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**The epidemiology and pathology of  
*Paranannizziopsis australasiensis* in New Zealand  
reptiles**

A thesis presented in partial fulfilment of the requirements

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## Abstract

*Paranannizziopsis australasiensis*, has recently been diagnosed in tuatara at two captive facilities in New Zealand. This newly emerging fungal pathogen, is a member of the onygenalean fungal group formally known as *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV). Fungi of this genera are thought to be obligate primary pathogens in reptiles, and closely related species such as *Ophidiomyces ophiodiicola*, and *Nannizziopsis guarroi* have caused significant morbidity and mortalities in captive and wild reptile populations. The detection of this disease raised concerns for wild and captive population health and resulted in a temporary cessation of tuatara breed and release programmes from affected facilities. Similar lesions have been reported in tuatara at multiple other captive facilities in New Zealand, but lack of veterinary assessment and, until recently, inadequate diagnostic capabilities has led to an inability to confirm the presence or absence of *P. australasiensis* in these populations.

This research aimed to investigate the epidemiology of *P. australasiensis* in New Zealand wild and captive endemic reptiles. Skin samples were collected from nine captive, six wild and two ecosanctuary populations of tuatara across New Zealand. Skin samples from in contact geckos and skinks were opportunistically collected to determine the possible cross species infection of *P. australasiensis*. Samples were tested for presence of *P. australasiensis* by fungal culture followed by PCR, and by loop-mediated isothermal amplification (LAMP). Soil samples were collected from burrows, basking areas and captive enclosures and analysed by LAMP to determine the presence of *P. australasiensis* within the environment.

*Paranannizziopsis australasiensis* was found to be wide spread in New Zealand captive and wild reptile populations. In populations where the pathogen was detected prevalence varied between 6.7% and 44.4% for tuatara, 3.8% and 40% for geckos and 6.7% and 66.7% for skinks. A low virulence of disease associated with infection was seen in tuatara across New Zealand, with many LAMP positive tuatara being asymptomatic. Increased severity of disease was seen in two captive tuatara, where

other concurrent disease was present. One fatality was reported. In other reptile hosts, no disease was identified, and it is suspected these species act as reservoirs for the transmission of this organism to tuatara. *Paranannizziopsis australasiensis* was detected multiple times in soil samples and may survive as an environmental saprophyte.

*Paranannizziopsis australasiensis* appears to have a close association with New Zealand reptiles. The prevalence, distribution and pathology of *P. australasiensis* observed in this study suggests that this organism is not a threat to tuatara or other endemic reptile populations in New Zealand. The findings of this study have enabled restrictions placed on tuatara translocations, based on *P. australasiensis* status, to be removed.

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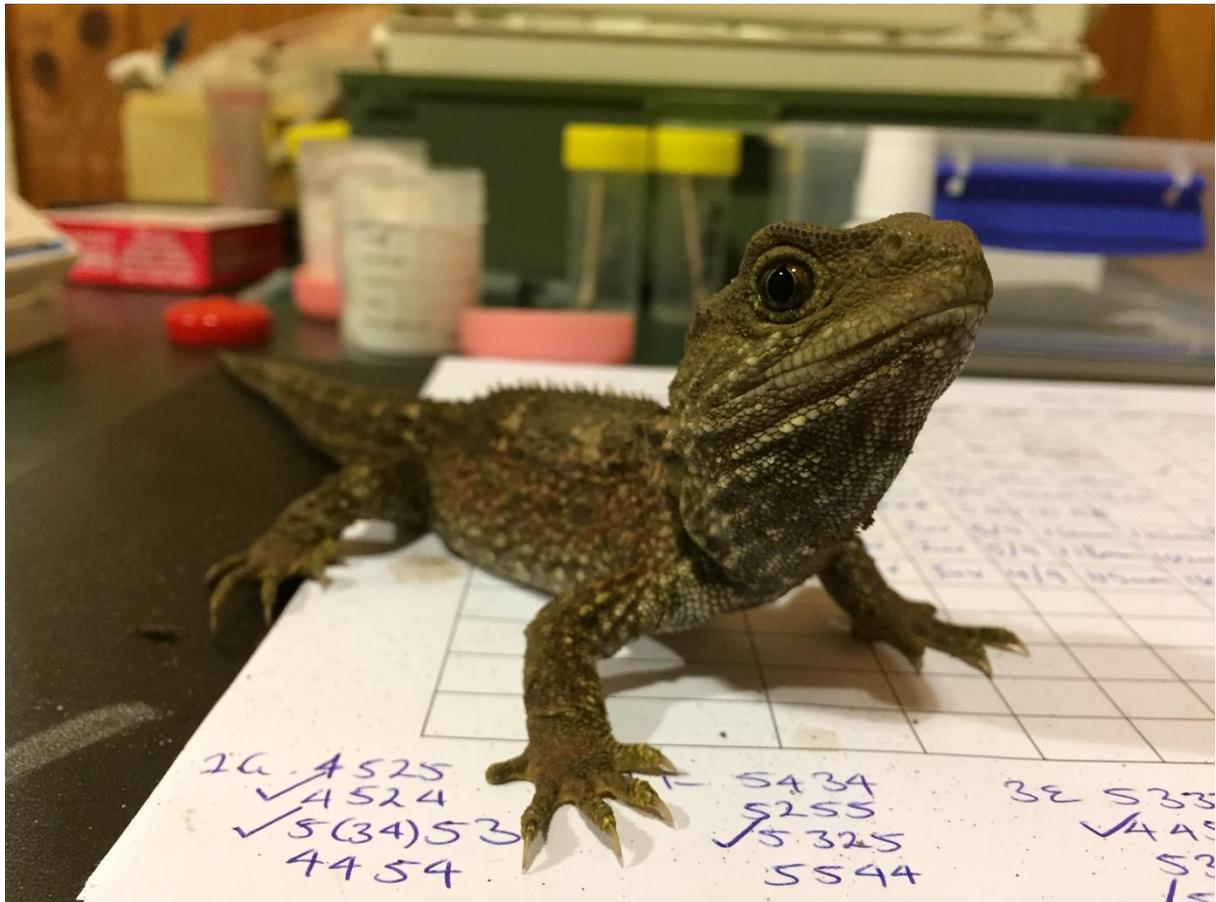
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# CHAPTER ONE

## Introduction, literature review and research aims



## 1.1 Introduction

The tuatara is an iconic reptile endemic to New Zealand and is the only extant species of the order *Rhynchocephalia*. The emerging fungal pathogen, *Paranannizziopsis australasiensis* has recently been diagnosed in tuatara, at two captive facilities in New Zealand. *Paranannizziopsis australasiensis* has been identified as a key threatening disease process to captive and wild tuatara and other endemic terrestrial New Zealand reptiles. The population of captive tuatara that *P. australasiensis* has been reported in are involved in a breeding for release programme. This has raised concerns regarding the potential release of this disease into wild populations, from both historical and proposed future release events. The consequences of mycotic pathogen emergence in naive wild host populations has been demonstrated multiple times with diseases such as Chytridiomycosis (Skerratt et al. 2007), white nose syndrome (Blehert et al. 2009) and snake fungal disease (Allender et al. 2011) resulting in population declines and extinctions.

*Paranannizziopsis australasiensis* is a member of the onygenalean fungal group formally known as the *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV). Infections in tuatara result in superficial brown-yellow exudative to ulcerative dermatitis lesions on the ventrum and gular regions (Masters et al. 2016). Fungi of this grouping are thought to act as primary pathogens in reptiles, and infections have been reported in both wild and captive populations without any identified predisposing stressors (Bowman et al. 2007; Sigler, Hambleton, and Paré 2013; Allender, Baker, et al. 2015). Closely related fungal species such as *Ophidiomyces ophiodiicola* and *Nannizziopsis guarroi*, have caused significant morbidity and mortalities in wild and captive reptile populations, respectively (Sigler, Hambleton, and Paré 2013; Allender, Raudabaugh, et al. 2015). The impact of this disease on tuatara and other reptile species health is unknown, and thus the detection of this disease is of concern to wild and captive reptile populations. The finding of *P. australasiensis* in captive tuatara has resulted in a temporary cessation of tuatara breed for release programs. The origins, epidemiology and significance of *P. australasiensis* in New Zealand is currently unknown. Decisions regarding the captive management of tuatara cannot be made until more is known about this emerging mycotic disease.

## 1.2 Emerging Mycotic Diseases

Over the past several decades emerging infectious diseases have been of increasing threat to the conservation of biodiversity globally. Fungal diseases have been implicated as the major pathogen driven cause of species decline and extinctions, of both animal and plant hosts (Fisher et al. 2012). Pathogens such as *Batrachochytrium dendrobatidis*, the cause of chytrid disease in amphibians (Skerratt et al. 2007), *Pseudogymnoascus destructans*, the cause of white-nose syndrome in bats (Blehert et al. 2009), and *Ophidiomyces ophiodiicola*, the cause of snake fungal disease (Allender, Raudabaugh, et al. 2015), highlight the devastating effect mycotic disease can have on wild populations. Often these hosts survive in remote, un-monitored populations, allowing the host-pathogen relationship to go undetected until declines are significant (Lorch et al. 2015).

The key drivers of emerging fungal diseases are related to the pathogen, the host and the environment as well as anthropogenic factors. Fungi have persistent environmental stages, either as durable spores or free-living saprophytes and remain infectious for long periods, facilitating spread. Fungi often have very broad host ranges and different host species will have differing susceptibilities to infection. Asymptomatic carrier animals may occur, resulting in super shedders contaminating the environment with large quantities of organism. Environmental changes, such as increasing temperature and deforestation, can be responsible for distribution changes of both the pathogen and the host, and subsequent exposure to novel pathogens. Increased host stress, immunocompromise, and increased disease susceptibility may also result in response to environmental change. Pathogen spread has been augmented by international animal and plant trade, both regulated and unregulated. Introduced novel pathogens may be of high virulence in naïve hosts. Movement of pathogens can create a condition of accelerated virulence when previously allopatric fungal species mix and recombine (Rachowicz et al. 2005; Fisher et al. 2012).

### 1.3 Mycotic disease in reptiles

Mycotic disease in reptiles is common. Infections frequently involve the integument, respiratory or gastrointestinal system, and fatal systemic mycosis can result (Schumacher 2003; Harvey et al. 2010). Most mycotic infections result from opportunistic environmental saprophytes, causing disease in animals under immune stress. Predisposing factors are generally associated with captive conditions and include poor husbandry, malnutrition, overcrowding, concurrent disease, prolonged antibiotic therapy, trauma to the skin and mucous membranes, and overwhelming environmental load. Infections are often mixed (Schumacher 2003; Harvey et al. 2010). Common fungal isolates from lesions in reptiles include *Paecilomyces*, *Penicillium*, *Fusarium*, *Geotrichium*, *Mucor*, and *Aspergillus* spp. (Jacobson, Cheatwood, and Maxwell 2000). It is often unclear if these organisms are the primary cause of morbidity and mortality, secondary invaders or environmental contaminants (Paré et al. 1997; Gartrell 2016).

Primary mycotic pathogens occur less frequently. Fungi of the families *Onygenaceae* and *Clavicipitaceae* are the only known obligate mycotic pathogens of reptiles (Schmidt 2015). The family *Onygenaceae* includes the fungal group formally known as the *Chrysosporium* anamorph of *Nannizziopsis vriesii*. This has recently been reclassified into sixteen known species of three phylogenetic lineages (Paré and Sigler 2016). These pathogens are emerging globally within many different reptile groups. *Ophidiomyces ophiodiicola* and *Nannizziopsis guarroi* are two significant examples, that cause high rates of morbidity and mortality in most affected species (Schmidt 2015).

#### 1.3.1 Mycotic Disease in New Zealand Reptiles

Of post mortems performed between 1991 and 2015 in New Zealand, fungal disease was reported as the third most common cause of death in tuatara and endemic geckos, the sixth in endemic skinks and the second in captive exotic reptiles. Fungal isolates cultured from lesions at biopsy and post mortem include *Alternaria*, *Aspergillus*, *Beauveria*, *Candida*, *Cladosporium*, *Mucor*, *Paecilomyces*, *Paranannizziopsis*, *Penicillium*, and *Rhizopus* spp. (Gartrell 2016).

Black spot disease is a seasonal superficial mycotic dermatitis of captive New Zealand geckos. It is believed to be related to a sudden drop in temperature and increased humidity, resulting in increased environmental fungal load, and decreased host immunity (Twentyman 1999; Harvey et al. 2010). *Mucor ramosissimus*, *Cladosporium cladosporioides* and *Alternaria alternata* have been implicated in this disease (Twentyman 1999; Gartrell and Hare 2005). Clinical signs are multifocal to coalescing black superficial skin discolouration, lethargy and anorexia. Resolution often occurs with subsequent ecdysis. Infrequently infections invade deeper tissues, and cause systemic mycosis, and can be fatal (Gartrell and Hare 2005).

There is a single case report of a microsporidium infection *Plistophora sp.*, in a pair of captive tuatara held at the Bronx Zoo, New York. Microsporidia have been recently recognised as an early diverging clade of fungi, rather than protozoa as previously classified (Capella-Gutiérrez, Marcet-Houben, and Gabaldón 2012). Clinical signs included anorexia and lethargy eight to twelve weeks prior to death. Histologically a severe granulomatous inflammatory reaction in skeletal muscle and the tongue with what were erroneously interpreted as protozoan cysts within the muscle fibres (Liu and King 1971).

## **1.4 Reptile pathogens of the genera *Nannizziopsis*, *Paranannizziopsis* and *Ophidiomyces***

### **1.4.1 Classification**

The group of fungi previously known under the appellation *Chrysosporium* anamorph of *Nannizziopsis vriesii* are increasingly being reported as the cause of mycotic lesions across a broad range of reptilian species. This fungal group has recently been reclassified based on molecular characterisation, into three genera *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces* belonging to the family *Onygenaceae* order *Onygenales* (Sigler, Hambleton, and Paré 2013; Stchigel et al. 2013). This order also includes significant fungal pathogens of other vertebrates, such as *Blastomyces*, *Coccidioides*, *Trichophyton*, *Microsporum*, *Epidermophyton*, *Histoplasma*, and

*Paracoccidioides* (Cabañes, Sutton, and Guarro 2014). Three genera *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces* with thirteen species (*N. guarroi*, *N. arthrosporioides*, *N. barbata*, *N. chlamydospora*, *N. crocodili*, *N. dermatitidis*, *N. draconii*, *N. pluriseptata*, *P. australasiensis*, *P. californiensis*, *P. crustacea*, and *P. longispora* and *O. ophiodiicola*) associated with reptiles have been described to date. Three additional species of *Nannizziopsis* have been isolated from immunocompromised humans (*N. hominis*, *N. infrequens*, and *N. obscura*). These species have not been associated with reptiles, and there is no known zoonotic risk (Sigler, Hambleton, and Paré 2013; Paré and Sigler 2016).

The fungi in this group are all thought to be primary pathogens of reptiles, with the exception of human isolates. This has been demonstrated with *N. dermatitidis* in veiled chameleons (*Chamaeleo calytratus*), and *O. ophiodiicola* in corn snakes (*Pantherophis guttatus*) and Cottonmouths (*Agkistrodon piscivorous*) (Paré et al. 2006; Lorch et al. 2015; Allender, Baker, et al. 2015). They are keratophilic, causing mycotic dermatitis, which can progress to deeper invasion and rarely systemic spread (Bowman et al. 2007; Han, Lee, and Na 2010; Masters et al. 2016). Infections have been the cause of significant morbidity and mortality of both captive and wild reptile groups (Paré and Sigler 2016). Each species of fungi appears to have close associations to specific reptile hosts. *Nannizziopsis* spp. is associated with the lizards chamaeleonids, gekkonids, cordylids, teiids, agamids, and iguanids, as well as crocodiles, *Paranannizziopsis* spp. has been reported in semi aquatic snakes, agamids and tuatara, and *Ophidiomyces* sp. is reported only in terrestrial and semiaquatic snakes (Sigler, Hambleton, and Paré 2013; Paré and Sigler 2016). Disease emergence is global with reports of infections from most continents including, Asia, North America, Europe and Australia (Paré and Sigler 2016).

#### 1.4.2 Diagnostics

Over the last two decades reports of *Nannizziopsis* spp, *Paranannizziopsis* spp. and *Ophidiomyces ophiodiicola*, infections have been increasing. This is likely attributable to disease emergence, increased surveillance and historical under reporting and inadequate identification. Lack of appropriate diagnostic techniques have previously led to misidentification of these pathogens (Sigler, Hambleton, and Paré 2013;

Masters et al. 2016). Microscopic identification is difficult, as conidia appear similar to other anamorphic genera. *Blastomyces*, *Emmonsia*, *Geomyces*, *Malbranchea*, *Myceliophthora*, *Trichophyton*, *Chrysosporium* have all been confused with these fungi (Paré et al. 1997; Sigler, Hambleton, and Paré 2013; Cabañes, Sutton, and Guarro 2014). The current gold standard for diagnostic testing is full thickness skin biopsies taken from the edge of a lesion that is utilised for fungal culture, to isolate the organism, in combination with histology, to establish the pathogenic role of the fungi. In addition, polymerase chain reaction (PCR) or molecular sequencing is warranted to identify the pathogen down to a species level. (Mitchell and Walden 2013). All diagnostic tools should be utilised in combination, for studies investigating the prevalence of these fungal diseases, to enable increased sensitivity and specificity, and accuracy of reporting.

*Nannizziopsis* spp, *Paranannizziopsis* spp and *Ophidiomyces* sp can be cultured under aerobic conditions at 25-30°C degrees on potatodextrose agar or Sabouraud agar. Culture medium should contain cycloheximide. Gentamycin or chloramphenicol can be used to decrease bacterial growth. Colonies should be checked frequently for growth for up to three weeks. The growth is restricted by temperature, with no growth occurring over 35-37 °C, or under 15 °C. Colonies for all species are yellow to white with a velvety appearance. Culture is a highly specific but poorly sensitive diagnostic technique. The fungi are slow growing and large samples containing viable organisms are required (Bohuski et al. 2015; Paré and Sigler 2016). Bacterial contamination of samples is often heavy. Differentiation of the fungi to species level or between lineages is difficult based solely on gross or microscopic fungal characteristics. (Mitchell and Walden 2013; Schmidt 2015). All species have poorly differentiated fertile hyphae. Aleurioconidia are broad and arise terminally and laterally from hyphae or on short stalks. Arthroconidia are also formed in most species in adjacent chains (Sigler, Hambleton, and Paré 2013; Cabañes, Sutton, and Guarro 2014).

