura 2-parameter model with gamma distribution. Green circles and silhouettes indicate environmental and ferret isolates, respectively. **(B)** Higher resolution tree showing the relationships of the *C. gattii* VGI isolates. Bars = number of nucleotide substitutions per site.

DISCUSSION

This study identified a hollow eucalypt log, used for shelter and environmental enrichment, as the likely point source of infection for *C. gattii* VGI in a ferret with cryptococcosis and the likely source of a nasal colonising isolate in another asymptomatic ferret. A mixed mating type *C. gattii* VGI population (both α and **a**) was identified in this environmental niche, raising the possibility of sexual production of basidiospores in the pathogenesis of cryptococcosis in this setting. The findings of this study suggest that careful consideration of environmental

enrichment items for use by ferrets (and potentially other animal species susceptible to cryptococcosis) may be indicated.

18-month survival in a ferret with widely disseminated cryptococcosis is a good clinical outcome, considering the patient had a good quality of life before developing a terminal malignancy, with two concurrent age-related co-morbidities (dilated cardiomyopathy and chronic renal disease). The prognosis for disseminated cryptococcosis in ferrets is not well-established, but could reasonably be considered guarded, based on a review of prior case reports (Wyre et al., 2013). The decision to use itraconazole was based on the ease with which itraconazole capsules can be opened and mixed in with palatable food, and because its pharmacokinetics in other carnivores permit once daily dosing (compared to fluconazole, which is generally given twice daily). Ideally, antifungal susceptibility testing should have been undertaken prior to treatment to determine minimum inhibitory concentrations (MICs) for itraconazole and fluconazole, but financial constraints made this impossible. Retrospective testing of both disease isolates (nasal exudate isolate collected at the time of diagnosis and brain isolate collected postmortem) suggested that they were susceptible to all antifungals tested (including itraconazole and fluconazole). This is consistent with the expectation that cases of cryptococcosis on the east coast of Australia are usually susceptible to both agents, as the *C. gattii* VGII molecular type [more associated with higher MICs for some antifungals (Chong et al., 2010)] is rare in this area (McGill et al., 2009). Antifungal pharmacokinetics are not reported for the ferret and it is possible that therapeutic failure was related to pharmacokinetic variations.

With the benefit of hindsight, it seems likely that this ferret had asymptomatic CNS disease at the time of initial diagnosis, but that long term itraconazole therapy was enough to prevent this developing further, and indeed the substantial clinical improvement suggested efficacy of therapy. Infusions of amphotericin B given subcutaneously (Malik et al., 1996a) or intravenously (Richardson & Perpiñán, 2017) two to three times weekly in concert with an oral triazole antifungal would have represented a more aggressive approach and likely been more effective but was also beyond the financial and time constraints of the owner. The dose of itraconazole could also have been increased, with successful treatment reported in a case of localised disease using a dose of 25 mg/kg once daily orally (Malik et al., 2002). Given that the clinical signs largely resolved and the dose of 10 mg/kg was well tolerated, this was not

thought worthwhile at the time. Fluconazole is generally considered the drug of choice for treating cryptococcal meningitis due to better penetration of the blood brain barrier (Felton et al., 2014), but it requires twice daily therapy in most species, and for ferrets this would probably have required use of a compounded palatable liquid formulation. Ideally, therapy should also have been monitored with ongoing serological testing, and indeed our findings of microscopic foci of ongoing infection (despite full resolution of clinical signs) emphasise the value of sequential antigen titres. Unfortunately, sequential LCAT titre determination was beyond the financial resources of the owner. Therapeutic drug monitoring would have been ideal to ensure dosing was producing effective itraconazole levels in blood, given that the pharmacokinetics of no antifungal drugs have been determined for ferrets.

Most of the environmental *C. gattii* VGI strains collected from the enclosure log were ST 154, with one *C. gattii* VGI ST 541 isolate detected also (these two STs differing by a SNP in the IGS1 allele). MLST also showed that both disease isolates, collected approximately 18 months apart, were *C. gattii* VGI ST 154. A similar pattern was observed amongst the *C. neoformans* VNI isolates, with a colonising isolate from one asymptomatic ferret identical to most environmental *C. neoformans* VNI isolates (ST 13). One *C. neoformans* VNI ST 23 strain was identified, which differed from ST 13 at five of the seven MLST loci. Phylogenetic analyses of the MLST sequences confirmed the molecular typing results, grouping all isolates with their appropriate standard strain and further confirming a high degree of similarity between *C. gattii* VGI STs 154 and 541, but a greater difference between *C. neoformans* VNI STs 13 and 23 (Figure 4.3.5).

The MLST results, along with the absence of any positive cultures from other environmental samples, provide high support for the notion that this single eucalypt log was the point source of disease and nasal colonisation for two in-contact ferrets. The log was therefore removed from the enclosure. Aerosolisation of *Cryptococcus* spp. from a source external to the enclosure cannot be excluded, but in the absence of any large trees nearby and no significant soil disturbances, this is unlikely. The hollow decaying log, which had been present in the enclosure for three years prior to the time of the initial diagnosis, likely provided an ideal substrate for the growth of pathogenic *Cryptococcus* spp. This may have been enhanced by ongoing scarification by the ferrets and regular soiling with organic matter (urine and faeces). This is very similar to a phenomenon observed in captive koalas, where it

is presumed that their use of enclosure furniture helps to greatly amplify the amount of *Cryptococcus* spp. in the environment (Krockenberger et al., 2002b, Malik et al., 2011). The log, sourced by the ferret's owner from the locale of Bogan Gate, New South Wales, Australia, is of unknown tree species, but originates from an area within the natural range of *Eucalyptus camaldulensis* (Brooker & Kleinig, 2006) [a tree with well-established associations with *C. gattii* VGI (Ellis & Pfeiffer, 1990)]. Approximately two years after its removal, a follow-up swab sample was collected from the log and bird seed agar culture revealed a persistent heavy growth of *Cryptococcus* spp. RFLP analysis confirmed that the mixture of *C. gattii* VGI and *C. neoformans* VNI isolates remained.

Our findings are similar to a study where a likely environmental source of infection for *C. gattii* VGI was identified in a large flight aviary (Schmertmann et al., 2018a). In addition, our study provides comparable findings to another publication where an environmental source of infection for *C. gattii* VGI in a domestic ferret was identified, although this publication used amplified fragment length polymorphism typing, a less discriminatory typing technique than MLST, to establish the connection (Morera et al., 2014). Another study reported two sibling ferrets residing in the same environment both developing cryptococcosis, but separated temporally, due to disease isolates shown to be identical using PCR fingerprinting. This technique is again less discriminatory than MLST, however, and the exact source of infection was not identified (Malik et al., 2002). Our findings raise a similar question to prior studies: why has only one of nine ferrets in contact with this source of infection developed cryptococcosis? In this case, the answer remains unclear. Genetic susceptibilities could play a role, as suggested by the report of cryptococcosis in two sibling ferrets (Malik et al., 2002). In the avian case mentioned previously, a high stress period was considered the likely cause of increased susceptibility (Schmertmann et al., 2018a). In one ferret case, concurrent lymphoma was reported as a likely cause of immunosuppression, leading to cryptococcosis (Erdman et al., 1992). It is also possible that more of the in-contact asymptomatic ferrets were colonised but this remained unidentified due to either transient colonisation or the relatively superficial swabs able to be collected safely while under manual restraint. General anaesthesia could have allowed for the collection of potentially more representative samples (either deeper swabs or nasal flushes). It may also have been of interest to test the human owners for cryptococcal antigenaemia and nasal colonisation, as

colonised humans were identified in association with another case of ferret cryptococcosis (Morera et al., 2011), but this was not possible.

The mixed mating type population (9 type α and 2 type **a**) amongst the *C. gattii* VGI isolates in this study, despite identical MLST results, indicates that genetic differences outside of the seven MLST loci exist between these ST 154 isolates. Mixed mating types amongst *C. gattii* VGI isolates of identical MLST type have also been reported in a prior study (Hagen et al., 2012). Whole genome sequencing would provide further insights into this but was beyond the scope of this study. Sexual reproduction (both mixed mating type and same-sex mating) is the possible source of basidiospores as an infectious propagule in this scenario (Fraser et al., 2005). Mating type **a** is also considered relatively rare in Australia, and a finding of 2/11 (18%) *C. gattii* VGI isolates being of this type is unusual, although similar results have been reported in *E. camaldulensis* tree hollows in New South Wales, Australia (Halliday et al., 1999).

Based on the findings of this study, and our knowledge of the environmental niches of pathogenic *Cryptococcus* spp., we recommend the use of other objects for environmental enrichment in ferrets and other species, such as unused porcelain or clay drainage pipes. If hollow logs, particularly from eucalypts, are to be used, the risk of cryptococcal infection might be minimised by regular manual cleaning followed by the application of an appropriate disinfectant. For example, a quaternary ammonium compound (such as F10®SC Veterinary Disinfectant, Health and Hygiene Pty Ltd, Roodepoort, South Africa) used at the manufacturer's recommended standard concentration (1 : 500) and contact time (at least 30 seconds) might provide a reasonable option based on a prior study (Krangvichain et al., 2016), but further testing is needed. Accelerated hydrogen peroxide disinfectants remain untested against *Cryptococcus* spp. but have shown great potential against other fungi and infectious agents of animals, including as an environmental disinfectant (Moriello & Hondzo, 2014, Holtkamp et al., 2017). A topical antifungal agent such as enilconazole (e.g. Imaverol, Elanco Pty Ltd, West Ryde, New South Wales, Australia) used as a rinse could also be an effective means of controlling environmental *Cryptococcus* spp., although this remains untested and could theoretically promote antifungal resistance. Systematic studies comparing the utility of various disinfectants for managing environmental *Cryptococcus* spp. are lacking, and this would be a highly useful area for future research. Such knowledge would allow for more

definitive recommendations regarding environmental decontamination, and this would benefit a variety of captive and domestic animal species prone to cryptococcosis.

This study successfully identified an environmental point source of infection for *C. gattii* VGI in captive ferrets, which led to the removal of the enclosure log in question, as it posed an ongoing risk of infection to the other ferrets and the owners. This emphasises both the importance of environmental investigations into cases of captive animal cryptococcosis and the utility of ferrets as sentinels for disease in humans and other animal species.

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DATA ACCESSIBILITY

Allele type sequences are available in GenBank (accession numbers MK331932-MK331951).

SUPPLEMENTARY TABLE S4.3.1 Accession numbers, multi-locus sequence typing results and strain information for *Cryptococcus gattii* species complex isolates identified from domestic ferrets and their environment in western Sydney, Australia. Red colour indicates allele and sequence type differences. Two standard strains (grey rows) used in phylogenetic analyses are also included.

							Allele and sequence type numbers							
WM number	**Mol. type**	**Date**	**Location**	**Category**	**Specific source**	**MT**	***CAP59***	***GPD1***	**IGS1**	***LAC1***	***PLB1***	***SOD1***	***URA5***	**ST**
17.116	VGI	Apr 2016	Sydney, Australia	Vet	Ferret (nasal exudate)	α	16	5	3	5	5	45	12	154
17.118	VGI	Sep 2017	Sydney, Australia	Vet	Ferret (brain)	α	16	5	3	5	5	45	12	154
18.117	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	109	5	5	45	12	541
18.118	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	3	5	5	45	12	154
18.119	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	3	5	5	45	12	154
18.121	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	3	5	5	45	12	154
18.122	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	**a**	16	5	3	5	5	45	12	154
18.123	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	**a**	16	5	3	5	5	45	12	154
18.125	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	3	5	5	45	12	154
18.127	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	3	5	5	45	12	154
18.130	VGI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	16	5	3	5	5	45	12	154
179	VGI	1993	Sydney, Australia	Clin	CSF	α	16	5	3	5	5	32	12	51
779	VGIV	1995	Johannesburg, SA	Vet	Cheetah	α	17	10	8	18	3	37	11	70

Apr = April; Clin = clinical; CSF = cerebrospinal fluid; Enviro = environmental; Mol. = molecular; MT = mating type; SA = South Africa; Sep = September; ST = sequence type; WM = Westmead Medical Mycology Culture Collection; Vet = veterinary.

SUPPLEMENTARY TABLE S4.3.2 Culture collection numbers, multi-locus sequence typing results and strain information for the *Cryptococcus neoformans* species complex isolates identified from domestic ferrets and their environment in western Sydney, Australia. Red colour indicates allele and sequence type differences. Two standard strains (grey rows) used in phylogenetic analyses are also included.

							Allele and sequence type numbers							
WM number	**Mol. type**	**Date**	**Location**	**Category**	**Specific source**	**MT**	***CAP59***	***GPD1***	**IGS1**	***LAC1***	***PLB1***	***SOD1***	***URA5***	**ST**
17.117	VNI	Apr 2016	Sydney, Australia	Vet	Ferret (nasal swab)	α	1	5	1	1	4	1	1	13
18.120	VNI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	1	5	1	1	4	1	1	13
18.124	VNI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	1	5	1	1	4	1	1	13
18.126	VNI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	1	5	1	1	4	1	1	13
18.128	VNI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	1	5	1	1	4	1	1	13
18.129	VNI	Apr 2016	Sydney, Australia	Enviro	Enclosure (log)	α	7	1	1	2	1	1	2	23
148	VNI	1989	Sydney, Australia	Clin	CSF	α	7	1	1	18	1	1	1	7
626	VNII	1993	Sydney, Australia	Clin	CSF	α	2	14	14	8	11	11	4	2

Apr = April; Clin = clinical; CSF = cerebrospinal fluid; Enviro = environmental; Mol. = molecular; MT = mating type; ST = sequence type; WM = Westmead Medical Mycology Culture Collection; Vet = veterinary.

4.4 Jet-setting koalas spread *Cryptococcus gattii* VGII in Australia

Authorship declaration:

This manuscript was published in *mSphere* in 2019 (volume 4, issue 3, e00216-19). L.J. Schmertmann is the primary author and contributed to all aspects of this study (designing and performing the research, analysing the data and preparing the manuscript). P. Danesi performed the research and analysed the data. J. Monroy-Nieto, J. Bowers and D.M. Engelthaler analysed the data. R. Malik, W. Meyer and M.B. Krockenberger designed the research and analysed the data. All authors were involved in the revision of the manuscript and support its inclusion in this thesis.

Laura J. Schmertmann _____ March 2019

Mark B. Krockenberger _____ March 2019

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RESEARCH ARTICLE
Clinical Science and Epidemiology

Jet-Setting Koalas Spread *Cryptococcus gattii* VGII in Australia

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ABSTRACT *Cryptococcus gattii* molecular type VGII is one of the etiologic agents of cryptococcosis, a systemic mycosis affecting a wide range of host species. Koalas (*Phascolarctos cinereus*) exhibit a comparatively high prevalence of cryptococcosis (clinical and subclinical) and nasal colonization, particularly in captivity. In Australia, disease associated with *C. gattii* VGII is typically confined to Western Australia and the Northern Territory (with sporadic cases reported in eastern Australia), occupying an enigmatic ecologic niche. A cluster of cryptococcosis in captive koalas in eastern Australia (five confirmed cases, a further two suspected), caused predominantly by *C. gattii* VGII, was investigated by surveying for subclinical disease, culturing koala nasal swabs and environmental samples, and genotyping cryptococcal isolates. *URA5* restriction fragment length polymorphism analysis, multilocus sequence typing (MLST), and whole-genome sequencing (WGS) provided supportive evidence that the transfer of koalas from Western Australia and subsequently between several facilities in Queensland spread VGII into uncontaminated environments and environments in which *C. gattii* VGI was endemic. MLST identified VGII isolates as predominantly sequence type 7, while WGS further confirmed a limited genomic diversity and revealed a basal relationship with isolates from Western Australia. We hypothesize that this represents a founder effect following the introduction of a koala from Western Australia. Our findings suggest a possible competitive advantage for *C. gattii* VGII over VGI in the context of this captive koala environment. The ability of koalas to seed *C. gattii* VGII into new environments has implications for the management of captive populations and movements of koalas between zoos.

IMPORTANCE *Cryptococcus gattii* molecular type VGII is one of the causes of cryptococcosis, a severe fungal disease that is acquired from the environment and affects many host species (including humans and koalas). In Australia, disease caused by *C. gattii* VGII is largely confined to western and central northern parts of the country, with sporadic cases reported in eastern Australia. We investigated an unusual case cluster of cryptococcosis, caused predominantly by *C. gattii* VGII, in a group of captive koalas in eastern Australia. This research identified that the movements of koalas between wildlife parks, including an initial transfer of a koala from Western Australia, introduced and subsequently spread *C. gattii* VGII in this captive environment. The spread of this pathogen by koalas could also impact other species, and these findings are significant in the implications they have for the management of koala transfers and captive environments.