Histology is performed on lesions to establish the pathogenic role of any fungi cultured. Proliferation of the fungi occurs within the keratinous epidermis and frequently, invasion of the dermis, underlying muscle and bone results. Granulomas associated with these mycotic infections consist of a central region of fungi, fibrin, and cell debris, surrounded by outer layers of macrophages, and connective tissue. There is frequently

necrosis and ulceration of the epidermis, with secondary bacterial contamination (Paré et al. 2006; Schmidt 2015).

Molecular tools are utilised to establish a diagnosis to species level. Sequencing and PCR usually target the internal transcribed spacer (ITS) gene or SSU rDNA gene (Sigler, Hambleton, and Paré 2013). PCR assays have been developed for a variety of pathogen species. PCR assays have greater sensitivity compared to culture alone and should be utilised to avoid false negative results from culture. (Mitchell and Walden 2013; Bohuski et al. 2015). Molecular speciation should be performed where feasible to allow for increased understanding of the epidemiology of these pathogens.

Specific diagnostic testing parameters have been established for *P. australasiensis*. This fungus is best cultured on mycobiotic agar containing cycloheximide at 30°C (as described by Humphrey, Alexander, and Ha 2016). *P. australasiensis* colonies are powdery yellowish-white and form single-celled conidia laterally on the hyphae. Arthroconidia are absent (Paré and Sigler 2016). As for other fungal species of this grouping, diagnosis should not be made based on morphological characteristics alone. PCR has been used to amplify the ITS region of fungal rRNA. Positive amplicons are subjected to DNA sequencing following procedures described in (Sigler, Hambleton, and Paré 2013).

#### 1.4.2i Loop Mediated Isothermal Amplification

Loop mediated isothermal amplification (LAMP) is a molecular test that has the advantage of being highly specific, rapid and more efficient than conventional PCR. It is performed at a constant temperature allowing equipment to be smaller and portable for field use. This method typically uses four primers that recognise six sequences on the target DNA. DNA is amplified in a stem loop structure and can detect as few as six copies of DNA (Notomi et al. 2000).

Loop mediated isothermal amplification has been used extensively in human diagnostic medicine to detect mycotic pathogens such as *Trichosporon asahii* (Zhou et al. 2015), *Candida spp.* (Inácio, Flores, and Spencer-Martins 2008), *Paracoccidioides brasiliensis*, (Endo et al. 2004) and *Histoplasma capsulatum* (Scheel et al. 2013) with high sensitivity and specificity. Loop mediated isothermal amplification

has not been used previously for the detection of *Nannizziopsis* spp, *Paranannizziopsis* spp or *Ophidiomyces* sp.

### 1.4.3 Transmission

Transmission is hypothesised to be via direct contact with infected individuals, and indirectly through fomites and environmental sources. The mode of transmission has been established for *N. dermatitis* in veiled chameleons and is assumed to be similar in all species of *Nannizziopsis*, *Paranannizziopsis* and *Ophidiomyces*. Transmission for *N. dermatitis* is via direct contact with infected hosts or indirectly via fomites. Abrasions of the skin facilitate infections of *N. dermatitis* and *O. ophiodiicola* but are not essential for infections to establish (Paré et al. 2006; Allender et al. 2011). Transmission via contact with exuvia, containing arthroconidia, in the environment is likely. Fungal spores of all three genera show resistance to environmental temperatures, and while growth is restricted above 37°C and below 15°C, survival can occur following freezing (Rajeev et al. 2009; Masters et al. 2016; Paré and Sigler 2016).

Environmental transmission has been postulated, however there have been few published reports investigating the source of these fungi (Thomas et al. 2002; Bowman et al. 2007; Allender et al. 2011). Environmental transmission of *O. ophiodiicola* is supported by the broad host range and annual infection cycles (Allender, Raudabaugh, et al. 2015). In vitro *O. ophiodiicola* can utilise environmental carbon and nitrogen sources, and can tolerate pH changes, sulphur compounds and low matrix potentials occurring in soil. Current evidence suggests that *O. ophiodiicola* can persist both as an environmental saprophyte and a keratophilic pathogen of reptilian hosts (Allender et al. 2015). One strain of *Nannizziopsis vriesii* has been isolated from soil in California, however no further research on this organism or other related species, as an environmental saprophyte has been undertaken (Paré and Sigler 2016).

For the fungus of primary interest to this thesis, the specific ability of *P. australasiensis* to survive and multiply in varying environmental conditions has not been determined, and our understanding of the role of environmental transmission of this fungal species is extrapolated from other closely related fungal organisms. The mode of transmission

has not been established but is believed to be via direct contact with infected individuals, and indirectly via fomites. Survival of *P. australasiensis* off the host in the environment is strongly suspected based on observations of seasonal variation in prevalence and recurrent *P. australasiensis* infection in individuals following treatment in a captive population (Masters et al. 2016, Pas 2017 pers. comm.).

#### 1.4.4 Clinical presentation

Disease caused by *Nannizziopsis*, *Paranannizziopsis* and *Ophidiomyces* species typically begins with superficial dermatitis. Clinical signs include, dermal colour change, skin flaking, vesicular lesions, exudation, ulceration and necrosis (Mitchell and Walden 2013; Schmidt 2015). Pyogranulomatous disease, causing myositis and osteomyelitis, often results from local invasion of the fungal pathogen. Systemic spread is rare. Pulmonary granulomas in a jewel chameleon (*Furcifer campani*) caused by *N. dermatitis* (Paré et al. 1997) and hepatic granulomas in multiple bearded dragons (*Pagona vitticeps*, *P. barbata*) caused by *N. guarroi*, and *P. australasiensis* (Bowman et al. 2007; Masters et al. 2016) have been reported. On clinical pathology the patient typically has anaemia, dehydration, leucocytosis and hyperglobulinaemia (Mitchell and Walden 2013).

Variation in disease severity for different host species is evident. *Ophidiomyces ophiodiicola* can cause high rates of morbidity and mortality and has been the cause of significant decline in already vulnerable snake populations (Clark et al. 2011; Allender, Raudabaugh, et al. 2015). However, mild disease caused by *O. ophiodiicola* presence in wild hosts exhibiting good body condition has also been documented (Guthrie et al. 2015), and almost full recovery from *O. ophiodiicola* occurred following shed, in experimentally infected captive bred corn snakes (*Pantherophis guttatus*) (Lorch et al. 2015). This suggests that the severity of disease caused by *O. ophiodiicola* is likely variable between individual hosts and host species (Guthrie et al. 2015). In addition, different species of fungi are likely to have differing levels of virulence on their hosts.

In two instances *O. ophiodiicola* has been detected on the skin of asymptomatic snakes (Paré et al. 2003; Bohuski et al. 2015). In a study of the mycoflora of healthy reptile skin *O. ophiodiicola*, was cultured from the skin shed of one out of 127

individuals (Paré et al. 1997). In a more recent report utilising PCR, *O. ophiodiicola* was detected in 6% of asymptomatic individuals (Bohuski et al. 2015). PCR has a greater sensitivity when compared to fungal culture alone, although false positive results may occur if the sequence used is not sufficiently specific to the organism in question. False negatives are likely when culture is used as the only means of diagnosis. These findings suggest that fungal load may be a factor in development of disease, and that asymptomatic carrier animals may play a role in disease transmission (Bohuski et al. 2015). The potential for reptilian hosts to act as asymptomatic carriers in the transmission of pathogens has not been investigated for any species of *Nannizziopsis*, *Paranannizziopsis* or *Ophidiomyces*.

#### **1.4.5 Predisposing Factors to Disease**

Outbreaks of *Nannizziopsis* spp. have been reported, in some cases, following periods of stress, such as inappropriate husbandry, overcrowding and transport (Bowman et al. 2007). Although this fungal group are generally thought to be primary pathogens, this has been proven in only two out of thirteen species of fungi. Caution should be exercised in extrapolating information from one fungal species to another. Disease outbreaks have often involved young, recently transported or wild caught animals, or animals subjected to overcrowding or poor husbandry (Thomas et al. 2002; Bertelsen et al. 2005; Toplon et al. 2013). In an outbreak of *N. dermatitis* in a captive colony of leopard geckos (*Eublepharis macularius*) disease occurred initially in rarer colour morphs prior to spreading to other individuals. It was proposed that these animals were more susceptible to infection due to immune incompetence induced by deliberate inbreeding in captivity for the skin colouration (Toplon et al. 2013). In snakes experimentally infected with *O. ophiodiicola*, an increase in frequency of lesion development occurred in individuals with abraded skin at the inoculation site, compared to those with non-abraded skin, suggesting compromise to the skin integrity increases risk of infection. (Lorch et al. 2015; Allender, Baker, et al. 2015). Other authors have reported disease outbreaks in the absence of any identified predisposing stressors, concluding that this group of fungal pathogens can transmit to non-compromised hosts through direct contact with infected hosts (Bowman et al. 2007; Hellebuyck et al. 2010).

#### 1.4.6 Treatment

There have been few published studies of pharmacokinetics of antifungal medications in reptiles. Most published reports describe the treatment of clinical cases based on response of clinical signs only. Minimum inhibitory concentrations (MICs) have been established for voriconazole, itraconazole, terbinafine and amphotericin B for thirty-two CANV isolates (Van Waeyenberghe et al. 2010). One isolate demonstrated acquired resistance to itraconazole in this study. Treatment should therefore be multimodal and consist of lesion debridement, topical antifungal application and systemic antifungal medication.

Topical medications that have been used to treat mycotic infections are 1% terbinafine (Masters et al. 2016), 10% povidone iodine (Johnson et al. 2011), 2% chlorhexidine (Hellebuyck et al. 2010), and miconazole (Bowman et al. 2007). Due to the high incidence of deeper tissue invasion, topical treatment should be used in combination with systemic medication for resolution.

The triazole antifungal drugs, ketoconazole, itraconazole and voriconazole are most frequently used in systemic antifungal treatment of reptiles. There is a single published case report demonstrating efficacy of ketoconazole against *N. vriesii* infection in two green iguanas (*Iguana iguana*) (Abarca et al. 2008). This drug is no longer commonly used as newer drugs with less associated toxicity are available. Side effects of systemic triazole use are depression, anorexia and mild degenerative hepatopathy. Regular monitoring of liver enzymes to detect hepatic damage including bile acids (BA), aspartate amino transferase, and gamma-glutamyl transferase should occur during treatment (Mitchell and Walden 2013).

Itraconazole has been used to treat superficial mycotic infections with mixed results. There is high mortality associated with itraconazole use due to toxic hepatic effects. Elevations in BA are seen with prolonged treatment with itraconazole, although histological evidence of hepatic damage is often lacking (Van Waeyenberghe et al. 2010). In healthy tuatara, itraconazole administered at 2.5mg/kg once daily for thirteen days resulted in elevations in BA, uric acid and weight loss (Alexander 2017). Doses of itraconazole at 5mg/kg once daily has resulted in clinical resolution in two out of seven bearded dragons (*Pagona vitticeps*) with *Nannizziopsis* sp. infection (Van

Waeyenberghe et al. 2010), and 10mg/kg once daily resolved infections in one out of two jewel chameleons with *N. dermatitis* (Paré et al. 1997). Itraconazole at 10 mg/kg once daily failed to resolve disease caused by *N. guarroi* in two bearded dragons, but 5mg/kg every 48 hours combined with surgical amputation of the effected limb was successful in another (Bowman et al. 2007). Judging the efficacy of itraconazole therapy in these patients is hampered by issues of inter-species variations in pharmacodynamics between reptile species, variation in the fungal pathogen species involved, and the nature of the disease process between cases.

Voriconazole has less reported mortalities from hepatotoxicity, associated with long term treatment regimes, than itraconazole (Van Waeyenberghe et al. 2010) Voriconazole has been used at 10mg/kg once a day in bearded dragons (Van Waeyenberghe et al. 2010) and a girdled lizard (*Cordylus giganteus*) (Hellebuyck et al. 2010) to successfully treat infections with *Nannizziopsis* spp. Individual variation in plasma concentrations of voriconazole, following administration, is high in both bearded dragons and tuatara (Van Waeyenberghe et al. 2010; Alexander 2017). This has also been reported in mammals, and birds (Schmidt et al. 2007; Howard, Hoffman, and Sheth 2008). The limited studies of voriconazole treatment for superficial mycoses in reptiles, show it to be a safer alternative when compared to itraconazole treatment, but with more interspecies and individual variation in absorption and response. Treatment should therefor involve regular assessment of plasma voriconazole concentrations to ensure therapeutic levels are being achieved, and the doses should be adjusted accordingly.

For treatment to be successful optimal immune function is required. Adequate access to ultraviolet B (UVB) light, and provision of optimal temperature and humidity is required for metabolism of systemic anti-mycotic medications and for optimal functioning of reptile immunity. As such all aspects of husbandry should be corrected where required during treatment. Strict quarantine protocols should be implemented to prevent further disease (Schmidt 2015). Suitable antiseptics and disinfectants for *O. ophiodiicola* include; bleach at 3%, quaternary ammonia products, lysol products, CLR, 70% ethanol, and benzalkonium chloride (trigene) (Rzadkowska et al. 2016)

### 1.4.7 *Paranannizziopsis australasiensis*

*Paranannizziopsis australasiensis*, has been found, to date, only in Australia and New Zealand. Infection has been reported in a central bearded dragon (*P. barbata*), aquatic file snakes (*Acrochordus sp.*) and tuatara. All affected animals were captive and housed in zoological institutions (Sigler, Hambleton, and Paré 2013; Masters et al. 2016). Two aquatic file snakes from Melbourne Zoo, in Australia, presented with multifocal skin discolouration and dermal necrosis caused by *P. australasiensis* (Sigler, Hambleton, and Paré 2013). In New Zealand, *P. australasiensis* has been isolated from tuatara and a bearded dragon from Auckland Zoo. The bearded dragon had rapid progression of fatal mycotic dermatitis and disseminated mycotic granulomatous hepatitis. The pathogen was isolated from a frozen biopsy retrospectively. Affected tuatara all had superficial dermatitis and no fatalities were reported (Masters et al. 2016). Isolates have since been obtained from an Australian water dragon (*Intellagama lesueurii*) at the same institution (Pas 2017 pers. comm) and multiple tuatara at Hamilton Zoo, New Zealand (Jensen 2015 pers. comm). All Australian and New Zealand isolates of *P. australasiensis* shared the same genotype. Trade between Auckland Zoo and Hamilton Zoo occurs regularly, however no direct link was found between Melbourne Zoo and New Zealand Zoological Institutions. It is thought the infections occurred independently (Sigler, Hambleton, and Paré 2013).

#### 1.4.7i *Paranannizziopsis australasiensis* in tuatara

Progression of clinical disease with *P. australasiensis* in tuatara occurs more slowly in comparison to other host species. No fatalities have yet been reported. Typical clinical signs include, raised yellow to brown hyperkeratosis and exudation of the epidermis that progress to necrotising ulcerative dermatitis. Histological findings consist of hyperplastic, heterophilic dermatitis with septate branching hyphae. No deep tissue invasion or systemic mycosis has yet been reported in tuatara (Masters et al. 2016). Tuatara have a lower body temperature than most other reptiles (7-24°C) (Cree et al. 1990) which is suspected to contribute to the reduced virulence of *P. australasiensis* observed in tuatara. Optimal growth of *P. australasiensis* in vitro is 25°C to 30°C (Masters et al. 2016).

Treatment with oral itraconazole at 2.5 to 5 mg/kg once daily combined with topical terbinafine, povidone-iodine or silver sulfadiazine, and surgical debridement has

resulted in resolution in all tuatara to date. In some cases, surgical excision alone has been curative. Increased frequency of ecdysis has been observed in infected individuals resulting in clinical improvement. Disease often reoccurs in the same individual over several years of monitoring (Masters et al. 2016). This suggests either; reexposure, incomplete resolution of the infection, incontact conspecifics are capable of carrying the *P. australasiensis* asymptotically, or the fungi is capable of surviving off the host in the environment. To date these factors of *P. australasiensis* epidemiology have not been investigated.

## 1.5 General Biology and Conservation of Tuatara

The tuatara (*Sphenodon punctatus*) is an endemic New Zealand reptile and the only extant species of the order *Rhynchocephalia* (Cree 2014). Originally reported as two species plus an additional extinct species, recent genetic work has shown tuatara to be of a single species with distinct geographic variation (Hay et al. 2010). Tuatara became extinct from mainland New Zealand following the introduction of mammalian predators, such as, rats (*Rattus exulans*, *R. rattus*, and *R. norvegicus*) mustelids (stoats (*Mustela erminea*), weasels (*M. nivalis*) and ferrets (*M. furo*)) and domestic cats (*Felis catus*), which occurred during human settlement starting around 800 years ago (Cree 2014). Tuatara currently survive on thirty-nine predator free islands, thirty-two of these are natural populations and seven exist as a result of island to island translocations. There are also tuatara populations in four recently created mainland reserves free from introduced predators (Gaze 2001). As a result of conservation efforts, pest eradication on many islands, translocations and captive breeding the conservation status of tuatara was reclassified in 1996 on the IUCN red list from rare to least concern (IUCN 1996). In New Zealand they are listed on the New Zealand Threat Classification System as range restricted (Hitchmough et al. 2013).