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The *Cryptococcus gattii* species complex comprises a group of environmentally acquired fungal pathogens that, along with the *Cryptococcus neoformans* species complex, are the etiologic agents of cryptococcosis (1, 2). A proposal to divide these species complexes into seven distinct species (according to genotypic differences) (3) remains under debate within the cryptococcal research community and has recently been reported as premature (4). Henceforth, the terms *C. gattii* and *C. neoformans* will be used to refer to their respective species complexes, with restriction fragment length polymorphism (RFLP) analysis molecular types (VGI to VGIV and VNI to VNIV) also used, when known. This disease affects a wide range of host species, from mammals to birds and reptiles (5). In Australia, the *C. gattii* population comprises mostly *C. gattii* VGI and VGII, with VGII largely confined to Western Australia (WA) and the Northern Territory (NT) (1, 6–8). Interestingly, *C. gattii* VGII has been implicated in several case clusters and outbreaks in various host species across several continents (most notably, Vancouver Island, Canada) (9–12), while *C. gattii* VGI disease tends to be more sporadic within a range of endemicity (although some outbreaks in animals have been reported) (5, 13, 14).

Koalas (*Phascolarctos cinereus*) appear particularly prone to developing cryptococcosis caused by *C. gattii*, with clinical disease, asymptomatic antigenemia (subclinical disease), and nasal colonization well documented among captive individuals (5, 15–19) and free-ranging populations (20, 21). This may in part be related to the preferred habitat and diet of the koala, eucalypt trees, also exhibiting a strong association with *C. gattii* VGI as an ecologic niche (12, 22–24). Seemingly healthy koalas are able to carry *C. gattii* VGI and VGII with them when translocated within Australia or internationally, either through colonization of the sinonasal mucosa or within constrained foci of infection (subclinical disease) (5, 25–27). Other animals can also carry *C. gattii* and *C. neoformans* in the nasal cavity and gastrointestinal tract, and there has been speculation that this presents a possible method of dispersal (28–34), especially in relation to birds. A prior study suggested that koalas may also seed new environments with *C. gattii* but did not genotype isolates or include confirmatory molecular evidence (16).

A case cluster of cryptococcosis largely attributed to *C. gattii* VGII was observed in captive koalas in eastern Australia, with five confirmed clinical cases over a 4-year period, including three mortalities (Table 1), and a further two suspected cases (based on cryptococcal antigen titers near the time of death). No known cases of cryptococcosis had occurred in any animals housed at these facilities prior to 2013. These koalas were regularly moved between three co-owned wildlife parks in Queensland, Australia (parks 1, 2, and 3), and an individual had previously been introduced into this population from a location in Western Australia (park 4) (Fig. 1). The involvement of *C. gattii* VGII prompted concerns regarding the emergence of this molecular type in eastern Australia and the implications this may have for managing the case cluster in these koalas (given that VGII may be less susceptible to fluconazole [35]). This scenario provided an opportunity to further characterize cryptococcosis in captive koalas while also observing the potential for koalas to translocate *C. gattii* into new environments. The aim of this study was to characterize the fine-scale molecular epidemiology of this case cluster by (i) determining the environmental burden of *C. gattii* and the prevalence of cryptococcal nasal colonization in the koalas housed at parks 1 to 3, (ii) using molecular epidemiologic tools to characterize environmental, colonizing, and disease strains from parks 1 to 4, and (iii) assessing the prevalence of subclinical disease (asymptomatic antigenemia) among the koalas domiciled in parks 1 to 3.

RESULTS

Koala nasal swabs. Cryptococcal nasal colonization was identified in 14 of 44 (32%) koalas on at least one occasion (Table 2). Of the colonized koalas, 9/14 were tested

TABLE 1 Koalas ($n = 5$) at co-owned captive facilities in the Cairns region of Queensland, Australia, with confirmed clinical cryptococcosis attributed to infection with the *Cryptococcus gattii* species complex from 2013 to 2016

Koala	Year	Antigenemia (LCAT titer)[a]	Primary lesion[b]	Molecular type	Outcome
1	2013	U[c]	Thoracic	VGIIb	Death
2	2014	+ (1:256)	Nasal	VGIIb	Death
3	2014	+[d]	CNS	VGI	Death
4	2016	+ (1:128)	Inguinal LN	VGIIb	Survival
5	2016	+ (1:512)	Nasal	VGIIb	Survival

[a]LCAT, latex cryptococcal antigen agglutination test.
[b]CNS, central nervous system; LN, lymph node.
[c]U, unknown.
[d]LCAT titer unavailable, cryptococcal antigen lateral flow immunoassay positive.

twice, with 5/9 progressing from negative to positive on nasal swab culture, 3/9 remaining consistently positive, and 1/9 progressing from positive to negative (Table 2). This resulted in a total of 17 positive culture result events among the 14 koalas. Low, moderate, and heavy cryptococcal colonization burdens were reported on five, three, and nine occasions, respectively. Among the three consistently positive koalas, one exhibited a heavy cryptococcal burden on both occasions, while the other two progressed from moderate to heavy.

In 2015, 14% (1/7), 14% (3/22), and 45% (5/11) of koalas were positive for nasal colonization at parks 1, 2, and 3, respectively. The single positive individual located at park 1 had recently been transferred from park 2. In 2017, following the closure of park 2 and transfer of all koalas to parks 1 and 3, 19% (3/16) and 33% (5/15) of koalas were positive at parks 1 and 3, respectively.

Environmental samples. In 2015, 56% (9/16) of all enclosures cultured positive for *Cryptococcus* spp. (colonies exhibiting the brown color effect were observed). At park 2, 86% (6/7) of enclosures were positive, while at park 3, 60% (3/5) were positive (Fig. 2). No enclosures at park 1 cultured positive for *Cryptococcus* spp. in 2015 (Table 3). Of the nine positive enclosures, six had heavy and three had low cryptococcal burdens. An isolate could not be obtained from one of the low-positive enclosures at park 3 due to overgrowth of filamentous fungi on the culture plate.

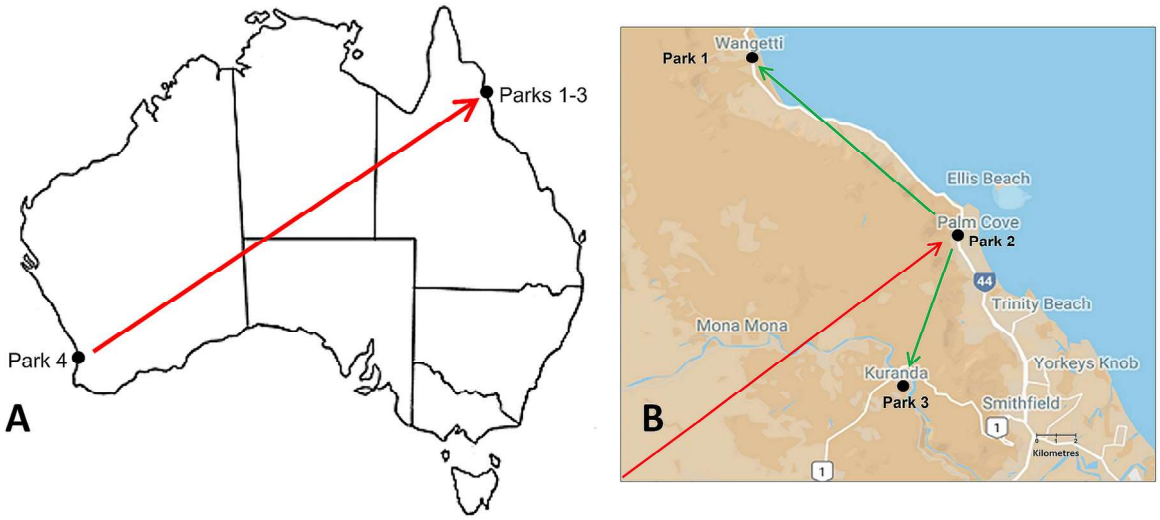

FIG 1 (A) Map of Australia showing the approximate location of the four wildlife parks (parks 1 to 4) studied. The red arrow indicates the movement of a koala from park 4 to park 2 approximately 10 years prior to this study. (B) Map showing the location of parks 1 to 3 within the Cairns region of Queensland, Australia. Green arrows indicate the movement of 20 koalas from park 2 to parks 1 and 3 in 2016 (approximately 10 koalas to each park).

TABLE 2 Koalas ($n = 14$), ordered by identification number, at three co-owned captive facilities in the Cairns region of Queensland, Australia, that tested positive for *Cryptococcus gattii* species complex nasal colonization on at least one of two time points

	2015		2017		
Koala number	Park[a]	Nasal colonization[b] (molecular type)	Park[a]	Nasal colonization[b] (molecular type)	Antigenemia (titer)[c]
LS40	1	+ (VGIIb)	1	NT[d]	+ (1:8)
LS49	2	+++ (VGI)	3	+++ (VGI)	+[e]
LS55	2	++ (VGIIb)	3	NT	+ (1:512)
LS56	2	++ (VGIIb)	3	+++ (VGI, VGIIb)	+ (1:8)
LS67	3	+++ (VGI)	3	NT	–
LS69	3	+++ (VGI)	3	NT	+ (1:8)
LS73	3	+ (VGI)	3	–	–
LS75	3	+ (VGI)	3	NT	–
LS77	3	++[f]	3	+++ (VGI)	+ (1:4)
LS291	2	–	1	+[f]	–
LS292	2	–	1	+++ (VGI)	–
LS298	2	–	1	+ (VGIIb)	–
LS307	2	–	3	+++ (VGIIb)	+[e]
LS311	2	–	3	+++ (VGI, VGIIb)	–

[a]Park 1 is located at 16°39′47.1′′S 145°33′51.9′′E, park 2 at 16°45′28.9′′S 145°39′46.4′′E, and park 3 at 16°49′′07.9′′S 145°37′58.3′′E (see Fig. 1).
[b]+, low degree of cryptococcal growth (1 to 10 colonies); ++, moderate (11 to 100 colonies); +++, heavy (>100 colonies).
[c]Antigenemia results reflect if these koalas tested positive (+) at any time between 2014 and 2018 and the highest recorded latex agglutination cryptococcal antigen test titer.
[d]NT, not tested.
[e]Lateral flow immunoassay only.
[f]No isolate available.

In 2017, 22% (5/23) of all enclosures tested positive for *Cryptococcus* spp., comprising two enclosures at park 1 and three at park 3. This meant that at parks 1 and 3, *Cryptococcus* spp. were cultured from 15% (2/13) and 30% (3/10) of enclosures, respectively (Table 3). The degree of cryptococcal growth was classified as low for two positive enclosures, moderate for two, and heavy for one. Isolates were obtained from all five positive enclosures. The change in enclosure numbers from 2015 (16) to 2017 (23) was due to the expansion of parks 1 and 3 (and sometimes the division of previously larger enclosures into several small enclosures) due to the transfer of all koalas from park 2.

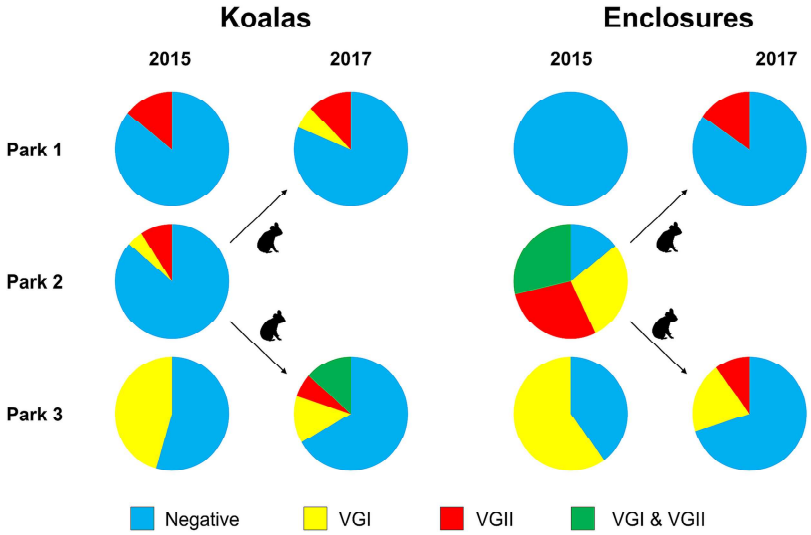

FIG 2 Proportions of koalas and enclosures colonized by members of the *Cryptococcus gattii* species complex across three wildlife facilities (parks 1 to 3) on two sampling occasions, 2015 and 2017. Blue indicates negative koalas/enclosures (not colonized), while green, red, and yellow indicate koalas or enclosures colonized by *C. gattii* VGI, VGII, or both VGI and VGII, respectively. Park 2 was not sampled in 2017 due to its closure; arrows indicate the movement of koalas from park 2 to parks 1 and 3 in 2016.

TABLE 3 Environmental sampling results for *Cryptococcus* spp., using bird seed agar culture and *URA5* restriction fragment length polymorphism typing, at three co-owned wildlife parks in the Cairns region of Queensland, Australia, across two sampling occasions

Park[a]	Year	No. of enclosures	No. (%) of enclosures culture positive for *Cryptococcus* spp.	*C. gattii* molecular type(s) identified (no. of enclosures)
1	2015	4	0	NA[b]
	2017	13	2 (15)	VGII (2)
2	2015	7	6 (86)	VGI (2); VGII (2); both VGI and VGII (2)
3	2015	5	3 (60)	VGI (3)
	2017	10	3 (30)	VGI (2); VGII (1)

[a]Park 2 closed in 2016, and parks 1 and 3 subsequently expanded (increased the number of enclosures) to accommodate koalas from park 2.
[b]NA, not applicable.

Cryptococcal antigenemia was detected in 20 of 58 (34%) koalas on at least one occasion during this study, with two testing positive by a lateral flow assay (LFA) only (including one case where a confirmatory latex cryptococcal antigen agglutination test [LCAT] could not be run due to insufficient sample), and the remaining 18 were positive using both tests (36). Confirmed clinical cases accounted for four koalas (with LCAT titers of 1:128, 1:256, 1:512, and one unknown). A further two were presumptive clinical cases, based on LCAT titers of 1:128 in both cases and their sudden deaths. Thus, six antigen-positive cases (10% of the 58 koalas sampled) were symptomatic. The remaining 14 exhibited asymptomatic cryptococcal antigenemia, resulting in a subclinical disease prevalence of 24% (14/58). One koala (koala 1) (Table 1) with confirmed clinical cryptococcosis (based on postmortem findings) was not tested for antigenemia. Among the 14 koalas with subclinical cryptococcosis, two were LFA positive only, while the remaining 12 returned positive LCAT results with titers of 1:2 (3/12), 1:4 (3/12), 1:8 (3/12), and 1:16 (3/12).

Nasal colonization results were available from 16/20 koalas with cryptococcal antigenemia (excluding two confirmed clinical cases, one suspected case, and one other individual). Of these 16 koalas, 9 were negative for nasal colonization on all occasions tested, while 7 were positive on at least one occasion (Table 2).

Molecular and mating type determination. A total of 71 *Cryptococcus* species strains were obtained, with 5 disease-associated, 33 nasal colonizing, and 33 environmental isolates. *C. gattii* VGI accounted for 39/71 isolates (one disease associated, 21 nasal colonizing, and 17 environmental). *C. gattii* VGI was not isolated from any samples collected (nasal or environmental swabs) at park 1 (Fig. 2). *C. gattii* VGII accounted for the remaining 32/71 isolates (4 disease associated, 12 nasal colonizing, and 16 environmental). All 32 *C. gattii* VGII isolates from parks 1 to 3 were determined to be mating type α.

At park 1, in 2015, two *C. gattii* VGII isolates (of the same sequence type [ST]) were obtained from a single nasal swab from a koala recently transferred from park 2. In 2017, seven isolates were obtained, with *C. gattii* VGI accounting for two nasal colonizing isolates from one koala and *C. gattii* VGII accounting for one nasal colonizing isolate and four environmental isolates. Both colonized koalas had been transferred from park 2.

At park 2, one disease isolate was identified as *C. gattii* VGII. Three koalas were colonized by *C. gattii*, with *C. gattii* VGI in one and VGII in the remaining two. Among the six positive enclosures at this park, 2/6 contained only *C. gattii* VGII, 2/6 contained only *C. gattii* VGI, and the remaining 2/6 had a mixed burden of both *C. gattii* VGI and VGII (Fig. 2).