In 1993 the first tuatara recovery plan was created detailing the conservation management of tuatara. Key conservation aims of this document include, to decrease the risk of rat incursions into remaining rat free islands, conserve at risk genetically distinct populations by translocations and captive breeding, and to establish several

pest free islands by eradication of introduced mammals. Captive breeding of tuatara from Cuvier (Repanga), Red Mercury (Whakau) and Stanley (Kawhiti) islands commenced at Hamilton Zoo and Auckland Zoo following uplifting of adult tuatara from these islands prior to mammalian pest removal. Tuatara from Little Barrier Island (Hauturu) are held in a rat proof enclosure in situ for the purpose of captive breeding and eggs are incubated off site prior to their release. Thirty-nine captive bred tuatara have been released to Cuvier Island between 2001-2010, seventeen have been released on Stanley between 2003 and 2010, thirty-five tuatara have been released onto Red Mercury between 1996 and 2001, and 132 tuatara have been released onto Little Barrier between 2006-2010 (Cree 2014). These populations were historically considered genetically distinct from the Cooks Strait tuatara and were previously described as a sub species *Sphenodon punctatus punctatus*. This has since been revised to represent a geographic variant rather than a true sub-species status (Hay et al. 2010). As a result, these breeding populations are no longer of such high conservation importance. The recent discovery of *P. australasiensis* in these captive populations of tuatara pose a risk to wild tuatara both at the proposed translocation sites and in other wild and captive locations. Previous release of *P. australasiensis* from captivity onto these islands is plausible, as only recently has appropriate diagnostic techniques been developed to identify this pathogen. The risk of pathogen transmission posed by these tuatara needs to be considered against their genetic importance. Release of these captive bred tuatara has currently ceased until more is known about the epidemiology of *P. australasiensis*.

In addition to the wild populations there is a large captive population of tuatara consisting of over 380 individuals, as of 2010. These are located in twenty-two facilities within New Zealand and seven facilities internationally. Most of these captive tuatara originated from Stephens Island stock, with the exception of the small number of individuals originating from diminishing island populations, maintained in captivity for conservation purposes, as discussed previously (Cree 2014; Eyre 2015 pers. comm).

## 1.6 Environmental characteristics of wild tuatara habitat

Tuatara are terrestrial burrowing reptiles. They can utilise a diverse range of habitat from dense forests to pastoral land, but prefer light forest cover, which provides protection from aerial predators such as the Australasian harrier (*Circus approximans*) (Cree 2014). Only one tuatara will inhabit a burrow at a time, however one burrow may be used by multiple tuatara at different times, allowing opportunities for pathogen transmission between individuals. Frequently on offshore islands the burrows of sea birds are utilised by tuatara (Walls 1978). Tuatara are primarily nocturnal, although individuals within forest habitat are frequently active for periods during the day to bask in patches of sunlight. Diurnal activity in tuatara from pastoral areas is observed less frequently, they may lay concealed in a burrow entrance for basking purposes but rarely come too far from the entrance. (Gillingham and Miller 1991).

On Stephen's Island where tuatara populations flourish, the mean ambient temperature ranges from 11 - 24°C. Temperatures have been recorded to be as low as 1°C and as high as 30°C (Blanchard 2002). Wild tuatara are able to avoid extremes in temperature by remaining in the more controlled environment of the burrow and decreasing activity. Tuatara exhibit short periods of torpor over winter during cold weather remaining in the burrow during this time (Cree 2014). Humidity is high on most islands inhabited by tuatara, and heavy rainfall is recorded at least every second week (Blanchard 2002). Humidity on Stephens Island was recorded in one study to vary between 48% and 100%, with high levels at or near 100% being most common. Tuatara in this study were found to be most active at higher levels of humidity and this behaviour is suspected to be a method of water conservation (Corkery, Bell, and Nelson 2014).

## 1.7 Captive Husbandry of Tuatara

The management of tuatara in captivity can create increased opportunities for pathogen transmission and multiplication. This can be through increased host animal density, prolonged contact with contaminated habitats, environmental conditions that

favour pathogen reservoirs, or sub-optimal husbandry conditions that reduce host immunity (Schumacher 2003, Harvey et al. 2010, Jakob-Hoff 2014). Husbandry recommendations are based on emulating the animal's natural environment, to provide conditions to which the animal is adapted. However, ideal husbandry and dietary requirements are not always known (Goetz and Thomas 1994; Burgess, Gartrell, and Blanchard 2009) and the closed environment of captivity prevents the animal from removing itself from unfavourable conditions, which may result in stress, immune compromise and disease (Huntingford et al. 2006; Mason 2010; Jakob-Hoff 2014).

Skin health in tuatara, as in other reptiles, is dependent on a variety of intrinsic and extrinsic factors (Hellebuyck et al. 2012). Extrinsic factors include environmental variables such as adequate UVB exposure, and exposure to a preferred optimal temperature and humidity range that will encourage regular ecdysis (Hellebuyck et al. 2012, Harvey et al. 2010). Tuatara, like other reptile species require direct exposure to UVB light, as dietary absorption of vitamin D is variable (Burgess, Gartrell, and Blanchard 2009). Inadequate UVB exposure can result in hypocalcaemia and nutritional secondary hyperparathyroidism (Burgess, Gartrell, and Blanchard 2009). Exact UVB light needs are not known, but the average intensity of UVB light at ground level on a cloudy day is 56  $\mu\text{W}/\text{cm}^2$  and this level has been recommended for tuatara housed indoors (Burgess, Gartrell, and Blanchard 2009). Outdoor enclosures generally have a fine wire-mesh roof to allow for adequate UVB entry, whilst preventing access to predators. Basking areas are provided near burrow entrances in areas that receive morning sunlight as this is the preferred basking time and location for adult tuatara (Blanchard 2002).

The preferred body temperature for tuatara is 20-22°C. Activity in wild tuatara on Stephens Island has been observed between 6-30°C. Under laboratory conditions loss of movement occurred below 4°C, and observable heat stress was recorded for most tuatara at temperatures 28°C and above (Cree 2014). In captivity temperature is routinely maintained between 4-15°C in winter and 10-25°C in summer. These temperatures are based on natural temperatures occurring on Stephens Islands, the source of most captive tuatara. Where temperatures over 30°C naturally occur, cooling by sprinkler systems are used to prevent heat stress (Blanchard 2002). Seasonal

variation in temperature, humidity and photo-period is provided, where possible, for indoor enclosures to allow animals to go through natural periods of torpor and reproductive activity (Blanchard 2002). Maintenance of the captive environment with the normal thermal range of tuatara enables optimal growth, metabolism and immune function (Jakob-Hoff 2014).

To enable normal ecdysis and hydration and to stimulate the natural environment, humidity is kept high (85-95%), by misting enclosures and the provision of leaf litter and plants. Outdoor enclosures usually have adequate ventilation, but indoor enclosures can become stagnant and additional active ventilation may be required to prevent high loads of fungal saprophytes developing within the enclosure. Ventilation of an enclosure is considered sufficient if the air is continuously moving (Blanchard 2002). The substrate should be well drained soft soil to allow for burrow formation. Artificial wooden burrows should also be supplied. Large shallow baths, which can be easily climbed out of, should be provided for hydration and to aid with ecdysis (Blanchard 2002). While many of these environmental conditions favour immunity and skin health in tuatara (Jakob-Hoff 2014), they can also provide a rich environment for the growth of saprophytic fungi (Coppock and Cookson 1951, Block 1953).

### **1.7.1 Diet**

One of the most important intrinsic factors in tuatara immunity and skin health is nutrition. However, the exact dietary needs for captive tuatara are unknown (Burgess, Gartrell, and Blanchard 2009). Wild tuatara consume a diverse range of invertebrate species, including tree weta (*Hemideina sp.*) and darkling beetles (*Mimopeus sp.*) (Cree 2014). Small lizards and the eggs and nestlings of small species of seabirds, such as fairy prions (*Pachyptila turtur*), are also consumed (Blanchard 2002). Captive tuatara should be provided with a mixed invertebrate diet including weta. Many captive institutions also add meat sources such as pinkie mice, mutton birds (*Puffinus griseus*) and ox heart to the diet (Blanchard 2002; Burgess, Gartrell, and Blanchard 2009). However, when these diets were analysed for their nutritional content deficiencies in calcium and vitamin D were found, and the addition of a reptile vitamin and mineral supplement powder was recommended (Burgess, Gartrell, and Blanchard 2009). While current information on the nutritional status of captive tuatara is limited, from Burgess (et al. 2009) we can conclude that many captive tuatara will have sub-optimal

nutrition, potentially increasing their susceptibility to pathogens such as *P. australasiensis*.

## 1.8 Research aims and thesis structure

The primary aim of this research was to investigate the epidemiology of *P. australasiensis* in New Zealand wild and captive endemic reptiles. This was achieved by investigating 4 major research questions:

- 1) Is *P. australasiensis* present in wild populations of tuatara? In particular, is *P. australasiensis* present on islands associated with the captive breed and release programmes for tuatara?
- 2) Is *P. australasiensis* present in tuatara in other captive facilities in New Zealand?
- 3) Can *P. australasiensis* infect other species of endemic New Zealand reptiles?
- 4) Describe the pathology of *P. australasiensis* in tuatara.

The hypothesis for this research is that *P. australasiensis* is of captive origin, introduced to New Zealand and tuatara through exotic reptile importation. It is possible that this disease has been released into the wild through captive breed and release programmes.

This thesis is organised into four chapters. This first chapter provides a review of fungal diseases of significance to wildlife globally, mycotic disease in reptiles, both exotic and native to New Zealand, and a brief overview of tuatara conservation and biology. The second chapter investigates the prevalence of *P. australasiensis* in tuatara and other endemic New Zealand reptiles in nine zoological captive centres, six wild island populations and two translocated eco-sanctuary populations. The third chapter investigates the pathology of *P. australasiensis* in both captive and wild tuatara. The final chapter presents a discussion on findings and addresses the conservation implication of these findings for the future management of tuatara populations.

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## CHAPTER TWO

### The prevalence of *Paranannizziopsis australasiensis* in New Zealand reptiles and environmental sources



## 2.1 Introduction

New Zealand is home to a unique assemblage of terrestrial reptiles most of which are found only in New Zealand. The current literature lists 102 species of lizards of the families *Diplodactylidae* and *Scincidae* and the tuatara (*Sphenodon punctatus*). 36% of these species are categorised as threatened or endangered, due to habitat loss and predation. Many of these reptile species have not yet been fully described (Jewell and Morris 2008; Hitchmough R et al. 2016). The tuatara is the only extant member of the order *Rhynchocephalia*. Following the introduction of mammalian predators approximately 800 years ago they became extinct across mainland New Zealand. They currently survive on thirty-nine off-shore islands and four predator-proof mainland ecosanctuaries. The conservation management of tuatara has involved pest eradication from off-shore islands and subsequent translocations of tuatara to these islands where they have flourished (Gaze 2001). In 1996 the conservation status of tuatara was changed from rare to least concern (IUCN 1996). The population is currently stable but restricted to many isolated offshore islands.

Recently the mycotic pathogen, *Paranannizziopsis australasiensis*, of the family *Onygenaceae*, order *Onygenales*, has been reported at two captive facilities in New Zealand (Masters, Alexander, et al. 2016, Jensen 2015 pers. comm). *P. australasiensis* is a member of a group of emerging mycotic pathogens of reptiles, formally known as the *Chrysosporium* anamorph of *Nannizziopsis vriesii*. To date, it has only been reported in captive reptiles from Australia and New Zealand, In New Zealand it has been isolated from multiple tuatara and a bearded dragon (*Pogona vitticeps*). Disease is reported to be mild in captive tuatara causing a slowly progressive superficial dermatitis that may extend to ulceration and necrosis. Clinical signs typically appear as yellow-brown skin discolouration and raised crusting nodules that are found on the ventrum, gular and tail. Lesions respond to debridement and treatment with topical terbinafine and systemic itraconazole. Progression of disease was rapid in the bearded dragon and death occurred from systemic dissemination (Sigler, Hambleton, and Paré 2013, Masters et al. 2016).

*Paranannizziopsis australasiensis* is closely related to the fungal pathogens *Ophidiomyces ophiodiicola* and *Nannizziopsis guarroi*. *Ophidiomyces ophiodiicola* has been identified as an agent responsible for population declines of wild snakes across the USA and Canada (Sigler, Hambleton, and Paré 2013; Lorch et al. 2015). *Nannizziopsis guarroi* is the aetiology of yellow fungus disease in captive Inland beaded dragons in North America and Europe and causes significant morbidity and mortality in this and other captive reptile species (Bowman et al. 2007; Sigler, Hambleton, and Paré 2013). Other emerging fungal pathogens such as *Batrachochytrium dendrobatidis*, the cause of chytrid disease in amphibians (Skerratt et al. 2007), and *Pseudogymnoascus destructans*, the cause of white-nose syndrome in bats (Blehert et al. 2009), highlight the devastating impacts fungal pathogens can have on wild populations. The discovery of *P. australasiensis* in tuatara in New Zealand is of concern for the health of captive and wild endemic New Zealand reptiles, many of which exist in small populations in remote isolated areas.

The two captive institutes which have reported *P. australasiensis* have been responsible for breed for release programs of genetically distinct tuatara for over twenty years. The origin of these infections and the prevalence of this organism in wild and captive reptile populations in New Zealand is currently unknown. We also have no knowledge of potential reservoirs for this fungus in other reptiles or environmental sources. The detection of this disease has resulted in a temporary cessation of tuatara translocations from captive institutes to the wild. There is concern that *P. australasiensis* may have already been translocated to the wild with its host. This study details the first report of active surveillance for *P. australasiensis* in New Zealand reptiles, in both captive and wild populations. I also investigate potential risk factors associated with the presence of this organism on reptile skin and its role in dermatitis in tuatara and other reptiles in New Zealand.

## 2.2 Materials and Methods

### 2.2.1 Location, capture and sample technique

A cross sectional survey was conducted with a total of 468 tuatara from six islands (Stanley Island (Kawhitu), Cuvier Island (Repanga), Hen (Taranga) Island, Coppermine Island, North Brothers Island and Lady Alice Island), two mainland ecosanctuaries (Zealandia and Cape Sanctuary), and nine captive facilities (Rainbow Springs Kiwi Encounter, Rotorua; Pukaha Mount Bruce, Masterton; Nga Manu Nature Reserve, Waikanae; Wellington Zoo, Wellington; Zealandia, Wellington; Victoria University, Wellington; Orana Wildlife Park, Christchurch; Southland Museum, Invercargill; and Kiwi Birdlife Park, Queenstown). For the purposes of this study, captive facilities are numbered randomly from one to nine for confidentiality. Sampling took place between February 2016 and March 2017 and excluded the winter months due to seasonal torpor of tuatara. Wild locations were selected to include populations with distinct genetic differences, and to capture captive bred, translocated and original wild populations. Captive populations were selected to represent a range of temperate zones across New Zealand. A total of seventy seven endemic New Zealand skinks of nine different species (*Oligosoma otagense*, *Oligosoma infrapunctatum*, *Oligosoma zelandicum*, *Oligosoma polychroma*, *Oligosoma aenea*, *Oligosoma lineocellatum*, *Oligosoma ornatum*, *Oligosoma alani* and *Oligosoma smithi*) and 122 endemic New Zealand geckos, of eight different species (*Dactylocnemis pacificus*, *Hoplodactylus duvaucelii*, *Hoplodactylus granulatus*, *Naultinus elegans*, *Naultinus rudis*, *Naultinus manukanus*, *Woodworthia chrysosireticus*, and *Woodworthia maculatus*) were also sampled opportunistically from these locations.

Table 2.1. Number of reptiles, organised by family, tested for *P. australasiensis* by LAMP and culture at each location. Numbers in brackets indicates number of culture samples where it differs from the number of LAMP samples.

	<i>Sphenodontidae</i>	<i>Diplodactylidae</i>	<i>Scincidae</i>
Stanley Island	0	26	29
Cuvier Island	11	38	4
Hen Island	62 (6)	0	0
Coppermine Island	65 (5)	0	0
Lady Alice Island	69 (8)	0	0
North Brothers	60 (2)	0	0
Zealandia	42	0	10
Cape Sanctuary	27	0 (1)	6 (28)
Facility 1	0 (8)	0 (5)	0 (5)
Facility 2	0 (25)	0	0
Facility 3	5	0	0
Facility 4	25	19	0
Facility 5	13	10	0
Facility 6	30	0	0
Facility 7	9	8	0
Facility 8	8	15	0
Facility 9	9	0	0
Total	435(233)	117 (122)	50 (77)

Wild tuatara were caught by hand at night. Skinks and geckos were caught opportunistically in locations with known tuatara populations by hand and using pit fall traps. Animals were held for up to one hour during sampling and were released back to the site of capture. Any dermatitis lesions were photographed and characterised for severity as mild (superficial skin discolouration or skin flaking), moderate (ulcerative or exudative lesions), or severe (deep invasion into tissues beneath the dermis).

Skin swabs were collected from all animals using a sterile cotton tip applicator moistened with sterile 0.9% saline rubbed vigorously over the ventral surface of the animal. Attention was paid to swabbing any skin lesions on the reptile. Cotton tip applicators were placed into sterile plastic sampling bags (Whirl-pak, Thermo Fisher

Scientific, Auckland, NZ) or sterile specimen containers and stored chilled (approximately 3°C) until time of processing. Where lesions were present portions of scales or exudate were removed and placed into a sterile sampling bags. The samplers' hands were cleaned with 70% ethanol between handling and equipment was cleaned with a disinfectant containing; n-alkyl dimethyl benzyl ammonium chloride, didecyl dimethyl ammonium chloride, and poly biguanide hydrochloride (SteriGene, Ethical Agents Ltd, Auckland) to prevent cross-contamination of samples and spread to other individuals.

### **2.2.2 Culture and PCR**

233 tuatara samples, and all gecko (122) and skink (77) samples were cultured for order *Onygenales* fungal isolates on mycobiotic agar containing cycloheximide at 30°C, as described by Humphrey, Alexander, and Ha 2016. Any colonies resembling *P. australasiensis* were subsequently analysed using a generic fungal polymerase chain reaction (PCR) which amplified the internal transcribed spacer (ITS) region of fungal rRNA using primers ITS1 and ITS4, as described by Humphrey, Alexander, and Ha 2016. Culture and molecular testing are performed at the Animal Health Laboratory (Ministry for Primary Industries, 66 Ward Street, Wallaceville, Upper Hutt, New Zealand). A subset of the total wild tuatara samples was selected for culture and PCR on the basis of presence of dermatitis lesions. Testing was limited by time and cost constraints.