At park 3, *C. gattii* VGII accounted for 3/4 disease cases (all three had a recent history of previously residing at park 2), while *C. gattii* VGI was identified as the etiologic agent in a single case. All isolates collected from park 3 in 2015 were identified as *C. gattii* VGI—this included nasal-colonizing isolates from four koalas and environmental isolates from two enclosures. In 2017, five koalas were colonized by *C. gattii* and either exhibited a *C. gattii* VGI burden (2/5), a *C. gattii* VGII burden (1/5), or mixed colonization

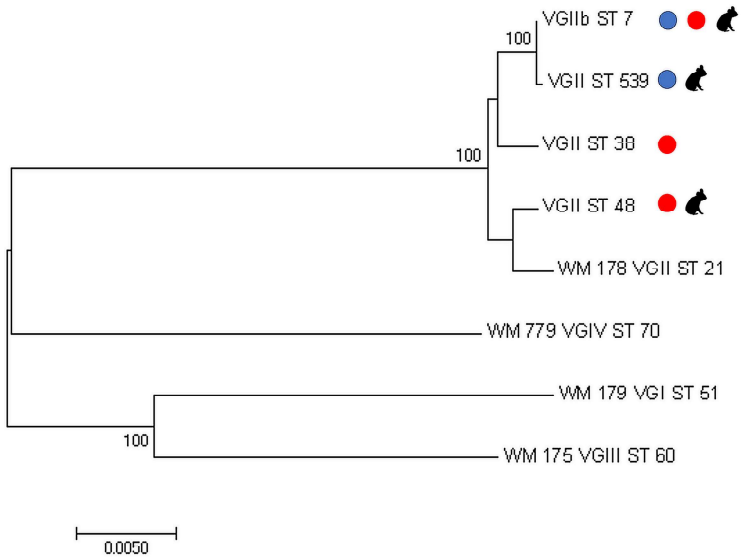

FIG 3 Maximum likelihood phylogenetic tree, using the Kimura 2-parameter model with gamma distribution, of the concatenated multilocus sequence typing sequences from four sequence types (STs) identified among koala disease, colonizing, and environmental isolates at three captive facilities in northern Queensland, Australia (blue circles), and a facility in Perth, Western Australia (red circles). STs found in koalas (as either disease or colonizing isolates) are identified by the koala silhouette. *Cryptococcus gattii* species complex standard strains (VGI, WM 179; VGII:, WM 178; VGIII, WM 175; VGIV, WM 779) were included for outgrouping. Bootstrap values were obtained from 1,000 replicates using a maximum likelihood model, with values over 70 shown. The scale bar indicates the number of nucleotide substitutions per site.

by both VGI and VGII (2/5). All koalas with any *C. gattii* VGII colonization had a history of relocation from park 2. Among the three enclosures identified as positive for *C. gattii* in 2017, two contained *C. gattii* VGI and one contained *C. gattii* VGII (Table 3).

A total of 34 *C. gattii* VGII isolates (16 environmental, 17 colonizing, 1 disease) previously collected from park 4 were identified for comparative inclusion in this study. Mating type α accounted for 33/34 isolates; a single mating type **a** environmental isolate was identified.

Multilocus sequence typing. Among the 32 *C. gattii* VGII isolates from parks 1 to 3 identified in this study, 30/32 were sequence type (ST) 7. The remaining two isolates were novel to the *C. gattii* multilocus sequence typing (MLST) database and were classified as ST 539; these were both nasal-colonizing isolates collected at the same time from a koala residing at park 2. Allele differences were observed only at the *URA5* locus, with two unique allele types identified (one for ST 7 and one for ST 539).

At park 4, 30/34 isolates were ST 7. The remainder were ST 38 and ST 48, accounting for one and three isolates, respectively. Allele type differences were observed at all seven MLST loci. Two unique allele types each were observed at the *GPD1*, *LAC1*, and *URA5*, whereas three allele types each were observed at *CAP59*, IGS1, *PLB1*, and *SOD1*.

The maximum likelihood analysis of concatenated MLST sequences revealed a highly limited genetic diversity among isolates from parks 1 to 3, with ST 539 settling in the *C. gattii* VGIIb clade with ST 7. The isolates from park 4, however, exhibited greater diversity, with ST 48 grouping closer to the *C. gattii* VGII standard strain (WM 178) than to ST 7 strains (Fig. 3).

Whole-genome sequencing. The phylogeny of the isolates based on whole-genome single nucleotide polymorphism (SNP) data separated the isolates into two major clades, with isolates from all parks present in both clades (Fig. 4). An overall low genetic diversity is seen across the entire tree, with each clade exhibiting few differences between isolates from parks 1 to 3. In both clades, isolates from park 4 are relatively basal.

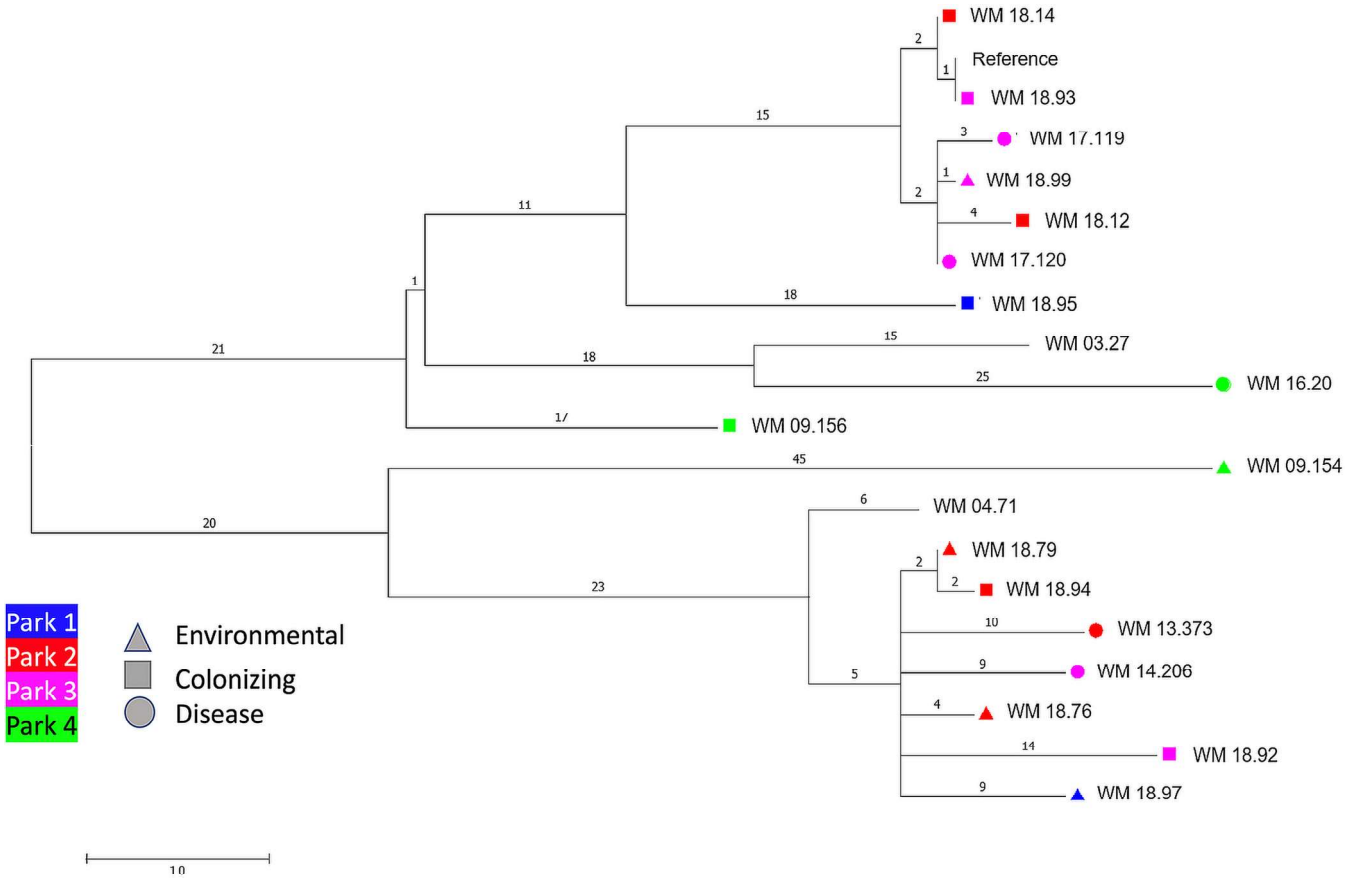

FIG 4 Maximum parsimony tree constructed with 95% consensus of 40 most parsimonious trees using whole-genome SNP data from 19 *Cryptococcus gattii* VGIIb isolates. Of these strains, 17 were isolated for the present study and 2 additional Australian isolates, WM 03.27 and WM 04.71, were included for comparison and are shown without leaf markers. The reference for SNP calling was the *de novo* assembly of WM 18.93. Colors and shapes represent the four parks and isolate type, as outlined in the key. A total of 302 high-confidence whole-genome SNP positions were considered. Polytomies represent less than 95% confidence. Branch lengths represent SNP distances.

A phylogenetic tree with additional relevant genomes from veterinary and environmental isolates displays the same overall topology with two major clades of VGIIb (Fig. 5). The isolates sampled from the koalas and enclosures at parks 1 to 3 remain clustered together within their respective clades, and all koala isolates demonstrate the same relationships as in Fig. 4.

DISCUSSION

This study provides evidence in support of the notion that koalas can seed environments with *C. gattii* VGII. The detection of an unusually high prevalence in an area where *C. gattii* VGII is considered to be nonendemic and the documented transfer of a single koala from a wildlife park in Western Australia where *C. gattii* VGII is endemic provided the opportunity to investigate what happens after *C. gattii* VGII is introduced into (i) an environment with no *C. gattii* (enclosures in park 1) or (ii) an environment with a moderate presence of *C. gattii* VGI (park 3). Our findings suggest that *C. gattii* VGII can indeed colonize a new environment (most likely originating from the animal transferred from park 4), be amplified and further dispersed by the presence of captive koalas, and compete successfully with an existing population of *C. gattii* VGI. This study also offered some insight into the effectiveness of environmental decontamination as a management tool to prevent cryptococcosis in captive scenarios.

The ability of koalas to translocate *C. gattii* VGII was made particularly apparent by its absence in the environment at parks 1 and 3 in 2015 and subsequent presence in 2017. This presumably was the result of the transfer of koalas from park 2, a facility with

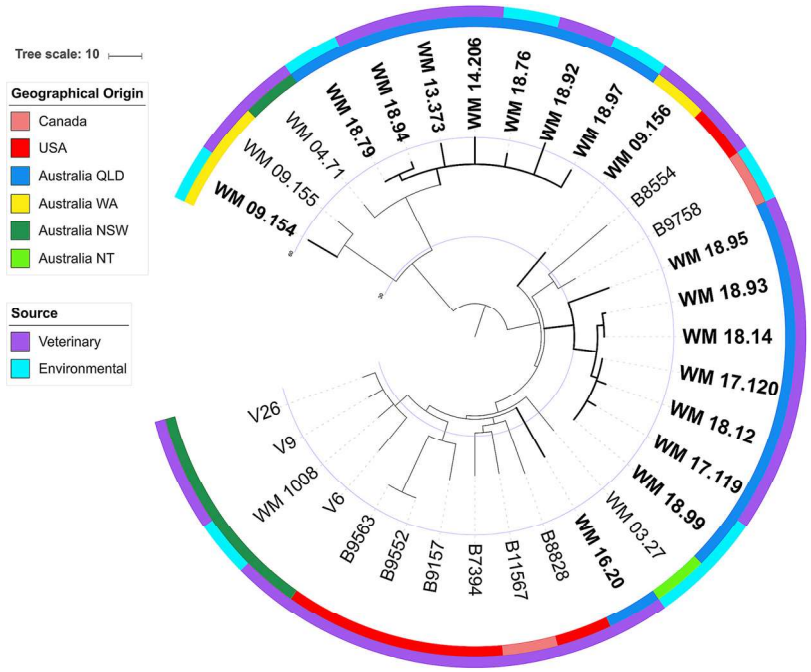

FIG 5 Maximum parsimony population tree for 32 environmental and veterinary isolates of *Cryptococcus gattii* VGIIb. Isolates from the koalas and enclosures in this study are shown in boldface font. A total of 712 high-confidence whole-genome SNP positions were considered. Outer rings display geographical origin and source of isolation. Polytomies represent consensus levels below 95%. The tree splits concordantly with Fig. 4, where the top clade represents the right split in this tree. NSW, New South Wales; NT, Northern Territory; QLD, Queensland; WA, Western Australia.

abundant environmental *C. gattii* VGII, to parks 1 and 3 in 2016. At park 1 in 2015, the only place we were able to detect *C. gattii* VGII was in the nasal cavity of one translocated koala from park 2; VGII was not detected in the environment. Although koalas were transferred between all three facilities prior to 2016, this was sporadic, typically involved just one individual at a time, and was predominantly between parks 2 and 3. Presumably by chance, this left park 1 as a low environmental presence zone for *C. gattii*, despite the potential amplifying effect of the captive koala population. Such low environmental presence in captive environments has been described previously (16).

In 2016, approximately 20 koalas from park 2 were transferred to parks 1 and 3, with roughly 10 individuals going to each of the two facilities, in short succession. This provided an opportunity for a mass introduction of *C. gattii* VGI and VGII into both environments, although park 3 already had a moderate presence of VGI. At the time, an environmental decontamination protocol was already in place at all parks. We also hypothesized that the transfer of a koala from park 4 in Western Australia, where *C. gattii* VGII is endemic (8), provided the initial introduction of *C. gattii* VGII into the environment at park 2. In further support of this hypothesis, no koalas at park 1 were positive for cryptococcal antigenemia prior to the 2016 closure of park 2 and translocation of numerous koalas, and when first sampled, no *C. gattii* VGI or VGII was isolated from the environment. Also, all koalas found to have *C. gattii* VGII disease or nasal colonization either resided at, or had a history of recent transfer from, park 2. Increased stress could play a role in this case cluster, due to concurrent transfer of large numbers of animals, but given that 3/5 confirmed cases (and one of the suspected cases) were diagnosed prior to the closure of park 2, it seems likely that this was a key site for the ongoing distribution of cases even prior to the major transfer event.

C. gattii VGII is difficult to find in natural environments in Australia, even in areas of endemicity (Western Australia and the Northern Territory) (12, 26, 37). This is in contrast with *C. gattii* VGI, which can be isolated with relative ease from well-established tree

hollows in an ever-expanding range of Australian tree species (12, 16, 20, 23, 24, 26, 38). The occasional isolation of *C gattii* VGII from cats with cryptococcosis in eastern Australia (and no travel history) (7) suggests that it must also be present in this environment, but successful isolations are rare (39). It therefore remains a possibility that the environmental *C. gattii* VGII in park 2 was already present and not introduced by a koala from Western Australia. Airborne dispersal of *C. gattii* VGII from external sources leading to colonization of the environment in park 3 is also theoretically possible, as hypothesized in a prior study (16). Given the apparent rarity of *C. gattii* VGII in the environment in eastern Australia, however, both scenarios seem unlikely.

MLST analysis showed that the *C. gattii* VGII population in these three facilities is almost exclusively ST 7, which was the most common type found in a prior study of Australian isolates (8). Greater genetic diversity is often seen in southwestern Western Australia, where *C. gattii* VGII is endemic, but ST 7 is still predominant (8, 26). Therefore, our finding of a mostly ST 7 population with a highly limited diversity (based on both MLST and whole-genome sequencing [WGS]) in the three eastern Australian facilities supports the hypothesis of a founder effect from the introduction of a colonized koala arriving from Western Australia. This seems more likely when considering that the strains of park 4 in Western Australia share an ancestor with the remainder of the sampled isolates from which they diverge early on, while the isolates from the rest of the sites remained closely related according to WGS.

The recent evolutionary histories of the two clades seen in Fig. 4 appear to be dissimilar. The top clade has splits with ≥95% consensus, indicating a more gradual formation of its population structure, whereas the polytomy of the bottom clade indicates a more radial spread of the fungus from this lineage. The inclusion of two other unrelated isolates (WM 03.27 and WM 04.71) for comparative purposes in the phylogenetic tree has also suggested a relative overall clonality for *C. gattii* VGIIb in Australia. In relation to the global population of VGIIb, Fig. 4 and 5 reflect the same topology as described in previous studies (40–42).