### **2.2.3 Loop mediated isothermal amplification**

A loop mediated isothermal amplification (LAMP) was developed and performed by the Institute of Fundamental Sciences, Massey University, Tennent Drive, Palmerton North, New Zealand. 435 tuatara samples, 117 gecko samples, and fifty skink samples were analysed via LAMP for *P. australasiensis*. Samples not analysed by LAMP were collected prior to the tests development. LAMP primers were designed based on comparisons of whole mitochondrial genome sequences of *P. australasiensis*, *P. californiensis* and eleven other representative fungi of the *Onygenales* (Winkworth et al., unpubl.). The primer set targeted a portion of the intergenic spacer between the genes of the ATP synthase F0 subunit six gene (*atp6*) and that encoding the tryptophan tRNA (tRNA-trp). Comparison of this intergenic spacer, which is 1487 nucleotides long in *P. australasiensis*, between the two representatives of

*Paranannizziopsis* indicates numerous nucleotide substitutions and several length differences between them. In particular, a section of 162 aligned nucleotides that contained two length differences (one of twelve nucleotides and one of sixty-three) and nineteen nucleotide substitutions was selected as a target for primer development (Winkworth et al., unpubl.).

LAMP amplification involves four to six oligonucleotide primers. LAMP primers for identification of *P. australasiensis* were designed using PrimerExplorer V4 (Eiken Chemical; <https://primerexplorer.jp/e/index.html>). The final primer set included external (commonly denoted F3 and B3) and internal (FIP and BIP) primer pairs as well as two loop primers (LF and LB) (Winkworth et al., unpubl.). Optimal reaction conditions as well as specificity and sensitivity were evaluated using a combination of PCR and LAMP amplification.

LAMP tests were conducted 25 µl reaction volumes containing 15 µl Isothermal Master Mix (Optigene ISO-DR001) along with 5 pM primers F3 and B3, 30 pM primers FIP and BIP and 10 pM primers LF and LB. An aliquot of total DNA extract (containing approximately 5 ng DNA) was added and the final volume adjusted to 25 µl using H<sub>2</sub>O. DNA was extracted from skin swabs and tissue samples using the Roche High Pure Template Preparation Kit and from soils using the Macherey-Nagel NucleoSpin Soil Kit; in both cases standard manufacturer recommendations were followed. Negative (adjusted to the 25 µl reaction volumes using only water) and positive (containing 5 ng of *P. australasiensis* DNA) controls were run with each set of LAMP reactions. Reaction mixtures were incubated at 60°C for 30 minutes using either a Diagenetix Smart-DART or BioRanger device. The Isothermal Master Mix contains a fluorescent FAM dye, the incorporation of which, and hence the test result, was monitored in real-time by the LAMP device. Additionally, for a subset of LAMP experiments results were confirmed visually following electrophoresis of reaction products on 1-2% (w/v) agarose-TAE gels at 100V for 60 minutes.

#### **2.2.4 Sex and age determination**

The sex and age of all reptiles were recorded where possible. Adult tuatara exhibit sexual dimorphism. The male is much larger, has a more prominent dorsal crest, and a narrower face and body (Cree 2014). An adult was defined as any individual where

the sex could easily be identified based on physical characteristics. The sex of captive juveniles was recorded when it had previously been determined by incubation temperature (Nelson et al. 2004) or laparoscopy (Cree 2014). The sex of wild caught juvenile tuatara was not able to be identified. The sex of adult geckos was distinguished by the presence (male) or absence (female) of a bulge at the tail base created by the inverted hemipenes (Todd 2003) Juvenile geckos were defined as an individual where the sex was not easily distinguished and measuring two thirds or less the snout-vent length of an adult of that species. Skinks do not exhibit external sexual dimorphism and the sex of individuals was not able to be determined. Juvenile skinks were defined as individuals measuring two thirds or less of the snout-vent length of the adult of the species.

### **2.2.5 Soil samples**

A pilot study was conducted with soil from the burrows of three captive tuatara with active *P. australasiensis* infections confirmed prior to this study by PCR and culture and not on medication at the time of sampling. Soil was collected from the entrance, middle and deep within the burrow. Approximately one teaspoon of top soil was collected into a sterile specimen container and stored chilled (3°C) until analysis. Soil samples were tested by LAMP for presence of *P. australasiensis* DNA. During the survey of reptiles eighty soil samples were collected from the entrance of tuatara burrows from two captive facilities, Facility 2 and Facility 4, and four wild sites, Stanley Island, Cuvier Island, Cape Sanctuary and Zealandia. Where burrows were not present, (e.g. in gecko enclosures) samples of top soil from high use areas were collected.

### **2.2.6 Statistical Methods**

Binomial logistic regression was used to assess risk factors associated with the presence or absence of *P. australasiensis* based on LAMP test results and the presence or absence of dermatitis in the reptiles examined. The risk factors assessed included animal location, management type (captive, wild, or ecosanctuary), reptile family, sex and age. Following this initial full model, a reduced model logistic regression was developed after manually evaluating possible confounding and interacting factors (Dohoo, Martin, and Stryhn 2009). For tuatara only, binomial logistic regression was then used to assess risk factors for detection of *P. australasiensis* by

LAMP test and again for the presence of dermatitis. Chi-squared analysis was carried out on selected parameters to assess frequency distributions.

## 2.3 Results

### 2.3.1 Tuatara

#### 2.3.1.i Culture and PCR

Two positive *P. australasiensis* samples were detected by culture and subsequent PCR from 233 tuatara samples (test prevalence = 0.86%). These were single animals from two separate captive institutes. No wild tuatara were positive on culture. Both culture positive tuatara had severe lesions present with multifocal mycogranulomas, and deep myositis. One tuatara also had associated osteomyelitis. The pathology of these cases is discussed in more detail in chapter 3. No culture positives were detected in individuals with moderate, mild or no lesions.

#### 2.3.1.ii LAMP

A total of sixty-five out of 435 tuatara tested by LAMP were positive for *P. australasiensis*, across all locations giving a test prevalence of 14.9%. Tuatara exist in isolated populations and as such each location was further evaluated separately. *Paranannizziosis australasiensis* was detected by LAMP in tuatara from four out of five wild populations, one out of two ecosanctuaries and four out of seven captive facilities (Figure 2.1). Prevalence varied between populations (Table 2.2) and location was identified as significant factor in both the frequency analysis and the logistic regression with significant differences in the odds ratios between locations (Table 2.6). Both tuatara that were positive on culture and PCR also tested positive on LAMP. No tuatara were found on Stanley Island, and samples from facilities 1 and 2 were not tested by LAMP due to processing prior to the tests development.

Table 2.2. LAMP results for *Paranannizziopsis australasiensis* in tuatara by location within New Zealand.

Location	n/N	Prevalence	95% C.I.
Zealandia	0/42	0	-
Cape Sanctuary	10/27	37%	22.7 – 51.3%
Coppermine Island	4/65	6.1%	1.4 – 10.8%
Cuvier Island	0/11	0	-
Hen Island	16/62	25.8%	20.6 – 36 %
Lady Alice Island	7/69	10.1%	2.9 – 17.3%
North Brothers	19/60	31.7%	20.9 – 43.0%
Facility 3	0/5	0	-
Facility 4	4/25	14.2%	-
Facility 5	0/13	0	-
Facility 6	2/30	6.7%	0 – 13.7%
Facility 7	0/9	0	-
Facility 8	1/8	12.5%	-
Facility 9	4/9	44.4%	-

\*No 95% CI is provided where all animals at a facility were sampled  
n = number positive. N = number tested C.I = confidence interval

### 2.3.1iii Dermatitis

112 tuatara were reported to have lesions consistent with mild to severe dermatitis. Only seventeen of these tuatara were positive for *P. australasiensis* by LAMP test. In the remaining ninety-five tuatara with lesions *P. australasiensis* was not detected. Of the sixty-five LAMP positive tuatara seventeen had lesions present. Fourteen had lesions characterised as mild, one had moderate lesions and two had severe multifocal lesions. 73.8% (n= 48) tuatara testing positive to *P. australasiensis* on LAMP had no lesions present.

### 2.3.2 Geckos and Skinks

There were no positive culture results for *P. australasiensis* in any of the 199 geckos or skinks sampled. On LAMP testing, *P. australasiensis* was detected in 30/167 (18.8%) geckos and skinks with positive results from four out of four wild and ecosanctuary sites and three out of four captive facilities. Mild dermatitis lesions were reported in six captive, and two wild geckos. No lesions were seen in any skink from any management type. No LAMP positive geckos or skinks had any skin lesions or other detectable health compromise. The reptile species that *P. australasiensis* was detected in include: Green Gecko (*N. elegans*), Pacific Gecko (*D. pacificus*), Common Gecko (*W. maculatus*), Duvaucel's Gecko (*H. duvaucelii*), Gold-striped Gecko (*W. chrysosireticus*), Forest Gecko (*H. granulatus*), Speckled Skink (*O. infrapunctatum*), Shore Skink (*O. smithi*), Spotted Skink (*O. lineocellatum*), Ornate Skink (*O. ornatum*), Copper Skink (*O. aenea*), and Brown Skink (*O. polychroma*) (Table 2.3).

Table 2.3. LAMP results for *Paranannizziopsis australasiensis* in New Zealand geckos and skinks by species and location.

<i>Location</i>	<i>Species</i>	<i>n/N</i>	<i>Species prevalence</i>	<i>Location prevalence</i>
<i>Cuvier Island</i>	<i>D. pacificus</i>	9/11	81.8%	28.9%
	<i>W. maculatus</i>	2/27	7.4%	
	<i>O. smithi</i>	0/4	0	0
<i>Stanley Island</i>	<i>H. duvaucelii</i>	1/26	3.8%	3.8%
	<i>O. smithi</i>	2/27	7.4%	6.9%
	<i>O. aenea</i>	0/1	0	
	<i>O. alani</i>	0/1	0	
<i>Zealandia</i>	<i>O. zelandicum</i>	1/4	25%	40%
	<i>O. polychroma</i>	0/2	0	
	<i>O. aenea</i>	1/1	100%	
	<i>O.</i>	1/1	100%	
	<i>lineocellatum</i>	1/2	50%	
	<i>O. ornatum</i>			
<i>Cape Sanctuary</i>	<i>O. infrapunctatum</i>	4/6	66.7%	66.7%
<i>Facility 4</i>	<i>N. elegans</i>	0/5	0	0%
	<i>H. granulatus</i>	0/10	0	
	<i>W.</i>	0/2	0	
	<i>chrysoisireticus</i>	0/1	0	
	<i>D. pacificus</i>			
<i>Facility 5</i>	<i>N. elegans</i>	2/3	66.7%	40%
	<i>N. rudis</i>	0/1	0	
	<i>N. manukanus</i>	0/1	0	
	<i>H. granulatus</i>	1/1	100%	
	<i>W. maculatus</i>	0/3	0	
	<i>W.</i>	1/1	100%	
<i>Facility 7</i>	<i>chrysoisireticus</i>			
	<i>N. elegans</i>	1/4	25%	25%
	<i>H. granulatus</i>	0/2	0	
<i>Facility 8</i>	<i>H. duvaucelii</i>	1/2	50%	
	<i>N. elegans</i>	2/12	16.7%	13.3%
	<i>H. granulatus</i>	0/3	0	

n = number positive. N = number tested

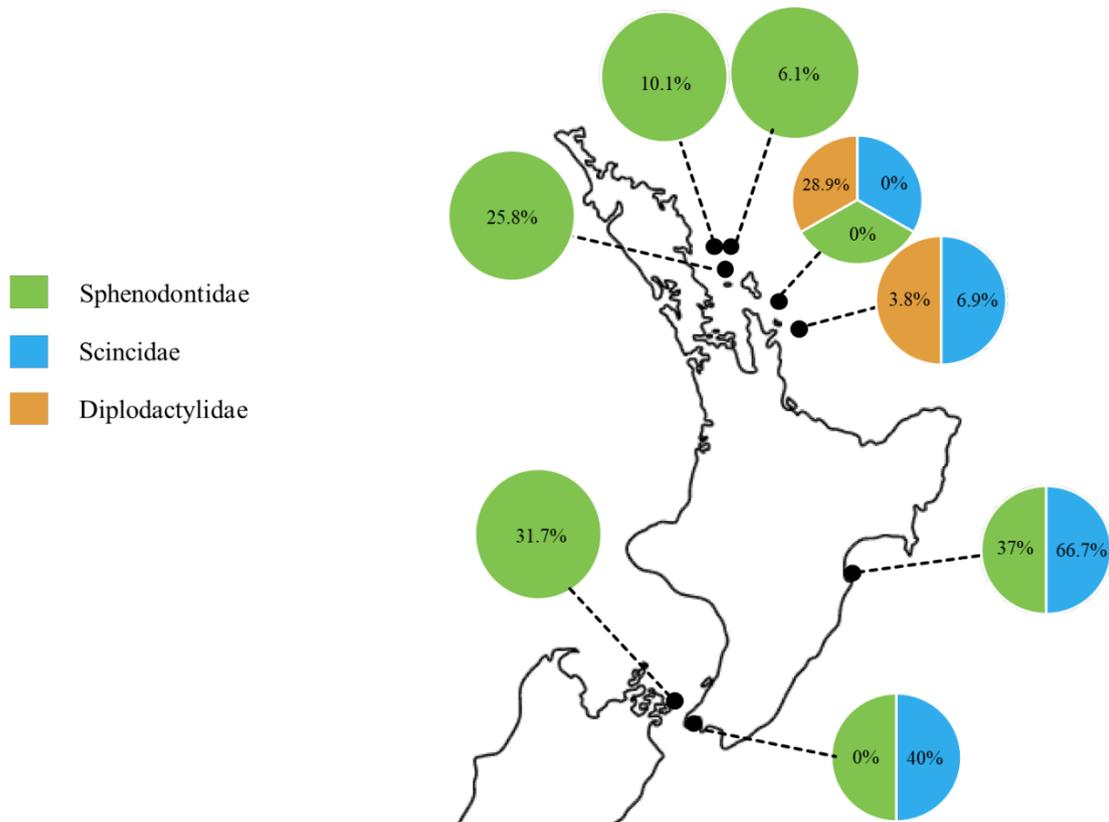


Figure 2.1. Prevalence of *Paranannizziopsis australasiensis* for tuatara, geckos and skinks at wild and ecosanctuary locations by LAMP test.

### 2.3.3 Association between *Paranannizziopsis australasiensis* presence and dermatitis

#### 2.3.3.i All reptiles combined

The total number of reptiles tested for *P. australasiensis* by LAMP and examined for clinical signs of dermatitis was 551 (Table 2.4). There was no significant association in the frequency of dermatitis lesions with a positive result on the *P. australasiensis* LAMP test (Chi-square 1.314, df = 1, p = 0.252).

Table 2.4. Contingency table for presence or absence of dermatitis (lesions) and the result of the LAMP analysis for *Paranannizziopsis australasiensis* in all reptiles studied

Lesions	PA LAMP		Total
	Negative	positive	
No	354	75	429
Yes	106	16	122
Total	460	91	551

### 2.3.3.ii Tuatara only

The total number of tuatara tested for *P. australasiensis* and examined for clinical signs of dermatitis was 437 (Table 2.5). There was no significant association in the frequency of dermatitis lesions with a positive result on the *P. australasiensis* LAMP test (Chi-square 0.016, df = 1, p = 0.901).

Table 2.5. Contingency table for presence or absence of dermatitis (lesions) and the result of the LAMP analysis for *Paranannizziopsis australasiensis* in tuatara only.

Lesions	PA LAMP		Total
	Negative	positive	
No	277	48	325
Yes	96	16	112
Total	373	64	437

## 2.3.4 Analysis of risk factors for presence of *Paranannizziopsis australasiensis*

### 2.3.4.i All reptiles combined

The results of a binomial logistic regression assessing potential predictors for the presence of *P. australasiensis* by LAMP test in all reptiles examined showed that lesion presence (p = 0.832), sex (p = 0.287) were non-significant factors and these were removed from the reduced model. Initially age cohort was included as a significant predictor but examination of the odds ratios of this factor revealed that this was due to a group of reptiles of unknown age. Therefore, this predictor was also removed from the final reduced model (Table 2.6). Significant predictors included in

the reduced model were location and reptile family. Assessment of the reduced model showed a Chi-square = 59.560, df = 16,  $p < 0.001$  compared to the null model with a -2 log likelihood ratio of 462.042 and the model predicted 84.7% of results correctly.

Table 2.6. Results of a reduced model binomial logistic regression for risk factors associated with a positive *P. australasiensis* LAMP test result in all reptiles in this study. Statistically significant predictors compared to the reference factor are in **bold**.

	B	S.E.	Wald	Df	Sig.	Odds Ratio	95% C.I. for Odds Ratio	
							Lower	Upper
<i>Location</i>								
Zealandia			46.620	14	0.000			
<b>Cape Sanctuary</b>	<b>2.463</b>	<b>0.691</b>	<b>12.713</b>	<b>1</b>	<b>0.000</b>	<b>11.741</b>	<b>3.032</b>	<b>45.470</b>
Coppermine Island	0.369	0.807	0.210	1	0.647	1.447	0.298	7.032
Cuvier Island	0.500	0.762	0.431	1	0.512	1.648	0.370	7.335
<b>Hen Island</b>	<b>1.973</b>	<b>0.688</b>	<b>8.236</b>	<b>1</b>	<b>0.004</b>	<b>7.195</b>	<b>1.870</b>	<b>27.689</b>
Lady Alice Island	0.913	0.737	1.533	1	0.216	2.491	0.587	10.564
<b>North Brothers Island</b>	<b>2.188</b>	<b>0.677</b>	<b>10.448</b>	<b>1</b>	<b>0.001</b>	<b>8.919</b>	<b>2.366</b>	<b>33.619</b>
Stanley Island	-1.419	0.838	2.865	1	0.091	0.242	0.047	1.251
Facility 3	-18.109	23205.422	0.000	1	0.999	0.000	0.000	
Facility 4	-0.022	0.853	0.001	1	0.979	0.978	0.184	5.207
Facility 5	0.758	0.868	0.764	1	0.382	2.135	0.390	11.692
Facility 6	0.455	0.959	0.225	1	0.635	1.576	0.240	10.329
Facility 7	0.224	1.020	0.048	1	0.826	1.251	0.169	9.239
Facility 8	0.120	0.941	0.016	1	0.899	1.127	0.178	7.126
Facility 9	1.841	1.014	3.300	1	0.069	6.304	0.865	45.955
<i>Reptile Family</i>								
Sphenodontidae			11.771	2	0.003			
<b>Diplodactylidae</b>	<b>1.417</b>	<b>0.540</b>	<b>6.882</b>	<b>1</b>	<b>0.009</b>	<b>4.127</b>	<b>1.431</b>	<b>11.899</b>
<b>Scincidae</b>	<b>1.841</b>	<b>0.604</b>	<b>9.285</b>	<b>1</b>	<b>0.002</b>	<b>6.304</b>	<b>1.929</b>	<b>20.605</b>
<i>Constant</i>	<i>-3.094</i>	<i>0.620</i>	<i>24.903</i>	<i>1</i>	<i>0.000</i>	<i>0.045</i>		

B = coefficient for the constant, S.E = standard error, Df = degrees of freedom, Sig = significance level, C.I = confidence interval

The frequency distributions of the identified predictors of a positive *P. australasiensis* by LAMP test in all reptiles examined are presented for location (Figure 2.2) and reptile family (Figure 2.3).