These results support a prior study that showed enclosures previously culturing negative for *C. gattii* could become positive after the introduction of koalas with cryptococcal colonization (skin or nasal) (16). The same study found that koalas appeared able to amplify the burden of cryptococcal environmental contamination in captive environments (16). The exact mechanism of environmental seeding is unknown, but it is hypothesized here that *C. gattii* could either be transferred directly to the enclosure environment from the skin of the koala (particularly through colonization of the feet and nail beds [16]) or from the sinonasal cavity by sneezing or snorting during vocalization. The amplification of environmental *C. gattii* in captivity is thought to be related to the scarification of enclosure "furniture" combined with regular soiling by urine and fecal material, providing an optimal high creatinine substrate for cryptococcal growth (5).

Given the evidence that *C. gattii* may be dispersed by anthropogenic disturbances (and has been detected on footwear and car wheels in Vancouver Island, Canada) (43), one factor that should also be considered is the possible involvement of fomites in this scenario. This is particularly relevant to the initial single koala transferred from park 4 to park 2, given that captive koalas are transported with fresh eucalyptus leaves as feed (referred to as leaf browse) for the journey. This material could theoretically have also provided the introduction of *C. gattii* VGII into the environment, but we consider this unlikely, given that fresh eucalyptus leaves are typically not heavily contaminated by *C. gattii* (even in environments where koalas and their enclosure furniture are heavily colonized) (16).

Environmental decontamination as a means of managing koala cryptococcosis has long been recommended (5), but its effectiveness remains largely anecdotal. A prior study found that quaternary ammonium compounds effectively killed all *C. neoformans* yeasts in pigeon droppings within 30 min of contact time at a concentration of 0.062%, while rapid killing was observed at a concentration of 0.5% (44). At the facilities in this study, a concentration of 0.4% was typically used, with a 30-min contact period.

However, it is unclear if this concentration is also effective against *C. gattii*. In the present study, the nasal colonization rate at park 3 decreased from 45% to 33% and the environmental contamination rate decreased from 60% to 30% of enclosures between 2015 and 2017 (Fig. 2), which suggests that the protocol implemented was effective. Further targeted and systematic environmental decontamination studies are required to draw conclusions about the usefulness of this as a management tool to reduce the prevalence of cryptococcosis in captive koala groups.

The current Australian requirement for koalas prior to travel or export is to test only for cryptococcal antigenemia (45). In the event of a positive result, further testing should be performed in an attempt to locate lesions and treatment should be considered, depending on the antigen titer and its persistence (21, 46). The present study brings into question whether koalas should also be tested for nasal and/or skin colonization prior to travel to attempt to prevent the introduction of novel *C. gattii* genotypes into new environments and/or to guide management practices at their destination. This would be hampered, however, by the unknown sensitivity and detection limit of nasal swabbing as a means of determining colonization status and by the difficulty in clearing nasal colonization with current treatments (19). Thus, preventing colonized koalas from seeding new environments with *C. gattii* is likely not feasible, but this possibility remains an important factor to consider in some scenarios (particularly when obtaining new koalas from areas of *C. gattii* VGII endemicity).

This study offers valuable insights into the management of captive koala cryptococcosis and the composition of the *C. gattii* VGII population in eastern Australia. The relative environmental competitiveness of *C. gattii* VGI and VGII remains uncertain, but our findings could suggest an increased fitness of VGII in this scenario. Confirmation of the capacity of koalas to translocate *C. gattii* to previously uncontaminated environments, and provision of compelling new evidence that this extends to *C. gattii* VGII, has implications for the prevention and management of potential outbreaks in Australia.

MATERIALS AND METHODS

Locations. All samples originated from four captive animal facilities in Australia: three related wildlife parks (under the same ownership) in the vicinity of Cairns, Queensland (park 1, 16°39′47.1′′S 145°33′51.9′′E; park 2, 16°45′28.9′′S 145°39′46.4′′E; and park 3, 16°49′07.9′′S 145°37′58.3′′E) and one near Perth, WA (park 4, 31°50′03.6′′S 115°57′01.2′′E) (Fig. 1). Koalas were regularly transferred between parks 1 to 3. Environmental samples and nasal swabs were collected from parks 1 to 3 on two occasions: December 2015 and September 2017. Four of the koalas with clinical cryptococcosis were diagnosed while domiciled at park 3 (koalas 1, 2, 4, and 5) (Table 1), with all four having a history of recent transfer from park 2. The fifth koala was diagnosed while residing in park 2 and had been located there for several years. A male koala from park 4 was translocated to park 2 approximately 10 years prior to this study (Fig. 1), and *C. gattii* VGII strains isolated previously from the environment and nasal cavities of koalas in park 4 (47) were therefore included in our analyses for comparative purposes, along with a disease isolate from another koala that contracted cryptococcosis while residing at park 4. Park 2 was closed in 2016, and most of its koalas were moved to parks 1 and 3, with a few transferred to external facilities.

The enclosure environment, including furniture and leaf browse, was similar across parks 1 to 3. All three parks also had similar protocols in place, enacted in March 2015, for the management of environmental *C. gattii* during this study. This included the changing of all koala perches every 6 months (more often if possible) and a decontamination protocol, enacted every 3 months (also more often if possible), during a "rest period" for each enclosure. This protocol included manual cleaning by scrubbing and hosing all surfaces with detergents followed by the dousing of the enclosure in a quaternary ammonium disinfectant (F10 SC; Health and Hygiene Pty Ltd., Roodepoort, South Africa) used at the manufacturer's recommended concentration for fungal disinfection and utilizing a 30-min contact period. After this, enclosures were thoroughly rinsed with water, allowed to dry, and then eventually returned to use. Prior to this protocol, the facilities changed the perches less frequently (approximately every 12 months), and while the floors and walls of each enclosure were often scrubbed, less attention was paid to the furniture (perches, etc.) unless there was visible soiling.

Koala nasal swabs. Nasal swabs from parks 1 to 3 were submitted to Veterinary Pathology Diagnostic Services (VPDS), The University of Sydney, for culture. A sterile, moistened cotton-tipped swab was inserted into the nasal vestibule on both sides and rotated gently, as per methods of prior studies (16, 18). This procedure was performed by a veterinarian as part of a systematic disease control and management plan instigated at these facilities. In December 2015, 40 koalas were sampled at parks 1, 2, and 3 (7, 22, and 11 koalas at each park, respectively). In September 2017, 31 koalas were sampled at parks 1 and 3 (16 and 15 koalas, respectively). Across both sampling occasions, a total of 44 individuals were swabbed, with 27 swabbed on both occasions and 17 on one occasion only. The sampling of 17 koalas once only was attributable to either external transfers (koala no longer at any of the co-owned

facilities or newly introduced in the time between the first and second samplings) or concerns regarding the stress of sampling from an individual (for example, mothers with young joeys).

Environmental samples. Samples were collected by moistening a sterile swab with sterile saline and running the tip thoroughly over the surface of perches and enclosure furniture, similar to previously described methods (16). In December 2015, samples were collected from all enclosures (16) at parks 1, 2, and 3 (4, 7, and 5 enclosures at each park, respectively). In September 2017, after the closure of park 2, 23 enclosures at parks 1 and 3 were sampled (13 and 10 at each park, respectively).

Culture. All swabs (koala nasal and environmental) were initially cultured on Staib's bird seed agar containing antibiotics (penicillin and gentamicin) by rolling the swabs gently across the agar. Plates were incubated at 27°C and examined at least once daily for 7 to 10 days. Samples were considered positive if yeast-like colonies exhibiting the brown color effect (consistent with *Cryptococcus* spp.) were observed. If no growth was observed by 7 to 10 days, the plates were considered negative and discarded. Positive samples were classified according to the number of cryptococcal colonies counted on the agar plates as exhibiting either a low (1 to 10 colonies), moderate (11 to 100 colonies), or heavy (>100 colonies) extent of growth. A minimum of one cryptococcal colony from each positive plate was subcultured onto Sabouraud's agar and incubated at 37°C for isolation and DNA extraction.

Cryptococcal antigenemia testing. Serum samples from 58 koalas were collected by veterinarians at the three facilities by cephalic venipuncture with the koalas gently restrained. Samples were collected at various time points between December 2014 and August 2018 as part of the ongoing disease investigation and surveillance program and submitted to VPDS. All samples underwent cryptococcal antigen testing using an LFA (CrAg LFA; IMMY, Norman, OK, USA). If the LFA was positive, an LCAT (CALAS; Meridian Bioscience, Inc., Cincinnati, OH, USA) was performed to confirm the result and establish a reciprocal antigen titer (36). Both procedures were performed according to the manufacturers' instructions by experienced staff and in the same laboratory.

Molecular and mating type determination. DNA was extracted from all isolates using an established protocol for fungi (48). PCR amplification of the *URA5* gene was then performed, with the resulting product undergoing RFLP analysis and comparison to known standards (VGI, WM 179; VGII, WM 178; VGIII; WM 175; VGIV, WM 779; VNI, WM 148; VNII, WM 626; VNIII, WM 628; VNIV, WM 629) as described previously (49). This was to provide molecular confirmation that all isolates were *C. gattii* or *C. neoformans* and to identify the molecular type.

Determination of the mating type of all *C. gattii* VGII isolates was performed by PCR amplification of the *MF***a** and *MF*α genes and comparison to known standards (**a** standard, WM 06.38; α standard, WM 179) as described previously (50, 51). *C. gattii* VGI isolates did not proceed to mating type analysis.

Multilocus sequence typing. MLST of all *C. gattii* VGII isolates was performed according to the ISHAM consensus scheme for *C. neoformans* and *C. gattii* (52). This involved the PCR amplification and sequencing of seven loci: *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*. The Fungal MLST database (http://mlst.mycologylab.org/) was then used to assign allele types and STs. *C. gattii* VGI isolates did not proceed to MLST analysis.

Multilocus phylogenetic analysis. Concatenated MLST sequences were aligned (MUSCLE), and a maximum likelihood phylogenetic analysis was performed (Kimura 2-parameter model [53] with gamma distribution) with 1,000 bootstrap replicates using the MEGA7 program (54). The following *C. gattii* standard strains were included for out grouping: WM 179 (VGI), WM 178 (VGII), WM 175 (VGIII), and WM 779 (VGIV).

Whole-genome sequencing. A representative group of 14 *C. gattii* VGII isolates collected in parks 1 to 3 (park 1, 2/14; park 2, 6/14; park 3, 6/14) were selected for WGS, including all four available disease isolates. Three isolates from park 4 (of the same multilocus sequence type as isolates from parks 1 to 3) were also included for comparative purposes (one environmental, one colonizing, and one disease isolate), making a total of 17 isolates.

DNA for WGS was extracted using the Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Genomic DNA then was fragmented using a Q800R2 sonicator (QSonica, Newtown, CT, USA) to approximately 500 bp, and genome libraries were prepared for paired-end sequencing using the NEBNext Ultra II DNA Library Prep kit (New England BioLabs, Ipswich, MA, USA) and quantified using the SequalPrep Normalization Plate kit (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were pooled and sequenced at 2 × 150 bp on a NextSeq (Illumina, Inc., San Diego, CA, USA).

Genomic data analysis. Read data from 19 *C. gattii* VGIIb genomes, 17 from the present study and 2 additional *C. gattii* VGIIb Australian genomes for comparison (WM 03.27 and WM 04.71; BioSamples SAMN02851029 and SAMN02851030, respectively) were included in data analysis. Reads were trimmed of adapter sequence and low-quality bases with Trimmomatic v0.32 (55). Parameters were set for sliding windows of 5 bp and required quality of 20. After quality trimming, no reads shorter than 65 bp were accepted.

The phylogenetic analyses of the whole-genome read data were conducted as described previously (42), with minor modifications. In short, we identified high-certainty SNPs in the samples using the NASP pipeline (v. 1.1.2) (56) against a *de novo* assembly created with read data from isolate WM 18.93 using SPAdes (v3.10.1) (57) with "careful" setting enabled. The pipeline was set to use BWA (v 0.7.15) (58) as the read aligner and GATK (3.7) (59) as the SNP caller. The pipeline also filtered out positions with coverage below 10×, those with base concordance below 90% among the aligned reads, and any positions that were not present in all samples of the set.

Phylogenetic analysis was conducted using MEGA7 (54). Tree structure was inferred using maximum parsimony and calculating a 95% consensus of 40 most parsimonious trees from the whole-genome SNP

data of the 19 *C. gattii* VGIIb isolates (Fig. 4). The trees were rooted on the basal most branch, inferred from analysis using outgroup *C. gattii* standard strains (VGI WM179, VGII WM178, VGIII WM175, and VGIV WM779). As an extended phylogeny, we also identified whole-genome SNP differences across 32 genomes of VGIIb veterinary and environmental isolates (17 from the present study, 7 other Australian isolates, and 8 from North America) following the same procedure described above (Fig. 5, strains described in Table S2 in the supplemental material).

Data availability. Each unique MLST allele type was submitted to GenBank (accession numbers MK133807 to MK133825). WGS data are available as BioProject PRJNA524387. All *C. gattii* VGII isolates collected for this study are available in the Medical Mycology Culture Collection at The Westmead Institute for Medical Research in Westmead, New South Wales, Australia (accession numbers and strain information in Table S1).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00216-19.

TABLE S1, PDF file, 0.3 MB.

TABLE S2, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

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Supplementary table S1: List of *Cryptococcus gattii* species complex isolates with multi-locus sequence typing data included in this study. Strain information, allele types and sequence types are listed. Standard strains are included at the top of the table (grey rows). Strains are subsequently ordered by sequence type number, then culture collection number. Isolates in bold were included in whole genome analyses.

								Allele and sequence type numbers							
WM number	**Species and molecular type**	**Year**	**Country (state)**	**Category**	**Park**	**Specific source**	**MT**	***CAP59***	***GPD1***	***IGS1***	***LAC1***	***PLB1***	***SOD1***	***URA5***	**ST**
175	*C. gattii* VGIII	-	USA	Enviro	-	Eucalypt (standard)	α	18	18	14	3	6	28	19	60
178	*C. gattii* VGII	1991	Australia (NSW)	Disease	-	Human - lung (standard)	α	1	17	16	16	14	19	7	21
179	*C. gattii* VGI	1993	Australia (NSW)	Disease	-	Human - CSF (standard)	α	16	5	3	5	5	32	12	51
779	*C. gattii* VGIV	1995	South Africa	Disease	-	Cheetah (standard)	α	17	10	8	18	3	37	11	70
03.27	***C. gattii* VGII**	**1999**	**Australia (NT)**	**Enviro**	**-**	**Eucalypt**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
04.71	***C. gattii* VGII**	**1991**	**Australia (NSW)**	**Disease**	**-**	**Cat - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
09.153	*C. gattii* VGII	2009	Australia (WA)	Enviro	4	New enclosure	α	2	6	10	4	2	15	2	7
09.154	***C. gattii* VGII**	**2009**	**Australia (WA)**	**Enviro**	**4**	**New enclosure**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
09.155	***C. gattii* VGII**	**2009**	**Australia (WA)**	**Colonising**	**4**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
09.156	***C. gattii* VGII**	**2009**	**Australia (WA)**	**Colonising**	**4**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
09.157	*C. gattii* VGII	2009	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
09.158	*C. gattii* VGII	2009	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
09.160	*C. gattii* VGII	2009	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
09.161	*C. gattii* VGII	2009	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
09.163	*C. gattii* VGII	2009	Australia (WA)	Enviro	4	Old enclosure	α	2	6	10	4	2	15	2	7
09.164	*C. gattii* VGII	2009	Australia (WA)	Enviro	4	Old enclosure	α	2	6	10	4	2	15	2	7
09.166	*C. gattii* VGII	2009	Australia (WA)	Enviro	4	Visitor area	α	2	6	10	4	2	15	2	7
09.167	*C. gattii* VGII	2009	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.198	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.200	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7