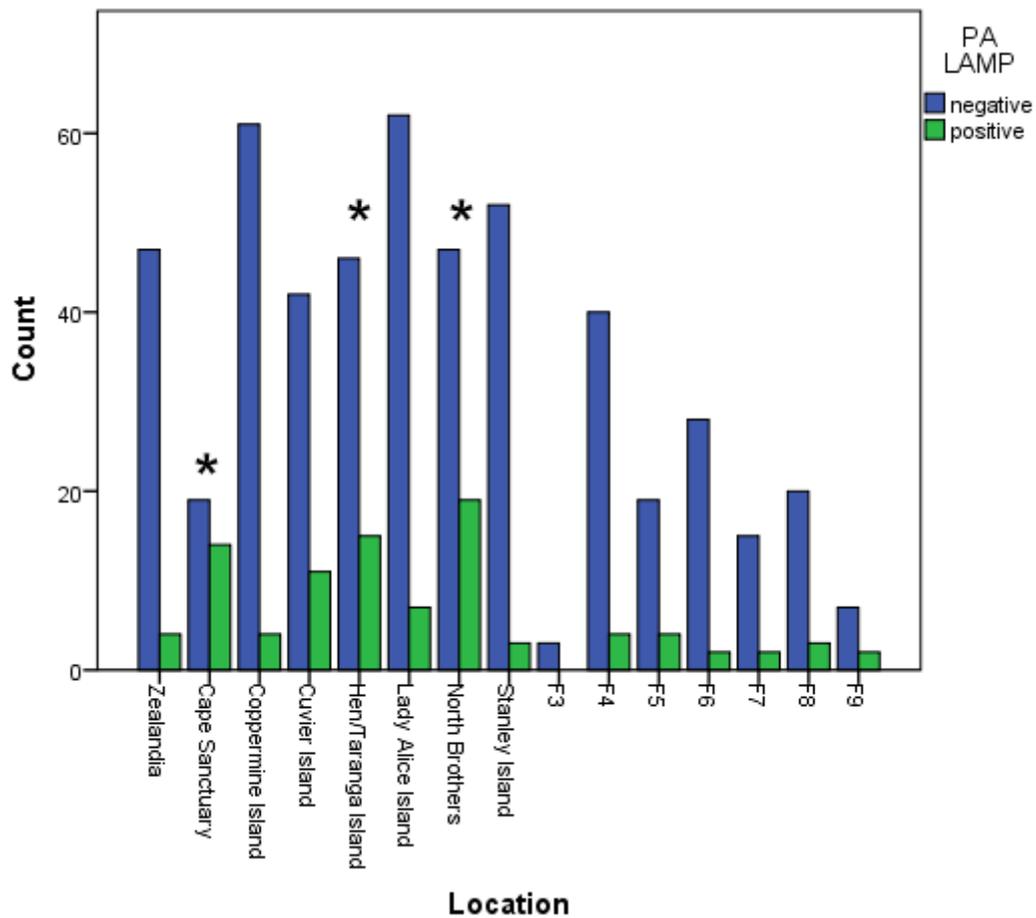


Figure 2.2. Frequency of *P. australasiensis* detection in all reptiles in the study by LAMP test in different locations in New Zealand. Locations with significantly increased risk of detection to the reference factor (Zealandia) are marked by an asterisk (\*).

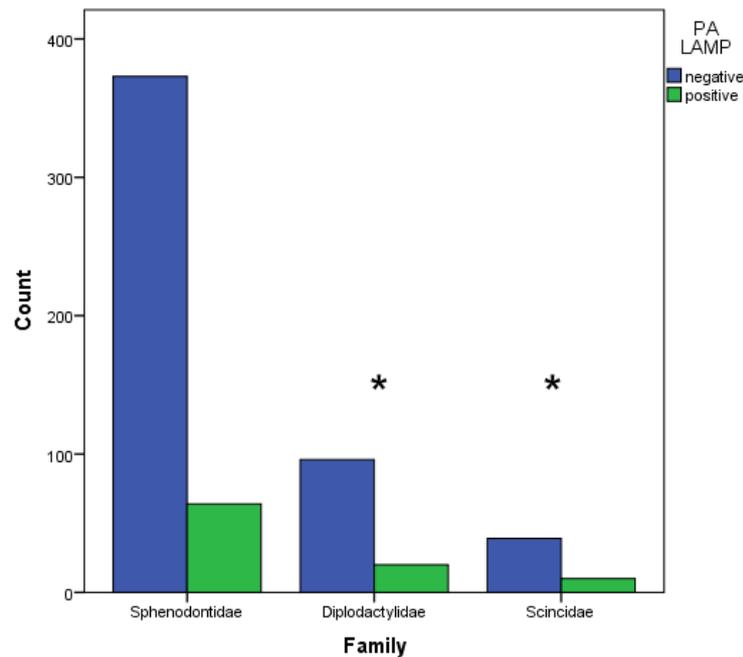


Figure 2.3. Frequency of *P. australasiensis* detection in all reptiles in the study by reptile family. Both Diplodactylidae and Scincidae have a significantly increased risk (\*) of a positive result compared to the Sphenodontidae.

#### 2.3.4.ii Tuatara only

The results of a binomial logistic regression assessing potential predictors for the presence or absence of *P. australasiensis* as detected by LAMP testing in tuatara examined showed that the following predictors were non-significant in the model: lesion presence ( $p=0.604$ ), sex (0.285), and age cohort (0.517). Therefore, only origin was included as a predictor in the reduced model, however the regression was unable to determine meaningful odds ratios for the different locations.

Analysis of the frequency distribution of presence or absence of *P. australasiensis* as detected by LAMP testing in tuatara showed a significant difference between locations (Chi-square = 46.302,  $df = 13$ ,  $P < 0.001$ ; Figure 2.4) but this difference was not able to be further defined.

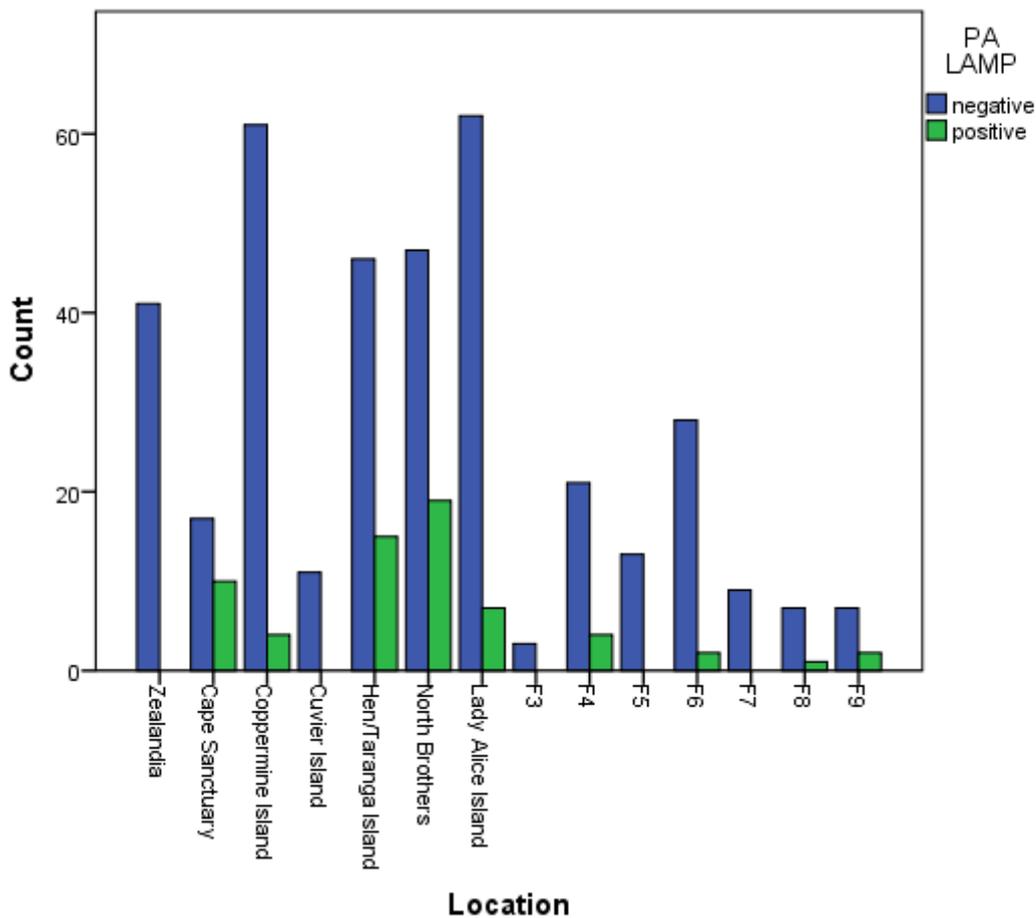


Figure 2.4. Frequency of *P. australasiensis* detection in tuatara by LAMP test in different locations in New Zealand.

### 2.3.5 Analysis of risk factors for presence of dermatitis

#### 2.3.5.i All reptiles combined

The results of a full model binomial logistic regression assessing potential predictors for the presence of dermatitis in all reptiles examined showed location, reptile family, age and sex as significant predictors in the model. However, further analysis of sex showed that this predictor was affected by a cohort of animals of unknown sex. However, the model fit was not improved by the removal of this factor, so the full model is presented below in Table 2.7 Assessment of the full model showed a Chi-square of 184.172,  $df = 22$ ,  $p < 0.001$  compared to the null model with a -2 log likelihood of 481.661 and the model predicted 83.5% of the results correctly.

Table 2.7. Results of a binomial logistic regression for risk factors associated with the presence of dermatitis lesions in all reptiles examined. Statistically significant predictors compared to the reference factor are in **bold** for positive predictor factors and *italics* for protective predictor factors compared to the reference factor.

	B	S.E.	Wald	Df	Sig.	Exp(B)	95% C.I. for EXP(B)	
							Lower	Upper
<b>Location</b>								
Facility 8			85.448	16	.000			
Cape Sanctuary	.395	.820	.232	1	.630	1.484	.298	7.399
<i>Coppermine Island</i>	<i>-1.808</i>	.836	<i>4.678</i>	<i>1</i>	<i>.031</i>	<i>.164</i>	<i>.032</i>	<i>.844</i>
Cuvier Island	.666	.884	.568	1	.451	1.947	.344	11.014
Hen Island	-1.215	.811	2.246	1	.134	.297	.061	1.453
<i>Lady Alice Island</i>	<i>-1.950</i>	.835	<i>5.451</i>	<i>1</i>	<i>.020</i>	<i>.142</i>	<i>.028</i>	<i>.731</i>
<i>North Brothers Island</i>	<i>-3.602</i>	<i>1.047</i>	<i>11.827</i>	<i>1</i>	<i>.001</i>	<i>.027</i>	<i>.004</i>	<i>.212</i>
Stanley Island	-.362	1.118	.105	1	.746	.696	.078	6.228
Zealandia	-.138	.794	.030	1	.862	.871	.184	4.132
Facility 1	-2.138	1.317	2.633	1	.105	.118	.009	1.559
Facility 2	-1.329	.991	1.800	1	.180	.265	.038	1.845
Facility 3	1.458	1.348	1.170	1	.279	4.300	.306	60.395
Facility 4	1.183	.767	2.383	1	.123	3.265	.727	14.668
Facility 5	-.514	.893	.332	1	.565	.598	.104	3.439
<b>Facility 6</b>	<b>1.663</b>	<b>.806</b>	<b>4.256</b>	<b>1</b>	<b>.039</b>	<b>5.275</b>	<b>1.087</b>	<b>25.606</b>
Facility 9	-1.449	1.286	1.270	1	.260	.235	.019	2.918
Facility7	-2.206	1.274	2.997	1	.083	.110	.009	1.338
<b>Sex</b>								
Male			4.674	2	.097			
Female	-20.019	4324.849	.000	1	.996	.000	.000	.
<i>Unknown</i>	<i>-1.305</i>	.603	<i>4.674</i>	<i>1</i>	<i>.031</i>	<i>.271</i>	<i>.083</i>	<i>.885</i>
<b>Age cohort</b>								
Juvenile			2.478	2	.290			
Adult	-.810	.597	1.846	1	.174	.445	.138	1.432

Unknown	-1.525	1.295	1.388	1	.239	.218	.017	2.752
<b>Reptile Family</b>								
Sphenodontidae			12.173	2	.002			
<i>Diplodactylidae</i>	-2.052	.610	11.329	1	.001	.129	.039	.424
Scincidae	-.456	.278	2.684	1	.101	.634	.367	1.094
Constant	.936	.953	.964	1	.326	2.549		

B = coefficient for the constant, S.E = standard error, Df = degrees of freedom, Sig = significance level, Exp B = exponentiation of the B coefficient, C.I = confidence interval

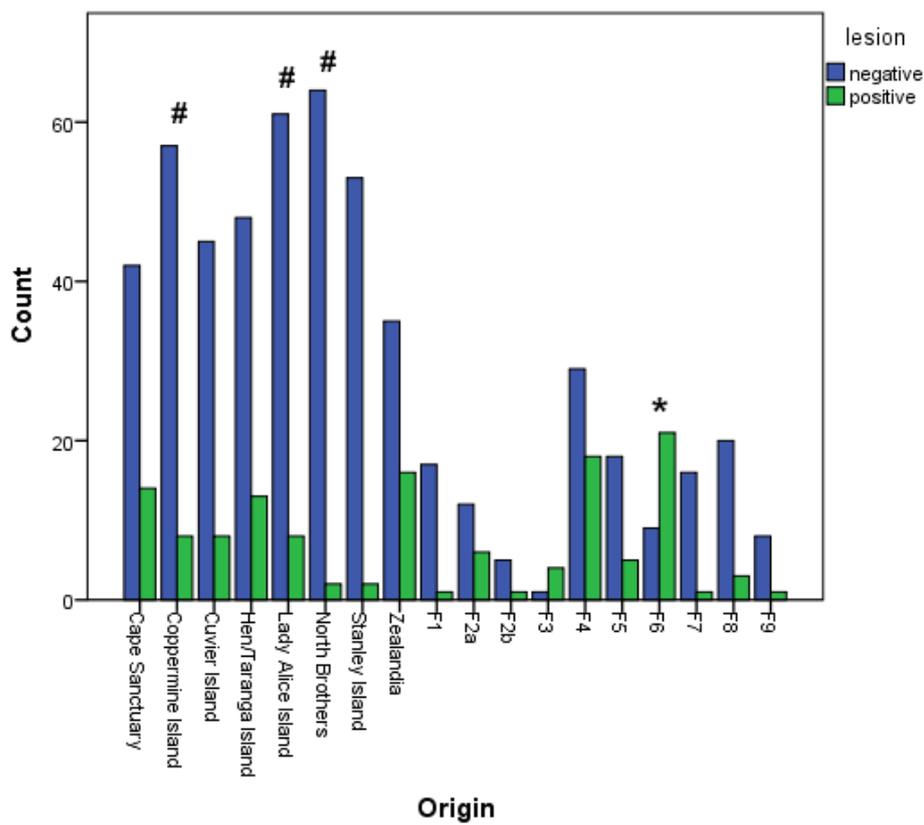


Figure 2.5. Frequency of the presence of dermatitis lesions in all reptiles examined by location. Locations with significantly increased risk of detection are marked by \*. Locations with a significantly reduced risk of detection of dermatitis are marked by #.

The type of management of the reptiles (wild, captive or translocated) was confounded with location, so this factor was not included in the logistic regression. Separate analysis of the frequency distribution of management type by lesion presence showed significant differences between management types (Chi-square = 65.961,  $df = 2$ ,  $p < 0.001$ ; Figure 2.6).

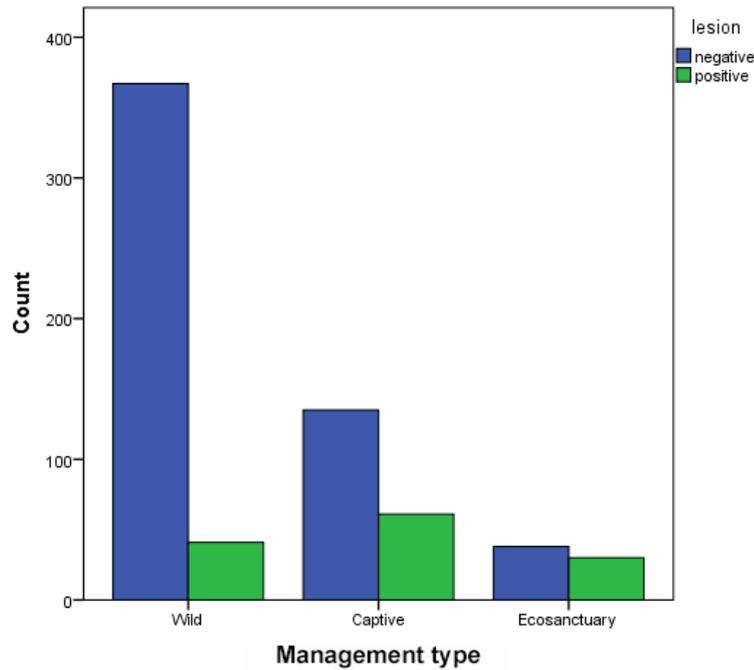


Figure 2.6. Frequency of presence of dermatitis lesions by management type (captive, ecosanctuary or wild population) in all reptiles examined. The wild population is significantly less likely to have dermatitis than the captive or ecosanctuary reptiles.