12.201	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.204	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.205	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.206	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.208	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
12.211	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	Main enclosure	α	2	6	10	4	2	15	2	7
12.212	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	Main enclosure	α	2	6	10	4	2	15	2	7
12.214	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	Nursery enclosure	α	2	6	10	4	2	15	2	7
12.215	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	QLD enclosure	α	2	6	10	4	2	15	2	7
12.216	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	QLD enclosure	α	2	6	10	4	2	15	2	7
12.217	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	VIC enclosure	α	2	6	10	4	2	15	2	7
12.219	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	VIC enclosure	α	2	6	10	4	2	15	2	7
13.222	*C. gattii* VGII	2013	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
13.223	*C. gattii* VGII	2013	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
13.244	*C. gattii* VGII	2013	Australia (WA)	Colonising	4	Koala - nasal	α	2	6	10	4	2	15	2	7
13.373	***C. gattii* VGII**	**2013**	**Australia (QLD)**	**Disease**	**2**	**Koala - thoracic mass**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
14.206	***C. gattii* VGII**	**2014**	**Australia (QLD)**	**Disease**	**3**	**Koala - nasal mass**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
16.20	***C. gattii* VGII**	**2005**	**Australia (QLD)**	**Disease**	**4**	**Koala - nasal mass**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
17.119	***C. gattii* VGII**	**2016**	**Australia (QLD)**	**Disease**	**3**	**Koala - nasal discharge**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
17.120	***C. gattii* VGII**	**2016**	**Australia (QLD)**	**Disease**	**3**	**Koala - lymph node**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.14	***C. gattii* VGII**	**2016**	**Australia (QLD)**	**Colonising**	**2**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.74	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA4	α	2	6	10	4	2	15	2	7
18.76	***C. gattii* VGII**	**2016**	**Australia (QLD)**	**Enviro**	**2**	**Enclosure KOA8**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.78	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA8	α	2	6	10	4	2	15	2	7
18.79	***C. gattii* VGII**	**2016**	**Australia (QLD)**	**Enviro**	**2**	**Enclosure KOA5**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.80	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA5	α	2	6	10	4	2	15	2	7
18.81	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA5	α	2	6	10	4	2	15	2	7
18.82	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA5	α	2	6	10	4	2	15	2	7
18.83	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA5	α	2	6	10	4	2	15	2	7

18.84	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA6	α	2	6	10	4	2	15	2	7
18.92	***C. gattii* VGII**	**2017**	**Australia (QLD)**	**Colonising**	**3**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.93	***C. gattii* VGII**	**2017**	**Australia (QLD)**	**Colonising**	**3**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.94	***C. gattii* VGII**	**2017**	**Australia (QLD)**	**Colonising**	**3**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.95	***C. gattii* VGII**	**2017**	**Australia (QLD)**	**Colonising**	**1**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.96	*C. gattii* VGII	2017	Australia (QLD)	Enviro	1	Group enclosure	α	2	6	10	4	2	15	2	7
18.97	***C. gattii* VGII**	**2017**	**Australia (QLD)**	**Enviro**	**1**	**Group enclosure**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.98	*C. gattii* VGII	2017	Australia (QLD)	Enviro	3	Enclosure 1A	α	2	6	10	4	2	15	2	7
18.99	***C. gattii* VGII**	**2017**	**Australia (QLD)**	**Enviro**	**3**	**Enclosure 1A**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**2**	**7**
18.152	*C. gattii* VGII	2017	Australia (QLD)	Enviro	1	GD Enclosure	α	2	6	10	4	2	15	2	7
18.158	*C. gattii* VGII	2017	Australia (QLD)	Colonising	3	Koala - nasal	α	2	6	10	4	2	15	2	7
18.160	*C. gattii* VGII	2017	Australia (QLD)	Colonising	3	Koala - nasal	α	2	6	10	4	2	15	2	7
18.161	*C. gattii* VGII	2016	Australia (QLD)	Colonising	1	Koala - nasal	α	2	6	10	4	2	15	2	7
18.162	*C. gattii* VGII	2016	Australia (QLD)	Colonising	1	Koala - nasal	α	2	6	10	4	2	15	2	7
18.163	*C. gattii* VGII	2016	Australia (QLD)	Colonising	2	Koala - nasal	α	2	6	10	4	2	15	2	7
18.164	*C. gattii* VGII	2016	Australia (QLD)	Enviro	2	Enclosure KOA8	α	2	6	10	4	2	15	2	7
18.165	*C. gattii* VGII	2017	Australia (QLD)	Enviro	1	Group enclosure	α	2	6	10	4	2	15	2	7
09.165	*C. gattii* VGII	2009	Australia (WA)	Enviro	4	Old enclosure	**a**	14	21	27	28	27	46	2	38
09.152	*C. gattii* VGII	2009	Australia (WA)	Enviro	4	New enclosure	α	27	6	4	4	1	43	7	48
12.197	*C. gattii* VGII	2012	Australia (WA)	Colonising	4	Koala - nasal	α	27	6	4	4	1	43	7	48
12.213	*C. gattii* VGII	2012	Australia (WA)	Enviro	4	Nursery enclosure	α	27	6	4	4	1	43	7	48
18.12	***C. gattii* VGII**	**2016**	**Australia (QLD)**	**Colonising**	**2**	**Koala - nasal**	**α**	**2**	**6**	**10**	**4**	**2**	**15**	**56**	**539**
18.13	*C. gattii* VGII	2016	Australia (QLD)	Colonising	2	Koala - nasal	α	2	6	10	4	2	15	56	539

Enviro = environmental; MT = mating type; NSW = New South Wales; NT = Northern Territory, QLD = Queensland; ST=sequence type; VIC = Victoria; WA = Western Australia; WM = Westmead Mycology Culture Collection. Locations of Parks 1-4: Park 1 = 16°39'47.1"S 145°33'51.9"E; Park 2 = 16°45'28.9"S 145°39'46.4"E; Park 3 = 16°49'07.9"S 145°37'58.3"E); Park 4 = 31°50'03.6"S 115°57'01.2"

Supplementary Table S2: List of all genomes included in the phylogenetic analysis of *Cryptococcus gattii* VGIIb in Figure 5.

Strain	Location	Category	Specific source	Year	Reference	BioSample number
B7394	Washington, USA	Veterinary	Cat	2008	[1]	SAMN02851018
B8554	Oregon, USA	Veterinary	Dog	2008	[2]	SAMN02851020
B8828	Washington, USA	Veterinary	Porpoise	2010	[1]	SAMN02851021
B9157	Washington, USA	Veterinary	Horse	2011	[2]	SAMN02851022
B9552	Washington, USA	Veterinary	Porpoise	2011	[2]	SAMN02851023
B9563	Washington, USA	Veterinary	Porpoise	2011	[2]	SAMN02851024
B9758	British Columbia, Canada	Environmental	Soil	2002	[2]	SAMN02851026
B11567	Nova Scotia, Canada	Veterinary	Deer - brain lesion	2015	[1]	SAMN07738531
V6	Sydney, NSW, Australia	Veterinary	Cat - CNS	1992	[3]	SAMN02906888
V9	Coogee, NSW, Australia	Veterinary	Cat – lesion aspirate	1992	[3]	SAMN02906887
V26	Rosemeadow, NSW, Australia	Veterinary	Cat – nasal lesion	1996	[3]	SAMN02906889
WM 1008	Blacktown, NSW, Australia	Environmental	*Eucalyptus tereticornis*	2000	[4]*	Pending
WM 03.27	Arnhem Land, NT, Australia	Environmental	*Eucalyptus* spp.	1992	[2]	SAMN02851029
WM 04.71	Chiswick, NSW, Australia	Veterinary	Cat – nasal lesion	1991	[2]	SAMN02851030
WM 09.154	Caversham, WA, Australia	Environmental	Koala enclosure	2009	This study	Pending
WM 09.155	Caversham, WA, Australia	Veterinary	Koala – nasal swab	2009	Unpublished	Pending
WM 09.156	Caversham, WA, Australia	Veterinary	Koala – nasal swab	2009	This study	Pending
WM 13.373	Palm Cove, QLD, Australia	Veterinary	Koala – thoracic mass	2013	This study	Pending
WM 14.206	Kuranda, QLD, Australia	Veterinary	Koala – nasal mass	2014	This study	Pending
WM 16.20	Currumbin, QLD, Australia	Veterinary	Koala – nasal mass	2005	This study	Pending
WM 17.119	Kuranda, QLD, Australia	Veterinary	Koala – nasal discharge	2016	This study	Pending
WM 17.120	Kuranda, QLD, Australia	Veterinary	Koala – lymph node	2016	This study	Pending
WM 18.12	Palm Cove, QLD, Australia	Veterinary	Koala – nasal swab	2016	This study	Pending
WM 18.14	Palm Cove, QLD, Australia	Veterinary	Koala – nasal swab	2016	This study	Pending
WM 18.76	Palm Cove, QLD, Australia	Environmental	Koala enclosure	2016	This study	Pending
WM 18.79	Palm Cove, QLD, Australia	Environmental	Koala enclosure	2016	This study	Pending
WM 18.92	Kuranda, QLD, Australia	Veterinary	Koala – nasal swab	2017	This study	Pending
WM 18.93	Kuranda, QLD, Australia	Veterinary	Koala – nasal swab	2017	This study	Pending
WM 18.94	Kuranda, QLD, Australia	Veterinary	Koala – nasal swab	2017	This study	Pending
WM 18.95	Wangetti, QLD, Australia	Veterinary	Koala – nasal swab	2017	This study	Pending
WM 18.97	Wangetti, QLD, Australia	Environmental	Koala enclosure	2017	This study	Pending
WM 18.99	Kuranda, QLD, Australia	Environmental	Koala enclosure	2017	This study	Pending

CNS = central nervous system; NSW = New South Wales; NT = Northern Territory; QLD = Queensland; WA = Western Australia.

*Genome unpublished. Reference acknowledges the source of initial isolation.

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[3] Billmyre RB, Croll D, Li W, et al. Highly recombinant VGII *Cryptococcus gattii* population develops clonal outbreak clusters through both sexual macroevolution and asexual microevolution. *mBio.* 2014; 5 (4): e01494-01414.
[4] Kidd SE, Sorrell TC, Meyer W. Isolation of two molecular types of *Cryptococcus neoformans* var. *gattii* from insect frass. *Med Mycol.* 2003; 41 (2): 171-176.

5. General discussion and future directions

Many aspects of the pathogenesis of cryptococcosis caused by the *C. gattii* species complex remain enigmatic, particularly the relationship between environmental exposure, mucosal colonisation and the development of disease. We have a reasonable grasp of this in some experimental animal models and in koalas kept in captivity (Krockenberger et al., 2002a, Krockenberger et al., 2010), but our understanding of the phenomenon in a broader context, and in relation to naturally-occurring disease in free-ranging animals, companion animals, birds and especially people, is much poorer.

This thesis investigated the central hypothesis, that in the context of cryptococcosis caused by the *C. gattii* species complex, '*disease is a random outcome of heavy environmental exposure'*, on several levels. These included exploring:

1. The notion that some environmental associations for these pathogens could affect their survival and abundance, and thus the likelihood of host exposure;
2. Some clinical aspects of cryptococcosis in koalas, including the tendency for free-ranging animals to exhibit cryptococcal colonisation or disease, and whether this is affected by any individual host or environmental factors; and
3. The utility of fine-scale molecular epidemiology to elucidate aspects of the host-pathogen-environment interactions in cases of animal cryptococcosis.

This section will discuss the major findings of the thesis, in the context of the hypothesis and aims, and suggest directions for future research.

5.1 General discussion

In **section 2.1,** the findings of this investigation into the mycobiome of Australian tree hollows were consistent with expectations of a decaying woody environment. Yet, no clear relationships between *Cryptococcus* spp. and any other fungi were established, thus addressing **aim 1**. The predominant genera detected were all fungi classically associated with ligneous breakdown and decay. The lack of a relationship between the presence of *Cryptococcus* spp. in a tree hollow and any other specific fungal genera was somewhat

surprising but consistent with previous findings (Vanhove et al., 2017). The differential heat tree analysis did suggest some differences in the relative abundance of other fungi in association with *Cryptococcus* spp., but no striking pattern was observed and thus no definitive conclusions could be drawn. This does not exclude the possibility of involvement in the rot process or associations with other organisms, such as insects or free-living amoebae. Further investigations of different aspects of the tree hollow 'biosphere' should be explored in order to determine the role of this niche in the ecology of the fungus.

Differences in the results of culture-dependent and culture-independent methods for detecting environmental *Cryptococcus* spp. suggested that neither was superior, but that these methods are complementary. The sensitivity of culture-based identification is unknown, however, and thus there is no gold standard to compare results with. Culture-dependent methods are advantageous in many ways, as they allow for the collection of isolates and thus further downstream analyses (including more in-depth genotyping), but NGS barcoding may provide a more sensitive methodology. The biological relevance of a positive result on NGS must be interpreted with care, however, given the possibility that such results could represent only residual DNA in a sample, rather than vegetative organisms or spores (Lindahl et al., 2013, Nguyen et al., 2015).

The major limitation of this study was the small sample size. The experiments were effectively a pilot study to determine whether the methodology was suitable for examination of the mycobiome, and samples collected as part of other investigations were relied upon. Consequently, metadata that could have been of interest was sometimes lacking. These factors made statistical inferences difficult to make, and thus no relationships between the mycobiome and e.g. tree species, tree age, number of hollows, hollow aspect, etc. could be established. With regards to the central hypothesis, since the findings of this study did not offer any predictors as to when a heavy environmental presence of members of the *C. gattii* species complex might occur, they can neither be considered to clearly support nor refute the hypothesis, and further work is needed (discussed in section 5.2). Indeed, the expansion of the known environmental niches of the *C. gattii* VGI in this study highlights the fact that this organism can likely be associated with any decaying wood, not just eucalypts (given its isolation from Australian trees of other genera). This suggests that any exposure to environments with a heavy burden of *Cryptococcus* spp. (such as tree hollows) could result in

an increased likelihood of disease, rather than a relationship to particular tree species (such as *E. camaldulensis* and *E. tereticornis*).

In **section 3.1**, two cases that acted as sentinels for a cluster of cryptococcosis (further characterised in section 4.1) are described, with the successful treatment of subclinical disease (to full resolution) in one koala and the presence of concurrent disease in both a mother and dependent juvenile also reported for the first time. This also presented a review of published cases of the treatment and management of cryptococcosis in koalas, and thus addressed **aim 2**. Important insights into serological testing in koalas were also identified, with the LFA remaining positive at least four months beyond the first negative LCAT. This suggests that the LFA should be used, particularly once the LCAT is negative, to ensure full resolution of antigenaemia. The primary, and strikingly severe, appendicular osteomyelitis lesion reported in this case expands the current knowledge of the clinical presentation of cryptococcosis in koalas.

These cases offered the opportunity to consider some aspects of the host-pathogen-environment interactions of koala cryptococcosis, namely how both koalas (the mother and the joey) developed cryptococcosis simultaneously. Host susceptibility could play a role given the relatedness between these two individuals, but with the joey recovering well, the mother testing negative for KoRV-B and no recrudescence of disease in the joey three years later, this seems less likely. The joey did later contract chlamydiosis as an adult, but this is a very common infectious disease of koalas (Blanshard & Bodley, 2008) and such a result is not necessarily indicative of immunocompromise. The most likely cause is the exposure of both the mother and the joey to a common heavily contaminated environment, and thus likely the same heavy inoculum of *C. gattii* VGI, offering some support to the overarching hypothesis. A disease isolate from the joey could have helped to confirm this, but unfortunately was not available.

Section 3.2 extends the preliminary data of the previous section by systematically confirming the utility of the LFA as a diagnostic tool for cryptococcosis in koalas (along with cats and dogs). This validated a crucial tool for addressing **aim 3**. The LFA achieved excellent sensitivity and negative predictive values in all species when compared to the LCAT, but specificity and positive predictive values were more variable and generally less impressive, particularly in koalas. These findings indicate that the LFA presents an excellent screening test for quickly ruling out cryptococcosis in these species, with the potential to be used ‘cage-side’

in veterinary hospitals or in the field, but that positive LFA results need to be followed up with further testing (such as the LCAT).

The large number of LFA-positive yet LCAT-negative results, particularly in koalas, was considered likely due to both the superior sensitivity of the LFA (Kozel & Bauman, 2012) allowing it to pick up low-level antigenaemia, and the greater tendency for koalas (compared to cats and dogs) to develop nasal colonisation and subclinical disease. The LFA may be detecting very limited subclinical disease, or even heavy nasal colonisation, in koalas that is indicative of early exposure to cryptococcal antigen (which is likely to be cleared quickly by the host). Such low levels of circulating antigen are unlikely to be of clinical relevance to the host and probably do not warrant treatment in most cases but should still be monitored with follow-up testing. On the other hand, the LCAT may only return a positive result when subclinical disease is well-established. Therefore, both tests remain relevant to the diagnosis of cryptococcosis in animals, with the LFA more useful as a screening test and to detect early exposure, but the LCAT (or other diagnostic testing, such as culture) still required to confirm positive LFA results.

A limitation of this study was the use of the LCAT as the gold standard against which to compare the LFA. In a veterinary context (particularly in general practice), a diagnosis of cryptococcosis is often made based on only LCAT results and consistent clinical signs (largely due to financial constraints). Many of the cases in this study were followed up with other confirmatory testing, but particularly for koalas, there are relatively few cases in which mycological culture results (the classic gold standard) were available to validate the LFA. The strength of this study lies in the robust sample size, with over 500 specimens included across the three different animal species.