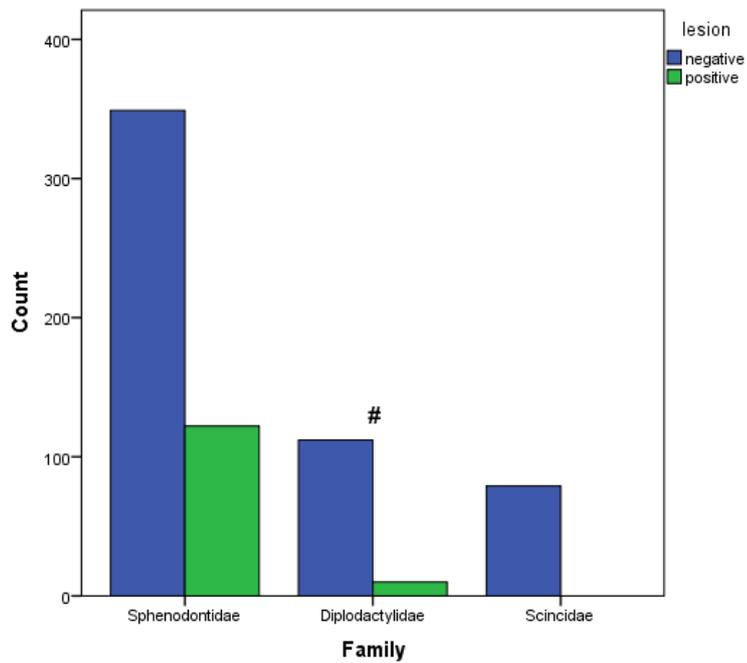


Figure 2.7. Frequency of presence of dermatitis lesions by reptile family in all reptiles examined. The Diplodactylidae (geckos (#)) have a significantly lower odds ratio for dermatitis presence than the Sphenodontidae (tuatara).

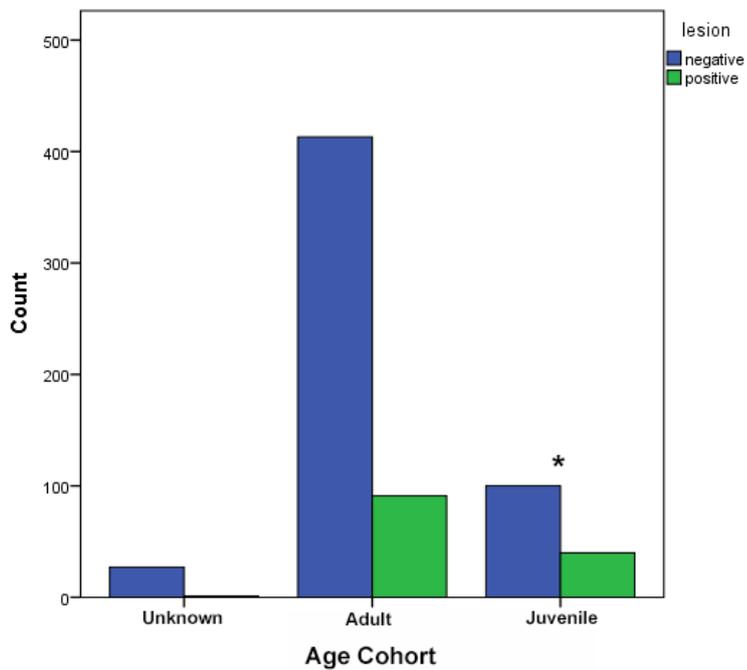


Figure 2.8. Frequency of presence of dermatitis lesions by age. The juveniles (\*) have a significantly higher odds ratio for dermatitis presence than the adults

### 2.3.5.ii *Tuatara only*

The results of a full model binomial logistic regression assessing all potential predictors for the presence of dermatitis in only the tuatara examined showed that location (Figure 2.8), sex and age cohorts (Figure 2.9) were all significant predictors in the model. However, further analysis of sex showed that this predictor was affected by a cohort of animals of unknown sex. When these reptiles were removed and the frequency distribution of sex was assessed independently there was no significant differences between the sexes (Chi-square = 1.182, df = 1,  $p=0.277$ ). Therefore, sex was removed from the final model (Table 2.8). Assessment of the reduced model showed a Chi-square of 111.498,  $df=16$ ,  $p<0.001$  compared to the null model with a -2 log likelihood of 421.338 and the model predicted 79.1% of the results correctly.

Table 2.8. Results of a reduced model binomial logistic regression for risk factors associated with the presence of dermatitis lesions in tuatara. Statistically significant predictors compared to the reference factor are in **bold** for positive predictor factors and *italics* for protective predictor factors compared to the reference factors.

	B	S.E.	Wald	df	Sig.	Odds Ratio	95% C.I. for Odds Ratio	
							Lower	Upper
<b>Location</b>								
Zealandia			73.225	15	0.000			
Cape Sanctuary	0.522	0.504	1.074	1	0.300	1.686	0.628	4.530
<i>Coppermine Island</i>	<i>-1.646</i>	<i>0.503</i>	<i>10.729</i>	<i>1</i>	<i>0.001</i>	<i>0.193</i>	<i>0.072</i>	<i>0.516</i>
<b>Cuvier Island</b>	<b>1.549</b>	<b>0.760</b>	<b>4.156</b>	<b>1</b>	<b>0.041</b>	<b>4.708</b>	<b>1.062</b>	<b>20.881</b>
<i>Hen Island</i>	<i>-1.068</i>	<i>0.463</i>	<i>5.328</i>	<i>1</i>	<i>0.021</i>	<i>0.344</i>	<i>0.139</i>	<i>0.851</i>
<i>Lady Alice Island</i>	<i>-1.672</i>	<i>0.500</i>	<i>11.185</i>	<i>1</i>	<i>0.001</i>	<i>0.188</i>	<i>0.071</i>	<i>0.500</i>
<i>North Brothers Island</i>	<i>-3.090</i>	<i>0.789</i>	<i>15.345</i>	<i>1</i>	<i>0.000</i>	<i>0.046</i>	<i>0.010</i>	<i>0.214</i>
F1	-1.460	1.120	1.700	1	0.192	0.232	0.026	2.085
F2	-0.164	0.583	0.079	1	0.778	0.849	0.271	2.660
F3	1.697	1.166	2.116	1	0.146	5.456	0.555	53.674
F4	0.516	0.557	0.857	1	0.355	1.675	0.562	4.989
F5	-0.043	0.658	0.004	1	0.947	0.958	0.264	3.475
<b>F6</b>	<b>1.509</b>	<b>0.537</b>	<b>7.900</b>	<b>1</b>	<b>0.005</b>	<b>4.521</b>	<b>1.579</b>	<b>12.946</b>
F7	-20.892	13397.657	0.000	1	0.999	0.000	0.000	
F8	-0.156	0.923	0.029	1	0.866	0.856	0.140	5.219
F9	-1.500	1.114	1.813	1	0.178	0.223	0.025	1.982
<b>Age Cohort</b>								
Juvenile								
Adult	0.533	0.345	2.384	1	0.123	1.704	0.866	3.350
Constant	-0.843	0.415	4.121	1	0.042	0.430		

B = coefficient for the constant, S.E = standard error, Df = degrees of freedom, Sig = significance level, C.I = confidence interval

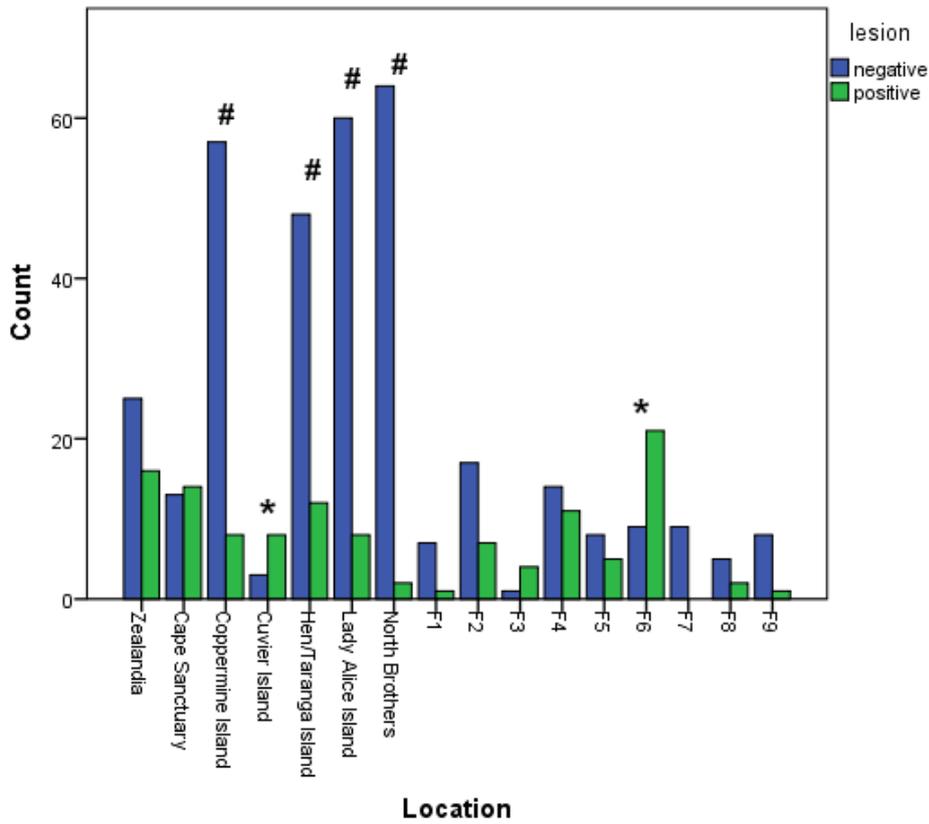


Figure 2.9. Frequency of the presence of dermatitis lesions in tuatara examined by location. Locations with significantly increased risk of detection are marked by \*. Locations with a reduced risk of detection of dermatitis are marked by #.

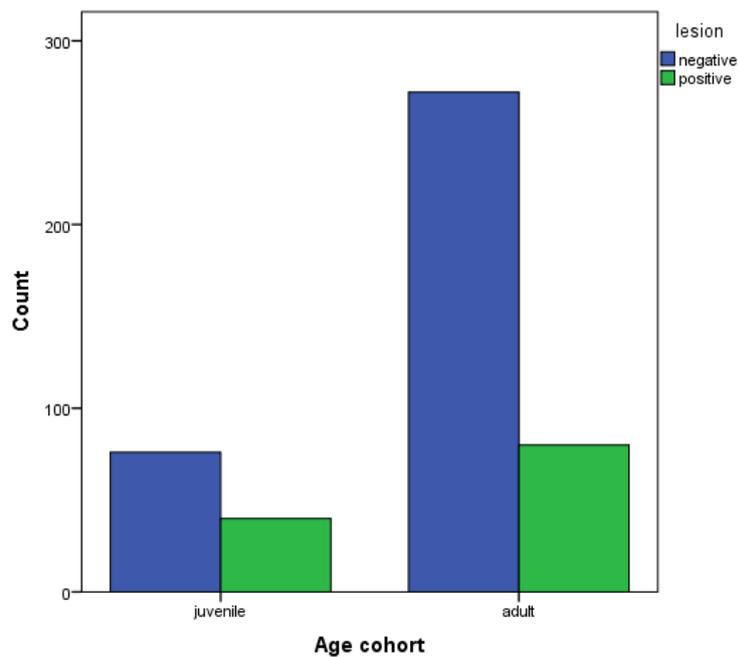


Figure 2.10. Frequency of the presence of dermatitis lesions in tuatara examined by age cohort.

### 2.3.6 Soil

In the pilot soil study, *P. australasiensis* was detected by LAMP in two of three samples from the entrance, one of three samples from the middle and one of three samples from deep within the burrow of three tuatara with active *P. australasiensis* infections. From this study, it was concluded that the optimal soil sample location is the burrow entrance. This was used for the collection of soil at other captive and wild sample locations. Positive soil samples were detected in two of eighteen soil samples from facility 4. One positive soil sample was from a tuatara enclosure with *P. australasiensis* positive individuals and one positive soil sample was from a Duvaucel's gecko enclosure where *P. australasiensis* was not detected in the captive animals. *P. australasiensis* was detected in one out of sixteen soil samples collected from Stanley Island. No tuatara were found on this island, however *P. australasiensis* was detected by LAMP assay in shore skinks and a Duvaucel's gecko. All soil samples from Zealandia (n 6), Cape Sanctuary (n 6), Cuvier Island (n 17), and Facility 2 (n 17) were negative for *P. australasiensis*.

## 2.4 Discussion

The detection of *Paranannizziopsis australasiensis* by culture, PCR and LAMP in tuatara at multiple captive facilities confirms that this disease is not confined to the two facilities within New Zealand that had previously reported it. *Paranannizziopsis australasiensis* was detected by LAMP in multiple species in multiple locations both captive and wild. The results of the LAMP test indicate that *P. australasiensis* is widespread throughout New Zealand. Prevalence varies between different sites, with an increased risk of detection at one ecosanctuary; Cape Sanctuary, and two wild sites; Hen Island and North Brother Island. *Paranannizziopsis australasiensis* is therefore not just a disease of captivity, but can maintain infection levels in isolated wild populations. Translocations have been an important conservation management tool for tuatara and numerous other reptile species in New Zealand for decades (Sherley, Stringer, and Parrish 2010) and there has been numerous opportunities to facilitate the spread of this disease to remote locations and isolated populations.

Validation of the LAMP test is in progress. Culture is currently considered the gold standard diagnostic technique. Culture is a highly specific but poorly sensitive technique. The fungi are slow growing and samples containing large numbers of viable organisms are required. Culture alone cannot be utilised to diagnose the organism to species level due to lack of morphological differences seen on cultured colonies. (Bohuski et al. 2015; Paré and Sigler 2016). Positive samples were only detected by culture on the two most severe cases where high quantities of viable fungal organisms were present. These cases were diagnosed by a biopsy of the lesion rather than a skin swab, providing optimal material for culture. The likelihood of false negatives on culture due to inadequate samples, containing insufficient or non-viable organisms, is high. LAMP has increased sensitivity over culture and PCR (Notomi et al. 2000; Mitchell and Walden 2013). The aim of this study was to investigate the presence of *P. australasiensis* in an environment, not the level of disease. LAMP was developed for this study to enable rapid detection of very small amounts of DNA and increased sensitivity of detection. Some reports of LAMP assays are associated with poor specificity (Senarath et al. 2014; Suleman, Mtshali, and Lane 2016) and the possibility of false positives occurring with primers reacting with environmental organisms cannot be excluded until further validation of the *P. australasiensis* LAMP assay occurs.

In this study dermatitis lesions were more likely to be seen in captive and ecosanctuary reptiles than in wild reptiles. Juvenile reptiles were more likely to have dermatitis lesions than adults, and tuatara were more likely to have dermatitis lesions than geckos or skinks. There are multiple aetiologies for dermatitis lesions on tuatara, and the presence of brown skin discolouration on a tuatara is not pathognomonic for *P. australasiensis*. Secondary dermatitis infections are common in captive reptiles (Schumacher 2003; Harvey et al. 2010) and the finding of increased lesion occurrence in captive and managed tuatara and other reptiles, suggests the husbandry of these animals requires improvements to prevent challenges to the hosts immune response.

LAMP positives were detected in both tuatara with lesions and tuatara without lesions present. As this was a cross sectional survey we cannot be sure what stage of the disease the individuals were at, however the high percentage (73.8%) of positive tuatara with no lesions suggests that tuatara can carry *P. australasiensis* asymptotically, or as a commensal organism. *O. ophiodiicola* has also been

detected by PCR and culture on asymptomatic reptiles (Paré et al. 2003; Bohuski et al. 2015). We cannot be certain of the significance of a positive LAMP result in reptiles with dermatitis. A biopsy of the skin lesion and analysis via histology and culture in combination with LAMP is required to be certain of the role of the fungus in the observed dermatitis lesions on tuatara in this study.

The geckos and skinks that were LAMP positive for *P. australasiensis* were all asymptomatic for signs of dermatitis. There have been no other reports of *P. australasiensis* in any species of native New Zealand geckos or skinks, and the progression of disease in these species is unknown. It would appear that *P. australasiensis* is part of the commensal skin mycoflora in these reptile families rather than a pathogenic organism. Both geckos and skinks have a greater risk of *P. australasiensis* detection on the dermis than tuatara. It is likely that they play a role in the transmission of the organism, as many of these reptile species share habitat, including burrows, directly with tuatara.

*Ophidiomyces ophiodiicola* has been found to survive in the soil under a number of conditions and can utilise environmental sources of energy (Allender et al. 2015). The environmental stability of *P. australasiensis* has so far not been investigated. The detection of *P. australasiensis* in soil samples from multiple locations on LAMP suggests either, environmental survival off the host in soil is possible, that there was recent fungal DNA contamination from direct contact with an infected animal or that the test is giving a false positive result, possibly due to cross-reaction with a soil borne fungus. As soil represents a heterogenous matrix of organisms a negative result is not truly significant and cannot rule out the possibility of *P. australasiensis* presence within an environment. A positive result is of significance for *P. australasiensis* presence. The detection of *P. australasiensis* on the skin of asymptomatic reptiles is further evidence that this fungus may be an environmental saprophyte or skin commensal, rather than a primary pathogen.

Little is known about the epidemiology of *P. australasiensis* in New Zealand. Studies investigating the transmission, environmental survival, and seasonal infection levels are warranted. The fungal load and conditions required for disease development should also be investigated to better understand the disease process. Association of

lesions with *P. australasiensis* positive reptiles can only be inferred, and there was no evidence for this association in my study. Koch's postulates need to be fulfilled before we can truly confirm that *P. australasiensis* is the causative agent of many of the cases of superficial dermatitis seen in the reptiles examined here. The LAMP test requires further validation for interpretation of these results and before being used more widely as a clinically useful diagnostic tool or for conservation management purposes such as disease screening for translocation.

It is unknown whether *P. australasiensis* has been released into the wild from a captive source or whether it has been brought into captivity with wild caught tuatara or other reptiles. *Paranannizziopsis australasiensis* was found on the two islands utilised by the breed and release programs from the captive facilities that had previously reported *P. australasiensis* cases. It was also detected in reptiles from all other wild locations sampled. This suggests that there has not been one single release event of *P. australasiensis* into the wild population as hypothesised. Instead there may have been multiple releases of *P. australasiensis*, or this pathogen may originate from the New Zealand environment. My study has confirmed that *P. australasiensis* is currently widespread both throughout wild and captive populations of New Zealand endemic reptiles. Based on this evidence, I recommend that translocations of healthy tuatara should be re-commenced in the support of the conservation management of tuatara.

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## CHAPTER THREE

### The pathology of *Paranannizziopsis australasiensis* in tuatara (*Sphenodon punctatus*)



### 3.1 Introduction

*Paranannizziopsis australasiensis* is an emerging mycotic pathogen of the family *Onygenaceae*, order *Onygenales*. It is a member of the fungal group formally known as the *Chrysosporium* anamorph of *Nannizziopsis vriesii*. This has recently been reclassified into three genera, *Paranannizziopsis*, *Nannizziopsis* and *Ophidiomyces* based on molecular analysis (Sigler, Hambleton, and Paré 2013). Most mycotic infections in reptiles result from opportunistic environmental saprophytes, causing disease in animals under immune stress. However, fungi of this grouping are unique in that they are all thought to be obligate pathogens, causing disease in reptiles with no concurrent stressors or immune compromise (Schmidt 2015). This has been demonstrated with *Nannizziopsis dermatitis* in Veiled chameleons (*Chamaeleo calytratus*), and *Ophidiomyces ophiodiicola* in Corn snakes (*Pantherophis guttatus*) and Cottonmouths (*Agkistrodon piscivorus*) (Paré et al. 2006; Lorch et al. 2015; Allender, Baker, et al. 2015). These fungi can have a significant impact on their hosts. *Ophidiomyces ophiodiicola* affects both wild and captive snakes and has had a devastating effect on some already vulnerable snake populations in the USA (Allender, Baker, et al. 2015; Lorch et al. 2015). *Nannizziopsis guarroi* causes significant morbidity and mortality in captive inland bearded dragons (*Pogona vitticeps*) (Bowman et al. 2007; Sigler, Hambleton, and Paré 2013).

The tuatara (*Sphenodon punctatus*) is an iconic New Zealand reptile and the only extant species of the order *Rhynchocephalia*. Due to mammalian predator introduction, their population is reduced to four predator proof sanctuaries and thirty-nine isolated off shore islands, many of which have restricted or difficult access. Translocations have been an important part of the recovery and ongoing conservation management of tuatara populations (Gaze 2001). *Paranannizziopsis australasiensis* has recently been detected in captive tuatara in New Zealand. To date this pathogen has only been isolated from captive reptiles in Australia and New Zealand (Sigler, Hambleton, and Paré 2013). The origin and time of pathogen establishment in New Zealand is unknown. *Paranannizziopsis australasiensis* is suspected to be a primary pathogen like other closely related fungal species. The close relationship of *P. australasiensis* to *O. ophiodiicola* raises concerns for the health impacts of the fungus on wild populations of tuatara. Breed for release programmes of tuatara have currently

ceased until more is known about the distribution and pathology of this pathogen to prevent the introduction of the fungus from captive to wild tuatara.

Fungi of the genera *Nannizziopsis*, *Paranannizziopsis* and *Ophidiomyces* are keratolytic (Paré and Sigler 2016). Disease caused by all three genera is typically a superficial dermatitis with rapid progression to ulceration and necrosis. Local invasion of the underlying muscle and bone is a common sequelae. Rarely systemic dissemination occurs. This has resulted in mycogranulomatous hepatitis and pneumonia in some cases (Bowman et al. 2007; Paré and Sigler 2016). Morbidity and mortality is high in most species but can be variable (Allender et al. 2011; Allender, Baker, et al. 2015). *Ophidiomyces ophiodiicola* has been documented as causing both significant decline in wild snake populations (Allender, Bunick, et al. 2015; Lorch et al. 2015; Allender, Raudabaugh, et al. 2015) and mild disease in wild hosts with good body condition (Guthrie et al. 2015). In two instances *O. ophiodiicola* has been detected on the skin of asymptomatic snakes (Paré et al. 2003; Bohuski et al. 2015). The severity of disease caused by this fungal group is likely variable between pathogen species, host species and individual hosts. (Guthrie et al. 2015). An asymptomatic carrier state has been postulated in bearded dragons, by one author, (Bowman et al. 2007) although this has not yet been investigated. Environmental factors and fungal load may also contribute to disease severity (Allender, Raudabaugh, et al. 2015; Langwig et al. 2016).

Reports of pathology associated with *P. australasiensis* in New Zealand is limited to a single coastal bearded dragon (*Pogona barbata*) and five tuatara from one captive institute. The bearded dragon presented with multifocal exudative dermatitis lesions on the chin, ventral abdomen and right tarsus. It was in poor body condition and had a leucocytosis with a monocytosis and a left shift. Disease progressed rapidly, and the bearded dragon was subsequently euthanised. Post mortem revealed multifocal hepatic granulomas, with central necrosis. Septate hyphae were seen in the granulomas on periodic acid-Schiff (PAS) stain, and *P. australasiensis* was confirmed with DNA sequencing (Sigler, Hambleton, and Paré 2013; Masters et al. 2016).

In the five tuatara disease was reported as mild superficial dermatitis, with slow progression to ulceration and necrosis of the dermis. Three out of five tuatara cases

presented or developed a leucocytosis with a monocytosis or azurophilia. No changes to tuatara body condition were reported. All tuatara responded to systemic itraconazole and topical terbinafine treatment. Reoccurrence of disease occurred in one out of five cases (Masters et al. 2016). The cause for the slow disease progression and mild pathology seen in tuatara has been postulated as due to the lower resting body temperature of tuatara (7-24 °C) (Cree et al. 1990) and the optimal growth of *P. australasiensis* above this temperature at 25-30 °C (Humphrey, Alexander, and Ha 2016)

*Paranannizziopsis australasiensis* is reported to cause mild pathology in tuatara in captivity but evaluation of the health of tuatara in the wild where additional pressures on survival exist have not yet been performed. In a prior study of the epidemiology of *P. australasiensis* in captive and wild tuatara (Chapter 2), *P. australasiensis* was found to be widespread in tuatara and other New Zealand reptiles in captivity, ecosanctuaries and wild populations across New Zealand. All fungi of this grouping are believed to be associated with specific reptile groupings (Paré and Sigler 2016) and a similar close association of *P. australasiensis* with tuatara and other New Zealand reptiles is apparent.

The aim of this study was to: 1. investigate the pathological effects of *P. australasiensis* by describing the gross lesions associated with positive LAMP tests, and the histopathology of significantly diseased cases, 2. document the successful treatment of two diseased tuatara with dermatitis using voriconazole and 3. evaluate the effects of *P. australasiensis* on the morphometrics and haematological parameters of tuatara by comparing the positive and negative tuatara tested for *P. australasiensis* by loop mediated isothermal amplification (LAMP).

### **3.2 Materials and Methods**

Tuatara from a prevalence study for *Paranannizziopsis australasiensis* (Chapter 2) were assessed for general health changes in response to presence of infection. A total of 437 tuatara; sixty-four positive and 373 negative for *P. australasiensis* on LAMP were included in this study. Tuatara were sourced from five islands (Cuvier Island

(Repanga), Hen (Taranga) Island, Coppermine Island, North Brothers Island and Lady Alice Island), two mainland ecosanctuaries (Zealandia and Cape Sanctuary), and seven captive facilities (Rainbow Springs Kiwi Encounter, Rotorua, Pukaha, Mount Bruce, Zealandia, Wellington, Victoria University, Wellington, Orana Wildlife Park, Christchurch, Southland Museum, Invercargill, and Kiwi Birdlife Park, Queenstown). Capture was performed for wild tuatara as described in Chapter 2. Sampling took place between February 2016 and March 2017 and excluded the winter months due to seasonal torpor of tuatara.



Figure 3.1. Map of New Zealand showing sampling sites

### 3.2.1 Skin samples and lesions

Skin samples were collected from tuatara as described in Chapter 2. Any lesions consistent with *P. australasiensis* were photographed and categorised as: mild (superficial skin discolouration or skin flaking); moderate (ulcerative or exudative lesions); or severe (deeper tissue invasion or granuloma formation). Tuatara with moderate to severe lesions were uplifted and taken to either Wildbase (Massey University, Palmerston North) or The Nest, Wellington Zoo (Wellington) for biopsy

under general anaesthesia, with alfaxalone (Alfaxan Jurox, Auckland, New Zealand) 10mg/kg intravenous injection into the ventral tail vein. Biopsies were performed by incising three full thickness skin samples (5mm x 3mm minimum) taken from the lesion margin. Two fresh tissue samples were stored chilled at 3°C and were submitted for fungal culture as described by (Humphrey, Alexander, and Ha 2016). and *P. australasiensis* LAMP assay as detailed in Chapter 2. The third sample was placed into 10% neutral buffered formalin and submitted for histologic analysis. Formalin fixed tissue were processed by standard techniques and stained with hematoxylin and eosin, grams stain and periodic acid-schiff stain (PAS) stains.

### **3.2.2 Treatment and quarantine of positive cases**

Tuatara positive for *P. australasiensis* with moderate or severe lesions were treated with voriconazole (Optimus Healthcare Compounding Pharmacy, Auckland, New Zealand) 2.5mg/kg orally once a day and 1% povidone iodine topically once a day. Treatment was continued for two weeks following the visible resolution of dermatitis lesions. Lesion swabs were collected weekly following treatment and the tuatara deemed clear of the infection after three consecutive negative LAMP results were obtained. These methods were implemented to prevent tuatara from re-entering the captive environment with low levels of fungi remaining on the dermis.

Positive individuals receiving medical treatment were kept isolated and handlers wore disposable protective clothing and gloves. Hands were cleaned with 70% ethanol and equipment and shoes were cleaned with a disinfectant containing; n-alkyl dimethyl benzyl ammonium chloride, didecyl dimethyl ammonium chloride, and poly biguanide hydrochloride (SteriGene, Ethical Agents Ltd, Auckland) following treatment and servicing to prevent spread to other individuals, and the environment, as recommended by Rzadkowska for the disinfection of *O. ophioidiicola* (Rzadkowska et al. 2016).

### **3.2.3 Morphometrics**

In all tuatara sampled body weight was measured using a spring balance (Pesola) and snout to vent length (SVL) was measured. Body condition index (BCI) was calculated by as the residuals of a linear regression of log-transformed mass and log-transformed SVL (Moore et al. 2007).

### 3.2.4 Haematology

Blood samples were collected from tuatara from seven captive facilities, one wild site and two ecosanctuaries. Approximately 0.1ml of whole blood was withdrawn from the ventral tail vein using a 1ml syringe and a 25G or 23G needle. Samples that were observed to be lymph contaminated, clotted or had noticeable haemolysis were discarded. Blood smears were made from whole blood and stained with Giemsa stain. Estimated total white blood cell counts (TWBCC) (n =127), differential white blood cell counts (n = 127), packed cell volume (PCV) (n = 111) and total solids (TS) (n = 108) were determined for the tuatara, where a viable sample was obtained, via manual methods as described in Fudge (2000). The differential TWBCC were made and read by a single veterinarian for consistency (R.K.E.W).

### 3.2.5 Statistical Methods

T-tests were used to assess for significant differences between reptiles with a positive LAMP test in relation to BCI, and haematological parameters including, PCV, TS and TWBCC, and absolute counts for heterophils, lymphocytes, eosinophils, basophils, monocytes and azurophils. All statistical analysis was carried out using IBM SPSS Statistics 24.

## 3.3 Results

### 3.3.1 Lesions

As previously reported in Chapter 2, a total of 14.6% (n=64) tuatara were positive for *P. australasiensis* on LAMP. Of these positive animals 75% had no lesions present (n=48). Mild lesions (Figure 3.2) were seen in 20.3% (n=13), moderate lesions were seen in 1.6% (n=1) and severe lesions were seen in 3.1% (n=2), of LAMP positive tuatara. There was no significant association in the frequency of dermatitis lesions with a positive result on the PA LAMP test (Chi-square 0.016, df = 1, p = 0.901). All lesions where *P. australasiensis* was detected were on the ventrum of tuatara, excluding one individual with severe multifocal granulomatous lesions on the dorsum. All moderate and severe cases reported were in captive individuals. These cases are referred to as Cases 1, (moderate), 2 and 3 (severe) and the pathology associated with these cases are described in more detail below.

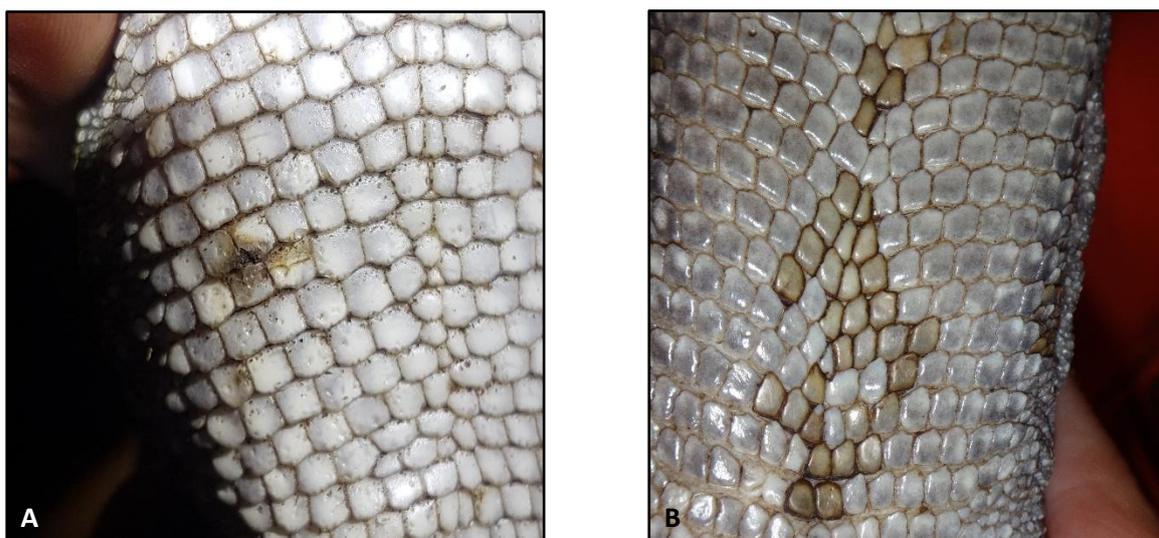


Figure 3.2. Mild dermatitis lesions. a. A focal area of skin discolouration and flaking on the ventrum of a tuatara from North Brothers Island (Photo supplied by Kate McInnes, Department of Conservation). b. Multifocal discolouration of scales on the ventrum of a tuatara from Curvier Island.

Table 3.1. Lesion presence in tuatara tested for *Paranannizziopsis australasiensis* (PA) on LAMP. There was no significant association in the frequency of dermatitis lesions with a positive result on the PA LAMP test (Chi-square 0.016, df = 1, p = 0.901).

Lesions	PA LAMP		Total
	Negative	Positive	
No	277	48	325
Yes	96	16	112
<b>Total</b>	373	64	437

### 3.3.1.i Case 1

A fourteen-year-old, adult captive male tuatara with no prior health issues presented to The Nest, Wellington Zoo with a single area of skin discolouration, flaking and ulceration mid body on the ventrum, measuring 20mm x 10mm (Figure 3.3). The tuatara was in moderate body condition, and haematological values fell within the mean ranges determined in this study (Table 3.2). On biopsy there was evidence of superficial inflammation and bacterial colonisation. Superficial ulceration of the dermis and hyperkeratosis of the lesion edges was present, with gram negative and occasional gram-positive coccobacillus embedded in this hyperkeratotic material

(Figure 3.6 A). No fungal hyphae were seen on periodic acid-schiff stain. The biopsy tissue was positive for *P. australasiensis* on LAMP, however culture of this sample failed to detect *P. australasiensis*.

Case 1 was treated with voriconazole orally once a day and 1% povidone iodine topically. Silver sulfadiazine (Flamazine, Smith and Nephew, Auckland, New Zealand) was also applied topically due to the detection of bacteria in the lesion. Lesions had visibly resolved after seven weeks of treatment. Treatment was continued for a total of nine weeks. All three swabs collected weekly following treatment were negative for *P. australasiensis* on LAMP assay. The tuatara remained bright and active during treatment. There was no change to appetite or body condition noted during this time.



Figure 3.3. Case 1. Ulceration of the mid ventral dermis of an adult male tuatara associated with a positive LAMP test result for *Paranannizziopsis australasiensis*. (Photo supplied by Anna-Karina Argandona-Gonzalez, Wellington Zoo)

### 3.3.1.ii Case 2

A captive, wild caught (approximately thirty years ago), adult female tuatara, with a chronic history of skeletal deformity of unknown cause presented to The Nest, Wellington Zoo, with multifocal deep pyogranulomas measuring 6 to 8 mm diameter, located on the right thoracic wall, between digits two and three of the right front foot, and on the dorsal and right lateral aspects of the tail base (Figure 3.4). Radiographs showed the underlying bone was unaffected. Biopsies of these lesions showed necrotic dermis and epidermis infiltrated with moderate numbers of septate parallel walled hyphae approximately 6mm wide with rare right angled non- dichotomous bud-like

branching (Figure 3.6 B). This sample was positive for *P. australasiensis* on culture and LAMP.