Section 3.3 offered the first large-scale and systematic insight into cryptococcosis in free-ranging koalas, partially by utilising a tool that was validated in the previous section. Overall, these findings characterised a finite prevalence of antigenaemia and nasal colonisation in free-ranging koalas that was much lower than that found in most captive populations (Connolly et al., 1999, Krockenberger et al., 2002a, Krockenberger et al., 2002b). This suggests that other factors in captivity inflate the prevalence of colonisation and antigenaemia in certain settings. A combination of confinement to a small area, regular soiling and scarification of wooden perches (providing a substrate for cryptococcal growth) and ongoing seeding of the environment by colonised koalas likely leads to an amplified

environmental presence in captivity, as speculated by Krockenberger et al. (2002b) and Malik et al. (2011). Given the observation that captive koalas can seed new environments with the *C. gattii* species complex (Krockenberger et al., 2002b) (further supported by the findings of section 4.4), evidence of nasal colonisation amongst free-ranging koalas indicates that this could present another method of natural dispersal in Australia. More Australian tree species with environmental associations to the *C. gattii* and *C. neoformans* species complexes were also identified, with *E. albens* a new environmental niche for *C. gattii* VGI and *E. populnea* for *C. neoformans* VNI.

The statistical analyses in this study found no significant relationships between antigenaemia or nasal colonisation and any of the individual koala or environmental characteristics tested, but there was a noticeable trend (that closely approached statistical significance) for antigenaemia-positive koalas to reside in areas with a high relative abundance of *E. camaldulensis*. While this may suggest an important role for *E. camaldulensis* in cryptococcal exposure and infection, this study did not sample enough trees in each area to assess the overall environmental prevalence. Thus, other differences between koalas and their habitat in the low and high abundance *E. camaldulensis* areas may have led to this result, and indeed the findings suggest that other tree species likely also act as important sources of exposure to *Cryptococcus* spp. in the region studied. Preliminary findings from another study (in which all of the authors of section 3.3 have also been involved) have suggested a reasonably high prevalence of environmental *C. gattii* VGI (approximately 50% of trees colonised) in this region, and that hollow size may be a significant predictor of the likelihood of colonisation (A. Kan, unpublished data). The significant relationship between nasal colonisation and antigenaemia provides strong evidence in support of previous studies and speculation that colonisation is a requisite precursor to tissue invasion and thus, antigenaemia (Krockenberger et al., 2002a, Malik et al., 2011).

This study, along with components of section 4, has addressed **aim 3**, and shown that this population of koalas has great potential to act as biological samplers for the presence of environmental *Cryptococcus* spp. and sentinels for human disease. With regards to the central hypothesis, the study offers some support, given the finding of no statistical significance associated with any of the individual koala or environmental factors tested and either antigenaemia or nasal colonisation. Further work is needed to confirm this, however, particularly since one relationship showed an obvious trend (despite not reaching

significance) and the environmental prevalence of *Cryptococcus* spp. was not determined. The weaknesses of this study primarily centre around the limited number of environmental samples collected and the lack of more in-depth molecular typing of isolates. Further strategies to address this will be discussed in section 5.2.

Section 4.1 identified and characterised a spatial and temporal case cluster of cryptococcosis amongst a group of free-ranging koalas and found that there was no apparent emergent outbreak strain responsible, addressing **aims 4** and **6**. This study was unable to identify an underlying cause for the outbreak, although the possibility of habitat destruction and other anthropogenic factors causing increased stress, and potentially immunosuppression, could not be ruled out. This, in isolation, could provide further support for the notion that these koalas randomly developed disease after heavy environmental exposure. The tendency for many koalas in this small area to exhibit nasal colonisation could suggest that other factors were at play, but only nine koalas were tested. Therefore, despite the significant difference reported between the two groups of koalas (with substantially more koalas from Soldiers Point and Salamander Bay colonised than from the other areas), it would be prudent to test more koalas from this region before drawing firm conclusions from this data.

The MLST data reported in this study indicates that: 1) koala nasal cavities are indeed excellent biological samplers, encompassing a broad range (phylogenetically speaking) of isolates and thus being representative of the local environmental *C. gattii* VGI population; and 2) *C. gattii* VGI disease and environmental isolates are fundamentally different. This was because disease isolates mostly clustered together (yet separate from most environmental isolates) in the phylogenetic analysis. Whether this difference is present due to random host contact with rare, higher virulence strains in the environment or from micro-evolution in the host remains undetermined, and WGS is needed to further elucidate this. Nevertheless, this brings the central hypothesis into question, as the involvement of specific, potentially more virulent strains could negate the randomness of *C. gattii* VGI cryptococcosis.

In **section 4.2** the likely source of infection for a case of cryptococcosis in a captive parrot was identified, thus successfully addressing **aim 5**. Although MLST could not unequivocally support this notion with an exact match between disease and environmental isolates, it was concluded that the tree in question must be the source of infection. This was based on a failure to identify any other environmental sources, the heavy and consistent

growth of *C. gattii* VGI from this tree, the tree's known heavy usage by parrots in this enclosure and the finding that disease and environmental isolates were very closely related using fine-scale epidemiology (with only one base pair insertion separating the disease strain from an environmental strain, according to MLST). This study highlighted the fact that animals (particularly those for which the range of domicile is strictly defined) offer an ideal opportunity to identify potential environmental sources of infection, when cases of disease or colonisation emerge. The enclosure in this case was regularly entered by staff, but also functions as a walk-through experience for members of the public. Thus, the identification of a tree heavily colonised by *C. gattii* VGI that had caused disease in one bird was important to minimise the risk of exposure for others, including humans and birds, and the tree was then carefully removed. This case also highlights the importance of animals as sentinels.

The underlying cause of disease in this parrot may have been related to stress (from its recent transfer to the enclosure and a change in the level of human contact it received). However, it may simply be that many birds were exposed to the source of infection but by chance, only this one went on to develop disease, which would support the central hypothesis of this thesis.

A similar scenario was reported in **section 4.3**, where one domestic ferret amongst a group residing in an outdoor enclosure developed cryptococcosis caused by *C. gattii* VGI. A hollow log used for enclosure enrichment (that was heavily and consistently colonised by both *C. gattii* VGI and *C. neoformans* VNI) was identified as the point source of infection, thus also addressing **aim 5**. Another ferret amongst the group was found to have cryptococcal nasal colonisation originating from the same log. These findings were supported by both culture and fine-scale epidemiology, but this time identical isolates to those found in the diseased and colonised ferrets were identified in a sample collected from the log. Again, and with parallels with section 4.2, other animals and humans (the remaining ferrets and their owners) were at risk of exposure to this source of infection, and these findings enabled this risk to be mitigated through the removal of the log. The results did suggest that at least one other ferret had already been exposed, thus again raising the question as to why no other in-contact animals developed disease. Unlike the previous section, there was no indication of increased stress or other potential causes of immunosuppression, and so this may represent the random development of disease in a host after incessant heavy exposure.

Sections 4.2 and 4.3 are both subject to similar limitations, namely that exposure of other in-contact animals to the source of infection could not be thoroughly investigated. Appropriate samples for this surveillance were not systematically collected by the attending veterinarians due to a combination of logistical difficulties (particularly in the free flight aviary), unnecessary risk to the animals, a lack of client consent and/or financial constraints. Also, in both cases, the diseased patients were not thoroughly assessed for underlying immunosuppression for similar reasons. Furthermore, clinical tools for immunological assessment, such as CD4 cell counts and measurement of antibody levels, are not well developed for most animal species.

Nevertheless, these studies remain ground-breaking in their successful identification of environmental point sources of infection, a rare achievement in the cryptococcosis literature that had not previously been defined beyond culture or basic genotyping (Sorrell et al., 1996, Bauwens et al., 2004, Singh et al., 2007, Morera et al., 2014). The successful identification of sources of infection using fine-scale molecular epidemiology (particularly in section 4.3, where an identical match was found between disease and environmental isolates) confirms that such investigations are worthwhile and can provide pertinent information for the management and prevention of further cases of disease.

Although sections 4.2 and 4.3 deal with different animal species, they still offer pertinent insights into the host-pathogen-environment interactions of cryptococcosis caused by the *C. gattii* species complex. They emphasise the commonalities between the disease in these species and koalas and thus highlight that any host has the potential to provide valuable insights into the pathogenesis of naturally-occurring cryptococcosis.

Section 4.4 examined a case cluster of cryptococcosis amongst a group of captive koalas and found that it was likely attributable to the relatively recent introduction of *C. gattii* VGII into this environment. The findings implicated koalas as being capable of translocating *C. gattii* VGII into new environments, including an initial introduction from WA to one of the wildlife parks in northern Queensland, and then subsequent spread from this facility to two other related wildlife parks. This presumably occurred when colonised koalas transferred organisms into a new environment via direct contact, sneezing, vocalisation, etc. Evidence of this phenomenon was found using a combination of culture and high-resolution genotyping. Overall, these results successfully addressed **aims 4, 6** and **7**.

The MLST results from this study indicated that the vast majority of VGII isolates (from the parks in eastern Australia) were the same ST. WGS offered a greater distinction between these isolates, but still confirmed that this is a closely related population, suggestive of a founder effect and clonal bloom after the initial introduction from WA (also supported by the basal relationship between a WA isolate and one clade of eastern Australian isolates). One limitation of this study was that only a representative portion of isolates proceeded to WGS, but this was still sufficient to provide insights into the molecular epidemiology, and largely correlated with MLST results. These results also maintain the previously established relationship between *C. gattii* VGII and western and northern central parts of Australia, with no evidence from WGS that this case cluster represented a long-term, established local population in eastern Australia. The inclusion of two other unrelated Australian *C. gattii* VGIIb isolates in the phylogenetic analyses (both were collected 15-20 years prior to this study; one was an environmental sample from the NT and the other from a cat in eastern Australia) also highlighted the overall low genetic diversity amongst Australian *C. gattii* VGIIb isolates and their stability over time.

The ability of koalas to translocate *C. gattii* VGII has implications for the management of outbreaks in Australia, and the transfer or export of koalas. Koalas are already routinely tested for cryptococcal antigenaemia prior to transfer or export, but these findings suggest that perhaps this protocol should extend to tests for nasal colonisation. The interpretation and outcome of such results, however, could be problematic. For example, a negative result on a nasal swab culture does not necessarily prove that a koala is not colonised (the sensitivity of this method is unknown, and deeper nasal washings may prove more fruitful than superficial samples from the nasal vestibule), and there is no established protocol for resolving nasal colonisation, thus a positive result would put all involved in a quandary. Given that in some captive populations, all koalas can also be colonised (Krockenberger et al., 2002b), this problem is likely to occur eventually. Therefore, the solution may be to monitor colonised animals and the new environment they are introduced in to closely (with baseline environmental samples collected prior to their introduction), institute a regular decontamination protocol and regularly monitor all koalas with serological testing and culturing of nasal swabs. A more aggressive stance might be to attempt to resolve the colonisation, particularly if *C. gattii* VGII is involved, using a combination of systemic and

topical antifungal therapy (potentially including nebulised AMB), but previous published attempts at this have yielded inconclusive results (Kido et al., 2012).

It is of note that the distinct phylogenetic difference between disease and environmental isolates, seen clearly in section 4.1, was not seen in sections 4.2 to 4.4. This may simply be because section 4.1 reflects a natural, free-ranging environment. The animals in the other sections were all either in captivity (4.2 and 4.4) or in a domestic scenario (4.3), where the environment is artificial, highly limited and may be regularly disturbed by cleaning, etc. These scenarios could also reflect single (or relatively few) introduction events of the *C. gattii* species complex into these environments and resulting clonal blooms of isolates. The natural environment likely represents a much more complex scenario. Therefore, section 4.1 may be more reflective of natural disease, but sections 4.2 to 4.4 still offer fascinating insights into the host-pathogen-environment interactions and provide important considerations for the management and prevention of this disease in captivity.

5.2 Future directions

The findings of this thesis have highlighted several areas for further study.

1) Broader-scale studies of the mycobiome of Australian tree hollows, including:
   a. The collection of more samples and the inclusion of metadata (e.g. size of tree, number of hollows, local climate, etc.) in statistical analyses;
   b. Choosing bigger genomic target regions (and thus using long-read technologies) to allow for more in-depth inter and intra-species distinctions; and
   c. Exploring possible relationships between *Cryptococcus* spp. and other microbes (including bacteria, protozoa, nematodes), and insects.

2) Further investigations into the treatment of cryptococcosis in koalas to improve case outcomes, including:
   a. Retrospective studies into all cases in which treatment has been attempted (as many remain unpublished);
   b. Systematic trials of treatment protocols; and
   c. Studies into the pharmacokinetics of AMB and itraconazole in koalas.

3) Exploring the relationship between koalas, *C. gattii* VGI and the environment in greater detail, utilising the Liverpool Plains population, through:

a. Systematic environmental surveys in this area to establish the environmental prevalence, and characterise different tree species and their individual characteristics;
b. Continuing to monitor this population to observe if differences between koalas in low and high relative abundance *E. camaldulensis* areas persist as the sample size increases;
c. Utilising tracking and home range data already obtained from some colonised koalas (as part of an ecological study) to determine which trees are used most heavily, and thus focus environmental sampling efforts on these trees;
d. Performing more discriminatory genotyping of *C. gattii* VGI isolates from koalas and the environment to further explore the interactions between koalas and individual trees;
e. Testing all koalas for KoRV and assessing if a relationship between KoRV (including different variants and insertion sites) and cryptococcal antigenaemia or nasal colonisation exists in this population;
f. Determining the prevalence of cryptococcosis, caused by *C. gattii* VGI, in humans and other species in this region; and
g. Performing similar systematic surveys in other free-ranging koala populations.

4) Further characterisation of the two case clusters observed in koalas through:

a. WGS (with phylogenetic analysis) of isolates from section 4.1;
b. Assessing the virulence of all isolates through analysis of WGS data (to determine if the infective strain does play a role in the development of disease), including:
    i. *In silico* examination of virulence genes;
    ii. *In vitro* virulence studies and the use of invertebrate or vertebrate animal models;
    iii. Comparing the genomes of environmental, disease and colonising isolates to highlight any changes to genes that may be involved in disease or virulence versus environmental survival;

c. Including more *C. gattii* VGII samples from WA, the NT and other parts of Australia in the phylogenetic analysis (section 4.4) to further test the basal relationship with eastern Australian isolates and to explore the population genomics of *C. gattii* VGII in Australia; and
d. Determining the prevalence of cryptococcosis, caused by *C. gattii* VGI, in humans and other species in the Port Stephens region (for the cluster described in section 4.1).

5) Exploring the biological control of environmental members of the *C. gattii* species complex, particularly in relation to captive koalas, through:

a. Determining the efficacy of disinfectants (including quaternary ammonium compounds and accelerated hydrogen peroxide) against *C. gattii* VGI and VGII in a laboratory setting; and
b. Using these results to develop and systematically test environmental cleaning and decontamination protocols in koala enclosures.

5.3 Final comments

This thesis has presented evidence largely in favour of the hypothesis that, with regards to naturally-occurring infections caused by members of the *C. gattii* species complex in animals in Australia, *'disease is a random outcome of heavy environmental exposure'*. Nevertheless, the findings in sections 3.3 and 4.1, where free-ranging animals were studied, indicate that the exact relationship between environmental exposure and eventual disease in a host is inevitably more complex and requires further investigation, with the infective strain potentially also playing a role.

The evidence that koalas (along with other animals) play a pivotal role in cryptococcosis caused by *C. gattii* VGI and VGII as sentinels, biological samplers, models for naturally-occurring infection and an environmental dispersal mechanism in Australia is compelling. A 'One Health' approach to *C. gattii* species complex cryptococcosis will inevitably lead to even greater insights to improve the prevention, treatment and management of this disease in all species.

6. References

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Appendix 1: Supplementary methods

Culture

Two mycological media were used for culture in this thesis, Staib's bird seed extract agar (containing penicillin and gentamicin) and SDA. The former was supplied by Veronica Ventura at the Sydney School of Veterinary Science, The University of Sydney while the latter was supplied by the Institute for Clinical Pathology and Medical Research, Westmead Hospital.

Primary cultures were performed by inoculating environmental samples on to bird seed agar plates. All swabs were gently rolled across the entire surface of the plate, while debris samples were inoculated by either: 1) inserting a pre-moistened sterile swab into the debris bag, and rolling this swab across the agar plate, or 2) agitating approximately 10 g of the debris in 50-100 ml of sterile saline solution, allowing it to stand for 30 minutes and then spreading a 1 ml aliquot of the supernatant across the agar plate. The agar plates were then sealed with a plastic paraffin film and incubated at 27°C for 10 days and observed daily for evidence of cryptococcal growth (yeast colonies exhibiting the brown-colour-effect). If no such growth was observed, the plates were discarded after 10 days.

If suspect cryptococcal growth was observed on a primary culture plate, 1-10 cryptococal colonies were subcultured to obtain a pure single colony isolate. This involved picking up an individual colony using a sterile plastic disposable inoculation loop and streaking it onto an SDA plate. These plates were sealed with a plastic paraffin film and incubated at 37°C. If any evidence of contamination was observed, a cryptococcal colony was subcultured onto another SDA plate. This process continued until there was confidence that a pure isolate, derived from a single colony, had been obtained.

Throughout this study, pure cultures of isolates were routinely stored as water cultures for easy access and regular use in the shorter term (typically several months). This involved collecting approximately one generous 10 µl inoculation loop of a pure, single colony culture and placing it into 1 ml of sterile distilled water in a sealed tube. For long term storage, isolates were submitted to the Medical Mycology Culture Collection at the Westmead Institute for Medical Research in Westmead, NSW, Australia where they were assigned accession numbers and stored at -80°C in 10% glycerol.

Serological testing

Two serological tests for cryptococcal antigen were used throughout this study, the LCAT and the LFA. Both were performed mostly according the manufacturer's instructions, with a few small modifications for the LCAT. The LFA was performed exactly according to the manufacturer's instructions. Minor adaptations to the manufacturer's LCAT protocol were used, which will be explained below. The LCAT was always used to determine antigen titres, barring a few examples shown in section 3.2.

LCATs were performed using the Cryptococcal Antigen Latex Agglutination System (CALAS®) manufactured by Meridian Bioscience, Inc., (Cincinatti, Ohio, USA). In this test, 200 µl of the serum sample is added to 200 µl of reconstituted pronase solution in a 1.5 ml sealed tube and incubated at 56°C for 15 minutes using a water bath. In the meantime, 50 µl of the supplied negative control is incubated at 56°C for 30 minutes and set aside for later use. The sample is removed from the water bath and immediately heated to 100°C for 5 minutes to inactivate the pronase. It is then allowed to cool to room temperature before being centrifuged on a mini benchtop centrifuge at approximately 1,000 g for 5 minutes (the exact specifications are not critical). The negative control (25 µl), positive control (one drop) and antibody control (one drop) (all provided in the kit) are then placed in their respective wells on a detection card while 25 µl of the sample to be tested is also placed in the top and bottom well of one column. One drop of detection latex is added to each well in the top row, while one drop of control latex is added to each well in the bottom row of the detection card. The wells are gently mixed using sterile applicator sticks. The card is then agitated gently for 5 minutes after which results are read immediately. Samples are considered positive if visible clumping is present and the controls have all behaved appropriately.

In the event of a positive result, a titration is performed to determine the antigen titre. Titration diluent (25 µl) is added to each well in one row of a 96 well plate. The pronase and heat-treated sample (25 µl) is added to the first well and mixed thoroughly. Half of contents of well 1 (25 µl) are transferred into well 2 and mixed. This continues to well 12. To determine the antigen titre, 25 µl of the contents of any well are placed on the top and bottom rows of one column of a detection card, and then processed as described in the previous paragraph. To determine the antigen titre, the endpoint of positivity must be determined. Typically, this

is done by testing wells 3, 6, 9 and 12. Then, for example, if well 3 is positive but wells 6, 9 and 12 are negative, wells 4 and 5 can be tested. Once the final positive well is confirmed, the antigen titre can be calculated. For example, if the positive endpoint is well 4, the titre is 1:32.

Genotyping

A minor adaptation of a fungal DNA extraction protocol developed by Ferrer et al. (2001) was used throughout this thesis. This involved collecting a generous 10 µl inoculating loop of pure, single colony culture, homogenising this in 250 µl of sterile water and freezing the mixture overnight at -20°C. After thawing, 2-mercaptoethanol (2 µl) and lysis buffer (0.5 g SDS, 1.4 g sodium chloride, 0.73 g EDTA, 20ml tris hydrochloride 1M, 80 ml sterile distilled water) (500 µl) were added. The mixture was vigorously vortexed then heated to 65°C for 1 hour in a heat block. During this time, the mixture was briefly and vigorously vortexed at least once (preferably more often). Phenol:chloroform:isoamyl alcohol (500 µl) was then added and the mixture was vigorously vortexed until it was homogenous (this took approximately 2 minutes). The mixture was then centrifuged at 14,000 g for 15 minutes and the upper aqueous layer was removed and mixed with an equal quantity of isopropanol (typically 500 µl of each). This new mixture was again stored overnight at -20°C then centrifuged at 4°C at 14,000 g for 15 minutes. The supernatant was discarded, and the pellet was washed with 70% ethanol (500 µl) then centrifuged at room temperature at 14,000 g for another 15 minutes. The supernatant was again discarded, and the pellet was dried in a concentrator at 45°C for 10 minutes. The pellet was resuspended in 50-100 µl of sterile distilled water. The DNA concentration was then determined and the 260:280 ratio checked using a spectrophotometer. The stock DNA was then diluted to 10 ng/µl for downstream PCR.

For WGS, initially a generous 10 µl inoculating loop of pure, single colony pure culture was collected and homogenised in 200 µl of sterile water and frozen overnight at -80°C. After thawing, this mixture underwent extraction using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. The DNA concentration was then measured using a spectrophotometer.

PCR amplifications were performed in 50 µl reaction volumes: 100 ng of DNA (10 µl volume), 10x reaction buffer (5 µl), 2 mmol dNTP solution (5 µl), 50 ng of forward primer (5 µl volume), 50 ng of reverse primer (5 µl volume), 50 mmol (3 µl) magnesium chloride and taq polymerase (0.5 µl). All reagents (BIOTAQ™ DNA Polymerase and dNTP Set) were sourced from Bioline (London, UK). The rest of the reaction volume consisted of sterile distilled water. Mating type PCR was the exception, where only 5 ng of DNA (5 µl volume), 12 ng (1.2 µl) of forward primer and 15.8 ng (1.58 µl) of reverse primer were used. PCR amplifications were performed using the SensoQuest Thermocycler (SensoQuest GMBH, Göttingen, Germany). A negative control was always included. The amplicons (10 µl) were then run on a 1.5% agarose gel (containing ethidium bromide) at 80 V for 40 minutes (with a 1 kb extension ladder). The gel was visualised under an ultraviolet lamp to determine if amplification had been successful, the product was of the appropriate size, and the controls had behaved appropriately.

RFLP analysis involved a *URA5* PCR amplification of each sample, with the inclusion of eight standard strains (VGI: WM 179; VGII: WM 178; VGIII: WM 175; VGIV: VM 779; VNI: WM 148; VNII: WM 626; VNIII: WM 628; VNIV: WM 629) and a negative control. Once amplification was confirmed by gel electrophoresis, 25.8 µl of amplicon was added to a mixture of buffer (3 µl), bovine serum albumin (0.3 µl) and the enzymes HhaI (0.3 µl) and Sau961 (0.6 µl) (all reagents sourced from New England BioLabs® Inc., Ipswich, Massachusetts, USA). The mixture was incubated at 37°C for three hours and then run on a 2.5% agarose gel (containing ethidium bromide) at 80 V for three hours with a 1 kb extension ladder and standard strains. The sample bands were compared to the standard bands to identify molecular type and species. This was an adaptation of a prior method develop by Meyer et al. (2003).

For MLST, PCR amplification of the *CAP59, GPD1,* IGS1, *LAC1, PLB1, SOD1* and *URA5* loci was performed (Meyer et al., 2009). Amplicons were sent to Macrogen, Inc. (Seoul, South Korea) for purification and sequencing of forward and reverse reads. Sequences were aligned to form contigs and trimmed to the appropriate lengths for the MLST scheme using Sequencher© (Gene Codes Corporation, Ann Arbor, Michigan, USA). ATs were assigned using a pairwise identification and local BLAST search on the Fungal MLST Database website (http://mlst.mycologylab.org/). The combination of AT numbers determined the ST. In the event of a novel allele or sequence type, sequences were submitted to the curators of the database.

Appendix Table 1.1: Kits, equipment and reagents.

Name	Use	Manufacturer	Location
BIOTAQ™ DNA Polymerase	PCR (standard)	Bioline	London, UK
CrAg® LFA	LFA	IMMY	Norman, OK, USA
CALAS®	LCAT	Meridian Bioscience, Inc.	Cincinnati, OH, USA
DNeasy PowerSoil Kit	DNA extraction	Qiagen GmbH	Hilden, Germany
dNTP Set	PCR	Bioline	London, UK
DS-11 Series Spectrophotometer	DNA concentration	Denovix®	Wilmington, DE, USA
Q800R2 Sonicator	Genomic DNA fragmentation	QSonica	Newtown, CT, USA
Quick-DNA™ Fungal / Bacterial Miniprep Kit	DNA extraction	Zymo Research	Irvine, CA, USA
Quick-Load® 1 KB Extend DNA Ladder	Gel electrophoresis	New England BioLabs®, Inc.	Ipswich, MA, USA
NEBNext® Ultra™ II DNA Library Prep Kit	WGS	New England BioLabs®, Inc.	Ipswich, MA, USA
NEBuffer™ 4, bovine serum albumin, HhaI, Sau961	RFLP	New England BioLabs®, Inc.	Ipswich, MA, USA
NextSeq	WGS	Illumina, Inc.	San Diego, CA, USA
SensoQuest Thermocycler	PCR	SensoQuest GMBH	Göttingen, Germany
SequalPrep™ Normalization Plate Kit	WGS	Thermofisher Scientific	Waltham, MA, USA
TaKaRa Taq DNA Polymerase	PCR (NGS)	Takara Bio USA, Inc.	Mountain View, CA, USA

Appendix Table 1.2: Software, programs, databases and other techniques.

Name	Use	Source
bcl2fastq 2.18.0.12	Mycobiome analysis	Illumina, Inc., San Diego, CA, USA
BWA v 0.7.15	WGS data analysis	(Li & Durbin, 2009)
Fungal MLST Database	MLST – allele and sequence type assignment	http://mlst.mycologylab.org (Meyer et al., 2009)
GATK 3.7	WGS data analysis	(McKenna et al., 2010)
ISHAM-ITS Database	Mycobiome analysis	http://its.mycologylab.org (Irinyi et al., 2015)
MEGA7	Phylogenetic analysis	(Kumar et al., 2016)
MetacodeR	Mycobiome analysis	(Foster et al., 2017)
MUSCLE	Sequence alignment	(Edgar, 2004)
NASP Pipeline v 1.1.2	WGS data analysis	(Sahl et al., 2016)
R	Mycobiome analysis	https://www.r-project.org/
Sequencher©	Aligning and trimming raw Sanger sequences	Gene Codes Corporation, Ann Arbor, Michigan, USA
SINTAX Classifier	Mycobiome analysis	(Edgar, 2016a)
SPAdes v 3.10.1	WGS data analysis	(Bankevich et al., 2012)
SparCC	Mycobiome analysis	(Friedman et al., 2012)
Trimmomatic v 0.32	WGS data analysis	(Bolger et al., 2014)
UNITE	Mycobiome analysis	(Kõljalg et al., 2013)
UNOISE	Mycobiome analysis	(Edgar, 2016b)
USEARCH 10.0	Mycobiome analysis	(Edgar, 2010)

Appendix Table 1.3: Primers and PCR amplification conditions.

Target	Primer sequences (5' to 3')	Amplification conditions	Source
CAP59	CAP59-F: CTCTACGTCGAGCAAGTCAAG *CAP59*-R: TCCGCTGCACAAGTGATACCC	94°C 3 min; 35 cycles: 94°C 30 s, 56°C 30 s, 72°C 1 min.	[1]
GPD1	GPD1-CG-F: CCACCGAACCCTTCTAGGATA GPD1-CG-R: CTTCTTGGCACCTCCCTTGAG GPD1-CN-F: ATGGTCGTCAAGGTTGGAAT GPD1-CN-R: GTATTCGGCACCAGCCTCA	94°C 3 min; 35 cycles: 94°C 45 s, 63°C 1 min, 72°C 2 min.	[1]
IGS1	IGS1-F: ATCCTTTGCAGACGACTTGA IGS1-R: GTGATCAGTGCATTGCATGA	94°C 3min; 35 cycles: 94°C 30 s, 60°C 30 s,72°C 1 min.	[1]
ITS1	ITS1-F: CTTGGTCATTTAGAGGAAGTAA ITS2: GCTGCGTTCTTCATCGATGC	95°C 7 min; 35 cycles: 94°C 30 s, 55°C 45 s, 72°C 1 min.	[2]
LAC1	LAC1-CG-F: AACATGTTCCCTGGGCCTGTG LAC1-CG-R: ATGAGAATTGAATCGCCTTGT LAC1-CG-F: GGCGATACTATTATCGTA LAC1-CG-R: TTCTGGAGTGGCTAGAGC	94°C 3 min; 30 cycles: 94°C 30 s, 58°C 30 s, 72°C 1 min.	[1]
MATα	MFαU: TTCACTGCCATCTTCACCACC MFαL: TCTAGGCGATGACACAAAGGG	94°C 5 min; 30 cycles: 94°C 1 min, 55°C 1 min, 72°C 1 min.	[3]
MATa	MFa2U: ACACCGCCTGTTACAATGGAC MFa2L: CAGCGTTTGAAGATGGACTTT	94°C 5 min; 30 cycles: 94°C 1 min, 55°C 1 min, 72°C 1 min.	[4]
PLB1	PLB1-F: CTTCAGGCGGAGAGAGGTTT PLB1-R: GATTTGGCGTTGGTTTCAGT	94°C 3 min; 30 cycles: 94°C 45 s, 61°C 45 s, 72°C 1 min.	[1]
SOD1	SOD1-CG-F: GATCCTCACGCCATTACG SOD1-CG-R: GAATGATGCGCTTAGTTGGA SOD1-CN-F: TCTAATCGAAATGGTCAAGG SOD1-CN-R: CGCAGCTGTTCGTCTGGATA	94°C 3 min; 35 cycles: 94°C 30 s, 52°C 30 s, 72°C 1.5 min.	[1]
URA5	URA5: ATGTCCTCCCAAGCCCTCGAC SJ101: TTAAGACCTCTGAACACCGTACTC	94°C 3 min; 35 cycles: 94°C 45 s, 63°C 1 min, 72°C 2 min.	[1]

[1] Meyer et al., 2009
[2] White et al., 1990
[3] Halliday et al., 1999
[4] Fraser et al., 2003

Appendix 2: Isolates and sequencing data

Appendix Table 2.1: BioProject numbers

BioProject number	Thesis section	Data type
PRJNA497337	2.1	ITS1 region amplicons from 23 tree hollows
PRJNA524387	4.4	WGS of 17 *C. gattii* VGII isolates

Appendix Table 2.2: GenBank accession numbers – *C. neoformans* VNI

Locus	Allele type number	Accession number	Thesis section
CAP59	1	MK331940	4.3
	7	MK331941	4.3
GPD1	1	MK331942	4.3
	5	MK331943	4.3
IGS1	1	MK331944	4.3
LAC1	1	MK331945	4.3
	2	MK331946	4.3
PLB1	1	MK331947	4.3
	4	MK331948	4.3
SOD1	1	MK331949	4.3
URA5	1	MK331950	4.3
	2	MK331951	4.3

Appendix Table 2.3: GenBank accession numbers – *C. gattii* VGI

Locus	Allele type number	Accession number	Thesis section
CAP59	16	MH286888	4.2
		MK092949	4.1
		MK331932	4.3
GPD1	5	MH286889	4.2
		MK092950	4.1
		MK331933	4.3
	14	MH286890	4.2
IGS1	3	MH286891	4.2
		MK092951	4.1
		MK331934	4.3
	12	MK092952	4.1
	20	MK092953	4.1
	97	MK092954	4.1
	109	MK331935	4.3
LAC1	5	MH286892	4.2
		MK092955	4.1
		MK331936	4.3
	57	MK092956	4.1
PLB1	5	MH286893	4.2
		MK092957	4.1
		MK331937	4.3
SOD1	32	MH286894	4.2
		MK092958	4.1
	45	MH286895	4.2
		MK092959	4.1
		MK331938	4.3
	65	MH286896	4.2
		MK092960	4.1
	111	MK092961	4.1
URA5	12	MH286897	4.2
		MK092962	4.1
		MK331939	4.3
	48	MK092963	4.1

Appendix Table 2.4: GenBank accession numbers – *C. gattii* VGII

Locus	Allele type number	Accession number	Thesis section
CAP59	2	MK133807	4.4
	14	MK133808	4.4
	27	MK133809	4.4
GPD1	6	MK133810	4.4
	21	MK133811	4.4
IGS1	4	MK133812	4.4
	10	MK133813	4.4
	27	MK133814	4.4
LAC1	4	MK133815	4.4
	28	MK133816	4.4
PLB1	1	MK133817	4.4
	2	MK133818	4.4
	27	MK133819	4.4
SOD1	15	MK133820	4.4
	43	MK133821	4.4
	46	MK133822	4.4
URA5	2	MK133823	4.4
	7	MK133824	4.4
	56	MK133825	4.4

Appendix Table 2.5: List of isolates with MLST data

This table begins on the next page.

WM number	Other number	Year	Species	Molecular type	Mating type	Category	Source	Location	*CAP59*	*GPD1*	IGS1	*LAC1*	*PLB1*	*SOD1*	*URA5*	Sequence type
13.223	-	2013	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
13.244	-	2013	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
13.373	13-4029	2013	*C. gattii*	VGIIb	α	Vet	Koala - brain	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
13.375	13-4219	2013	*C. gattii*	VGI	α	Vet	Koala - lymph node	Soldiers Point, NSW, Australia	16	5	3	5	5	45	48	364
14.206	14-3570	2014	*C. gattii*	VGIIb	α	Vet	Koala - nasal mass	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
14.340	14-7190	2014	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Salamander Bay, NSW, Australia	16	5	3	5	5	32	12	51
14.341	14-7197	2014	*C. gattii*	VGI	α	Vet	Koala - lung	Tanilba Bay, NSW, Australia	16	5	3	5	5	32	12	51
15.164	-	2015	*C. gattii*	VGI	α	Vet	Cockatoo - mass	Melbourne, VIC, Australia	16	14	3	5	5	45	12	159
15.169	14-8139N	2015	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Williamtown, NSW, Australia	16	5	3	5	5	32	12	51
15.170	14-8139H	2015	*C. gattii*	VGI	α	Vet	Koala - hock lesion	Williamtown, NSW, Australia	16	5	3	5	5	65	12	57
15.412	15-2658	2015	*C. gattii*	VGI	α	Vet	Koala - brain	Kuranda, QLD, Australia	16	5	3	5	5	32	12	51
15.422	E2652-D	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.423	E2655-A	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.424	E2655-B	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.425	E2655-C	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.426	E2655-D	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.427	E2655-E	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.428	E2656-A	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.429	E2656-B	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.430	E2656-C	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.431	E2656-D	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.432	E2656-E	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.433	E2657-A	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.434	E2657-B	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.435	E2657-C	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.436	E2657-D	2015	*C. gattii*	VGI	**a**	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	45	12	154
15.437	E2657-E	2015	*C. gattii*	VGI	α	Enviro	*E. pilularis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.438	E2659-A	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.439	E2659-B	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.440	E2659-C	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
15.441	E2659-E	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
16.01	E2660-A	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
16.02	E2660-E	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
16.03	E2665-A	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	111	12	365
16.04	E2665-B	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
16.05	E2665-C	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	12	5	5	32	12	188
16.06	E2665-D	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
16.07	E2665-E	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	111	12	365
16.08	E2666-B	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	12	5	5	32	12	188

WM number	Other number	Year	Species	Molecular type	Mating type	Category	Source	Location	*CAP59*	*GPD1*	IGS1	*LAC1*	*PLB1*	*SOD1*	*URA5*	Sequence type
16.09	E2666-C	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
16.10	E2666-D	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
16.11	E2666-E	2015	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
16.12	E2669-B	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
16.13	E2669-C	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
16.14	E2678-A	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	65	12	57
16.15	E2678-B	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	65	12	57
16.16	E2678-C	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	45	12	154
16.17	E2679-A	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	65	12	57
16.18	E2679-B	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	65	12	57
16.20	MK1012	2005	*C. gattii*	VGIIb	α	Vet	Koala - nasal mass	Currumbin, QLD, Australia	2	6	10	4	2	15	2	7
16.22	E2689-A	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	65	12	57
16.23	E2689-B	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	45	12	154
16.24	E2693-A	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	65	12	57
16.25	E2693-B	2015	*C. gattii*	VGI	α	Enviro	Large stag hollow	Melbourne Zoo, VIC, Australia	16	5	3	5	5	32	12	51
16.26	E2697-A	2015	*C. gattii*	VGI	α	Enviro	*E. robusta*	Salamander Bay, NSW, Australia	16	5	20	5	5	32	12	366
16.27	E2699-A	2015	*C. gattii*	VGI	α	Enviro	*Melaleuca* spp.	Salamander Bay, NSW, Australia	16	5	3	5	5	32	12	51
16.28	E2704-A	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Salamander Bay, NSW, Australia	16	5	3	5	5	32	12	51
16.29	E2704-B	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Salamander Bay, NSW, Australia	16	5	3	5	5	32	12	51
16.30	E2705-A	2015	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Salamander Bay, NSW, Australia	16	5	3	5	5	32	12	51
16.32	LS1-A	2015	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Salamander Bay, NSW, Australia	16	5	3	5	5	45	12	154
16.52	LS78	2015	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Bobs Farm, NSW, Australia	16	5	12	5	5	32	12	188
16.53	LS79	2015	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Corlette, NSW, Australia	16	5	3	5	5	32	12	51
16.54	LS81	2015	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Soldiers Point, NSW, Australia	16	5	12	57	5	32	12	395
16.55	LS82	2015	*C. gattii*	VGI	α	Vet	Koala - nasal swab	One Mile, NSW, Australia	16	5	20	5	5	32	12	366
16.56	LS87	2016	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Salamander Bay, NSW, Australia	16	5	97	5	5	32	12	396
16.57	LS90-A	2016	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Salamander Bay, NSW, Australia	16	5	3	5	5	65	12	57
16.58	16-0158-V	2016	*C. gattii*	VGI	α	Vet	Koala - vertebra	Salamander Bay, NSW, Australia	16	5	3	5	5	65	12	57
16.59	16-0158-NL	2016	*C. gattii*	VGI	α	Vet	Koala - nasal lesion	Salamander Bay, NSW, Australia	16	5	3	5	5	65	12	57
16.60	16-0158-B	2016	*C. gattii*	VGI	α	Vet	Koala - bronchi	Salamander Bay, NSW, Australia	16	5	3	5	5	65	12	57
16.61	16-0158-U	2016	*C. gattii*	VGI	α	Vet	Koala - urine	Salamander Bay, NSW, Australia	16	5	3	5	5	65	12	57
16.62	16-0158-M	2016	*C. gattii*	VGI	α	Vet	Koala - meninges	Salamander Bay, NSW, Australia	16	5	3	5	5	65	12	57
16.63	LS92	2016	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Soldiers Point, NSW, Australia	16	5	12	5	5	32	12	188
17.012	2000419115	2016	*C. gattii*	VGIIb	α	Vet	Koala - lymph node	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
17.112	LS98	2016	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
17.113	LS176-C	2016	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
17.114	E2704-C	2016	*C. gattii*	VGI	α	Enviro	*E. tereticornis*	Salamander Bay, NSW, Australia	16	5	3	5	5	32	12	51
17.116	Ambush 1	2016	*C. gattii*	VGI	α	Vet	Ferret - nasal lesion	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
17.117	Chewy	2016	*C. neoformans*	VNI	α	Vet	Ferret - nasal swab	Richmond, NSW, Australia	1	5	1	1	4	1	1	13

WM number	Other number	Year	Species	Molecular type	Mating type	Category	Source	Location	*CAP59*	*GPD1*	IGS1	*LAC1*	*PLB1*	*SOD1*	*URA5*	Sequence type
148	H39.1.1	1989	*C. neoformans*	VNI	α	Clinical	Human - CSF	Sydney, NSW, Australia	7	1	1	18	1	1	1	7
175	91B3214	-	*C. gattii*	VGIII	α	Enviro	*Eucalyptus* spp.	San Diego, California, USA	18	18	14	3	6	28	19	60
178	49435	1991	*C. gattii*	VGII	α	Clinical	Human - lung	Sydney, NSW, Australia	1	17	16	16	14	19	7	21
179	H33.1	1993	*C. gattii*	VGI	α	Clinical	Human - CSF	Sydney, NSW, Australia	16	5	3	5	5	32	12	51
626	CBS 10083	1993	*C. neoformans*	VNII	α	Clinical	Human - CSF	Sydney, NSW, Australia	2	14	14	8	11	11	4	2
779	CBS 10101	1995	*C. gattii*	VGIV	α	Vet	Cheetah	Johannesburg, South Africa	17	10	8	18	3	37	11	70
03.27	RAM 002	1999	*C. gattii*	VGIIb	α	Enviro	*Eucalyptus* spp.	Arnhem Land, NT, Australia	2	6	10	4	2	15	2	7
04.71	2500/91	1991	*C. gattii*	VGIIb	α	Vet	Cat - nasal lesion	Sydney, NSW, Australia	2	6	10	4	2	15	2	7
09.152	-	2009	*C. gattii*	VGII	α	Enviro	New enclosure	Caversham, WA, Australia	27	6	4	4	1	43	7	48
09.153	-	2009	*C. gattii*	VGIIb	α	Enviro	New enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.154	-	2009	*C. gattii*	VGIIb	α	Enviro	New enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.155	Sarah	2009	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.156	J	2009	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.157	Amie	2009	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.158	Amelie	2009	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.160	-	2009	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.161	-	2009	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.163	-	2009	*C. gattii*	VGIIb	α	Enviro	Old enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.164	-	2009	*C. gattii*	VGIIb	α	Enviro	Old enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.165	-	2009	*C. gattii*	VGII	**a**	Enviro	Old enclosure	Caversham, WA, Australia	14	21	27	28	27	46	2	38
09.166	-	2009	*C. gattii*	VGIIb	α	Enviro	Visitor area	Caversham, WA, Australia	2	6	10	4	2	15	2	7
09.167	-	2009	*C. gattii*	VGIIb	α	Enviro	Visitor area	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.197	MK1914	2012	*C. gattii*	VGII	α	Vet	Koala - nasal swab	Caversham, WA, Australia	27	6	4	4	1	43	7	48
12.198	MK1915	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.200	MK1917	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.201	MK1919	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.204	MK1923	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.205	MK1925	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.206	MK1926	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.208	MK1928	2012	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.211	E2606	2012	*C. gattii*	VGIIb	α	Enviro	Main enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.212	E2607	2012	*C. gattii*	VGIIb	α	Enviro	Main enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.213	E2609	2012	*C. gattii*	VGII	α	Enviro	Nursery enclosure	Caversham, WA, Australia	27	6	4	4	1	43	7	48
12.214	E2612	2012	*C. gattii*	VGIIb	α	Enviro	Nursery enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.215	E2618	2012	*C. gattii*	VGIIb	α	Enviro	QLD enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.216	E2619	2012	*C. gattii*	VGIIb	α	Enviro	QLD enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.217	E2620	2012	*C. gattii*	VGIIb	α	Enviro	VIC enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
12.219	E2622	2012	*C. gattii*	VGIIb	α	Enviro	VIC enclosure	Caversham, WA, Australia	2	6	10	4	2	15	2	7
13.222	-	2013	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Caversham, WA, Australia	2	6	10	4	2	15	2	7

WM number	Other number	Year	Species	Molecular type	Mating type	Category	Source	Location	*CAP59*	*GPD1*	IGS1	*LAC1*	*PLB1*	*SOD1*	*URA5*	Sequence type
17.118	17-7031	2017	*C. gattii*	VGI	α	Vet	Ferret - brain	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
17.119	16-9116	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal lesion	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.012	LS55-C	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Palm Cove, QLD, Australia	2	6	10	4	2	15	56	539
18.013	LS55-D	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Palm Cove, QLD, Australia	2	6	10	4	2	15	56	539
18.014	LS56-B	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.074	E2746-A	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA4	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.076	E2747-A	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA8	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.078	E2748-C	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA8	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.079	E2749-A	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA5	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.080	E2750-A	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA5	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.081	E2750-B	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA5	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.082	E2750-E	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA5	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.083	E2750-F	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA5	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.084	E2752-C	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA6	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.092	LS307-A	2017	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.093	LS310	2017	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.094	LS311-C	2017	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.095	LS298	2017	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7
18.096	E2966-B	2017	*C. gattii*	VGIIb	α	Enviro	Group enclosure	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7
18.097	E2966-C	2017	*C. gattii*	VGIIb	α	Enviro	Group enclosure	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7
18.098	E2994-A	2017	*C. gattii*	VGIIb	α	Enviro	Enclosure 1A	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.099	E2994-B	2017	*C. gattii*	VGIIb	α	Enviro	Enclosure 1A	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.117	E2775-A	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	109	5	5	45	12	541
18.118	E2775-B	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.119	E2775-C	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.120	E2775-D	2016	*C. neoformans*	VNI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	1	5	1	1	4	1	1	13
18.121	E2775-E	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.122	E2775-F	2016	*C. gattii*	VGI	**a**	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.123	E2775-G	2016	*C. gattii*	VGI	**a**	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.124	E2775-H	2016	*C. neoformans*	VNI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	1	5	1	1	4	1	1	13
18.125	E2775-J	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.126	E2775-K	2016	*C. neoformans*	VNI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	1	5	1	1	4	1	1	13
18.127	E2775-L	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.128	E2775-M	2016	*C. neoformans*	VNI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	1	5	1	1	4	1	1	13
18.129	E2775-N	2016	*C. neoformans*	VNI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	7	1	1	2	1	1	2	23
18.130	E2775-P	2016	*C. gattii*	VGI	α	Enviro	Eucalypt log	Richmond, NSW, Australia	16	5	3	5	5	45	12	154
18.131	16-1726	2016	*C. gattii*	VGI	α	Vet	Koala - nasal swab	Soldiers Point, NSW, Australia	16	5	3	5	5	65	12	57
18.136	E2666-F	2017	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
18.137	E2666-G	2017	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366

WM number	Other number	Year	Species	Molecular type	Mating type	Category	Source	Location	*CAP59*	*GPD1*	IGS1	*LAC1*	*PLB1*	*SOD1*	*URA5*	Sequence type
18.138	E2666-H	2017	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
18.139	E2666-J	2017	*C. gattii*	VGI	α	Enviro	*A. floribunda*	Soldiers Point, NSW, Australia	16	5	20	5	5	32	12	366
18.140	E3036-A	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.141	E3036-B	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.142	E3036-C	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.143	E3036-D	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.144	E3036-E	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.145	E3036-F	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.146	E3039-A	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.147	E3039-B	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.148	E3039-C	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.149	E3039-D	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.150	E3039-E	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.151	E3039-F	2017	*C. gattii*	VGI	α	Enviro	*Eucalyptus* spp.	Soldiers Point, NSW, Australia	16	5	3	5	5	32	12	51
18.152	E2958	2017	*C. gattii*	VGIIb	α	Enviro	GD enclosure	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7
18.158	LS307-B	2017	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.160	LS311-A	2017	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Kuranda, QLD, Australia	2	6	10	4	2	15	2	7
18.161	LS40-C	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7
18.162	LS40-D	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7
18.163	LS56-A	2016	*C. gattii*	VGIIb	α	Vet	Koala - nasal swab	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.164	E2748-A	2016	*C. gattii*	VGIIb	α	Enviro	Enclosure KOA8	Palm Cove, QLD, Australia	2	6	10	4	2	15	2	7
18.165	E2966-A	2017	*C. gattii*	VGIIb	α	Enviro	Group enclosure	Wangetti, QLD, Australia	2	6	10	4	2	15	2	7

Appendix 3: Other publications

I have been involved as a co-author in two other publications during my candidature that were either not directly related to, or unable to be included, in my thesis. These are:

Fernandez, C.M., **Schmertmann, L.J.**, Higgins, D., Casteriano, A., Irinyi, L., Mella, V.S.A., Crowther, M.S., Meyer, W. & Krockenberger, M.B. 2019. Genetic differences in *Chlamydia pecorum* between neighbouring sub-populations of koalas (*Phascolarctos cinereus*). *Veterinary Microbiology*, 231, 264-270.

Krockenberger, M., **Schmertmann, L.J.**, Canfield, P. & Malik, R. 2019. Cryptococcosis. *In:* Vogelnest, L. & Portas, T. (eds.) *Medicine of Australian mammals: current therapy*. Collingwood, Victoria: CSIRO Publishing.