Case 2 was treated with surgical debridement and lavage of granulomas plus oral voriconazole and topical iodine once a day for ten weeks in total. This includes two weeks of treatment post the resolution of clinical signs. All three swabs collected weekly following treatment were negative for *P. australasiensis* on LAMP assay. The tuatara remained bright and active during treatment. There was no change to appetite or body condition noted during this time.

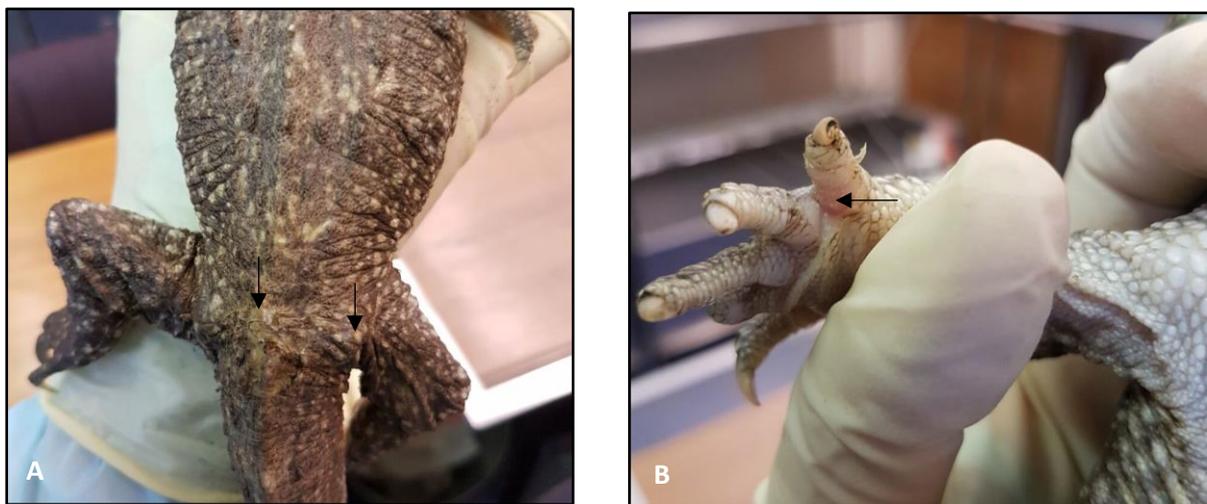


Figure 3.4. Case 2. Multifocal granulomatous lesions on A. the dorsal and right lateral aspects of the tail base (arrows), and B. between digits 2 and 3 of the right front foot (arrow) of an adult female tuatara with *Paranannizziopsis australasiensis* confirmed by LAMP test, culture and DNA sequencing.

### 3.3.1.iii Case 3

A captive bred, five year old, juvenile male tuatara with a history of slow growth from hatch presented to Wildbase with severe multifocal granulomatous lesions on the ventral chest and abdomen, ventral and lateral tail and the palmar carpal aspect of the right front foot (Figure 3.5). This individual died shortly following presentation. Post mortem and histology revealed a multifocal infiltrative mycotic granulomatous dermatitis and myositis. The epidermis was intact and contained areas of hyperkeratosis. The dermis and underlying skeletal muscle was largely replaced by heterophils and pyknotic debris containing large numbers of fungal elements characterised by thin, parallel and septate walls and occasional branching (Figure 3.6

C and D). The foot lesion was severe, with deep extension into the dermis and muscle, with large amounts of necrotic debris surrounding bones and joints, which while intact, showed signs of early inflammation (Figure 3.6 E and F). The tuatara had concurrent urolithiasis with secondary post obstructive dilation and mineralisation of the renal tubules and interstitial renal fibrosis. *Paranannizziopsis australasiensis* infection was confirmed with LAMP, culture, and sequencing of the mitochondrial and fungal ITS loci by the Institute of Fundamental Sciences, Massey University.



Figure 3.5. Case 3. A. Multifocal granulomatous lesions on the ventral chest, abdomen and tail (arrow heads) and B. transverse section through the palmar carpal aspect of the right front foot showing multiple granulomas (arrow heads) adjacent to the carpal bones of a juvenile male tuatara with *Paranannizziopsis australasiensis* confirmed by LAMP test, culture and DNA sequencing

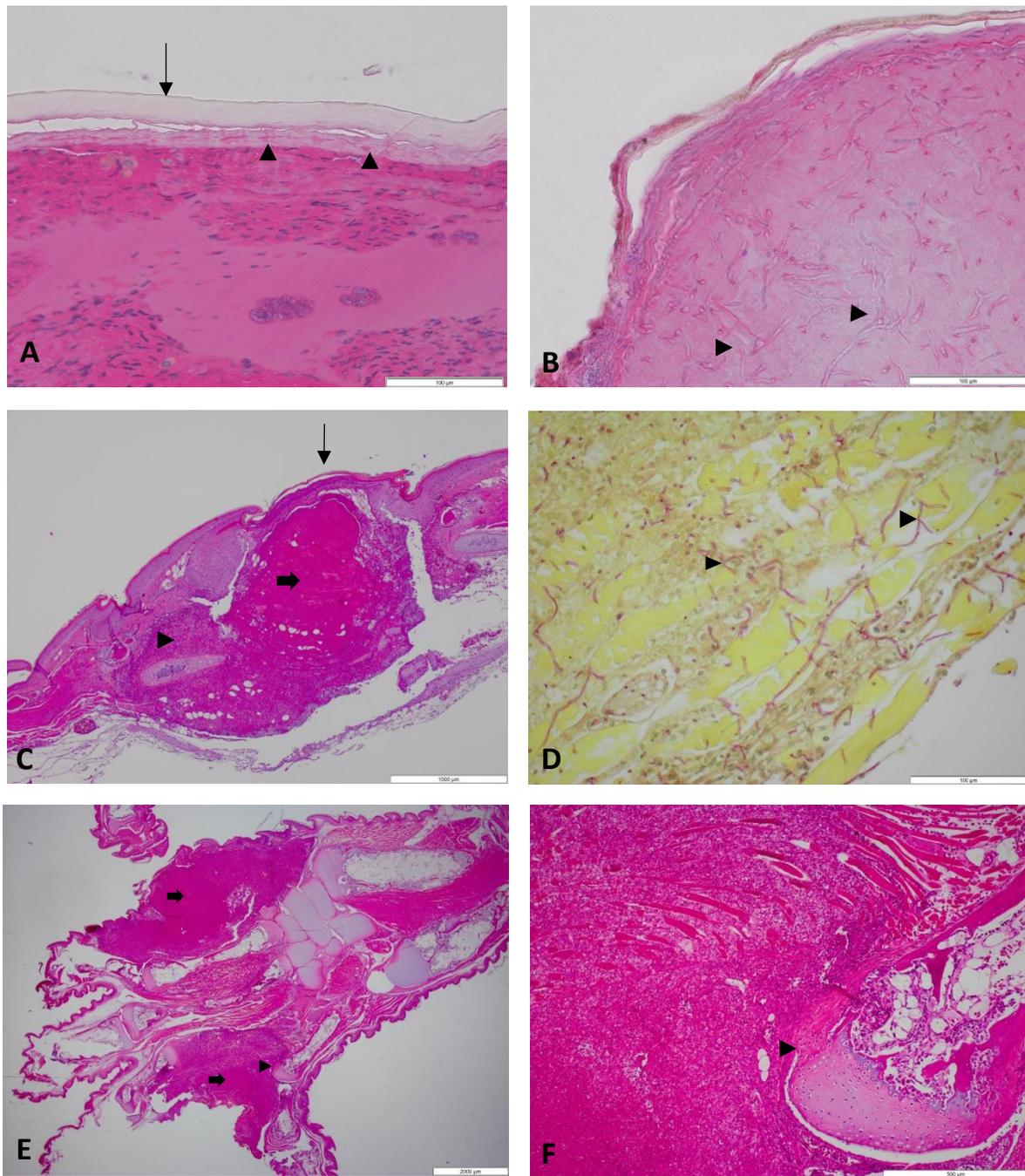


Figure 3.6. **A.** Case 1: Hyperkeratosis (arrow) of the lesion edges, with gram negative and occasional gram-positive coccobacillus embedded in this material (arrow head). **B.** Case 2: Necrotic dermis and epidermis infiltrated with moderate numbers of septate parallel walled hyphae with right angled branching (arrow heads) **C - F.** Case 3. **C.** Granulomatous dermatitis and myositis with heterophilic infiltration (arrow head) and pyknotic debris (bold arrow). The dermis is intact with areas of hyperkeratosis (arrow). **D.** A granuloma containing fungal hyphae with occasional branching (arrow heads) PAS stain. **E and F:** Right front foot, Case 3. Granulomas extend deep into the dermis and muscle, with large amounts of necrotic debris surrounding bones and joints (bold arrows), with early cortical inflammation (arrow head).

#### 3.3.1.iv Other lesions

112 tuatara were reported to have mild dermatitis lesions. Only 14.3% (n=16) of the tuatara with lesions were positive for *P. australasiensis* on LAMP. In the remaining 85.7% (n=96) of tuatara with lesions *P. australasiensis* was not detected. Further diagnostics to investigate other aetiologies for the observed dermatitis lesions were not performed.

#### 3.3.2 Effect of presence of *Paranannizziopsis australasiensis* on morphometrics and haematological parameters in tuatara

Analysis of all morphometric and haematological parameters (Table 3.2) for differences between tuatara testing positive and negative for *P. australasiensis* by LAMP test showed no significant differences for all parameters (Table 3.3 and 3.4)

Table 3.2. Descriptive statistics for morphological and haematological variables between tuatara testing positive and negative for *P. australasiensis* by LAMP test.

<b>Group Statistics</b>					
	Lesion	N	Mean	Std. Deviation	Std. Error Mean
TWBCC	0	81	4.376	2.114	.233
	1	73	4.475	2.550	.303
Het	0	80	1.333	.848	.0948
	1	73	1.572	1.456	.170
Lymp	0	80	.820	.932	.104
	1	73	.687	.754	.088
Eosin	0	80	.836	.540	.060
	1	73	.811	.548	.064
Baso	0	80	.238	.218	.024
	1	73	.265	.224	.026
Mono	0	80	.895	.712	.080
	1	73	.833	.569	.066
Azuro	0	61	.307	.440	.056
	1	65	1.32	7.905	.980
PCV	0	70	35.982	10.681	1.277
	1	64	36.344	11.041	1.380
TP	0	66	50.61	17.990	2.214
	1	62	45.92	18.494	2.349
BCI	0	100	.005	.029	.003
	1	13	.003	.047	.013

N = total number, Std = standard, TWBCC = total estimated white blood cell count, het = heterophil, Lymp = lymphocyte, Eosin = eosinophil, Baso = basophil, Mono = monocyte, Azuro = azurophil, PCV = packed cell volume, TP = total protein, BCI = body condition index

Table 3.3 and 3.4. Analysis of differences between morphological and haematological parameters in tuatara testing positive and negative for *P. australasiensis* by LAMP test.

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	Df	Sig. (2-tailed)
TWBCC	Equal variances assumed	.063	.802	-.038	125	.970
	Equal variances not assumed			-.044	19.919	.966
Het	Equal variances assumed	.012	.913	-.037	125	.971
	Equal variances not assumed			-.037	17.998	.971
Lymp	Equal variances assumed	.851	.358	-1.072	125	.286
	Equal variances not assumed			-1.063	17.871	.302
Eosin	Equal variances assumed	3.233	.075	.757	125	.451
	Equal variances not assumed			.605	16.148	.554
Baso	Equal variances assumed	1.455	.230	-.346	125	.730
	Equal variances not assumed			-.289	16.436	.776
Mono	Equal variances assumed	2.258	.135	.510	125	.611
	Equal variances not assumed			.756	26.465	.457
Azuro	Equal variances assumed	.364	.547	.336	121	.738
	Equal variances not assumed			.973	111.992	.333
PCV	Equal variances assumed	8.118	.005	.851	109	.397
	Equal variances not assumed			.519	10.621	.615
TP	Equal variances assumed	2.687	.104	-1.017	106	.312
	Equal variances not assumed			-.773	9.958	.458
BCI	Equal variances assumed	.728	.395	.187	111	.852
	Equal variances not assumed			.132	13.266	.897

Sig = significance level, Df = degrees of freedom, TWBCC = total estimated white blood cell count, het = heterophil, Lymp = lymphocyte, Eosin = eosinophil, Baso = basophil, Mono = monocyte, Azuro = azurophil, PCV = packed cell volume, TP = total protein, BCI = body condition index

### Independent Samples Test

		t-test for Equality of Means			
		Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
				Lower	Upper
TWBCC	Equal variances assumed	-.023	.604	-1.218	1.172
	Equal variances not assumed	-.023	.522	-1.112	1.067
Het	Equal variances assumed	-.0122	.332	-.669	.645
	Equal variances not assumed	-.0122	.331	-.707	.683
Lymp	Equal variances assumed	-.173	.161	-.492	.146
	Equal variances not assumed	-.173	.162	-.514	.169
Eosin	Equal variances assumed	.106	.140	-.171	.383
	Equal variances not assumed	.106	.175	-.265	.477
Baso	Equal variances assumed	-.019	.055	-.128	.090
	Equal variances not assumed	-.0190	.066	-.158	.120
Mono	Equal variances assumed	.096	.189	-.277	.470
	Equal variances not assumed	.096	.127	-.165	.358
Azuro	Equal variances assumed	.568	1.693	-2.784	3.921
	Equal variances not assumed	.568	.584	-.589	1.726
PCV	Equal variances assumed	2.954	3.470	-3.924	9.831
	Equal variances not assumed	2.954	5.695	-9.636	15.543
TP	Equal variances assumed	-6.100	6.001	-17.997	5.797
	Equal variances not assumed	-6.100	7.895	-23.702	11.502
BCI	Equal variances assumed	.002	.009	-.017	.020
	Equal variances not assumed	.002	.013	-.027	.030

Std = standard, TWBCC = total estimated white blood cell count, het = heterophil, Lymp = lymphocyte, Eosin = eosinophil, Baso = basophil, Mono = monocyte, Azuro = azurophil, PCV = packed cell volume, TP = total protein, BCI = body condition index

### 3.4 Discussion

This study details two additional confirmed cases of disease caused by *Paranannizziopsis australasiensis* in tuatara (Case 2 and 3) at two separate captive facilities with no prior confirmed history of this disease. Case 3 is the first confirmed mortality of a tuatara associated with *Paranannizziopsis australasiensis*. However, this case was complicated by a chronic history of poor health and concurrent urolithiasis. Case 1 had a single lesion consistent with disease caused by *P. australasiensis* as described by Masters et al 2016, and *P. australasiensis* was detected on LAMP assay from lesions swabs from this animal. However, the failure to culture *P. australasiensis* or find evidence of fungal invasion on histology, indicates the primary involvement of *P. australasiensis* in the lesion was unlikely. Chapter 2 describes a very poor statistical association of *P. australasiensis* with dermatitis lesions, leading to the conclusion that *P. australasiensis* may be a commensal organism rather than a pathogenic fungus. Care must be taken in the diagnosis of this disease, as detection of *P. australasiensis* with molecular tools does not confirm causation of the lesion present. It is likely the lesion in this case was caused by an undetermined bacterial infection and the detection of *P. australasiensis* with LAMP represents an asymptomatic carrier state. Gold standard diagnostics for confirming disease due to *P. australasiensis* should include culture, histology and molecular testing such as LAMP, PCR or genetic sequencing.

Antifungal therapy with the azole group of drugs can have a significant hepatotoxic effect in reptile patients, and these should be used with caution when indicated (Van Waeyenberghe, 2010). Voriconazole was selected for the treatment of clinically infected tuatara, as in other species of reptiles it has less reported mortalities from hepatotoxicity, associated with long term treatment regimes, in comparison to itraconazole (Van Waeyenberghe et al. 2010). The tuatara in our study undergoing treatment showed no adverse effects whilst on medication. However, biochemistry was not measured during treatment. Pharmacokinetic trials in tuatara by Alexander (2017), show adverse systemic effects in response to itraconazole therapy, indicated by increased bile acids, uric acid and weight loss. Voriconazole was used with less observed systemic effects but did not always reach therapeutic levels in plasma and is therefore of questionable efficacy in the treatment of *P. australasiensis*. Individual

variation in plasma concentrations of voriconazole, following administration, has also been reported in other reptile species, as well as in mammals, and birds (Schmidt et al. 2007; Howard, Hoffman, and Sheth 2008; Van Waeyenberghe et al. 2010). In our study one confirmed case (Case 2) of *P. australasiensis* resolved with a combination of oral voriconazole, debridement and topical iodine. Case 1, which was likely an asymptomatic carrier of *P. australasiensis* also resolved on this treatment with the addition of Silva sulfadiazine cream topically, and *P. australasiensis* was not detected on LAMP assay of skin and lesions swabs following treatment.

Unlike *O. ophiodiicola* which has caused significant population declines in wild snakes *P. australasiensis* appears to have low virulence in tuatara. As documented in this study and one previous report, tuatara suffer low morbidity from *P. australasiensis* (Masters et al. 2016). Our study showed no significant systemic effects in response to *P. australasiensis* presence on the dermis of tuatara. There was no significant change to PCV, TS, TWBCC and haematological values in tuatara with *P. australasiensis* detected by LAMP assay. *P. australasiensis* presence on tuatara did not result in measurable changes in BCI. Biochemical parameters were not measured. Our study shows that under optimal conditions little disease occurs in tuatara with *P. australasiensis*.

The two severe cases of *P. australasiensis* documented in this study were both in captive tuatara. Altered captive environmental temperatures occurring above the preferred body temperature (7-24°C) of tuatara may predispose to disease development. The average environmental temperature in January, the hottest month in New Zealand, ranges from 12-20°C. Temperatures over 25°C are uncommon (NIWA 2016). The optimal growth range of *P. australasiensis* is reported to be 25-30°C (Humphrey, Alexander, and Ha 2016, Masters et al. 2016). This may account for the lack of disease development in wild tuatara and the only occasional occurrence in captive animals. A captive environment can result in stressors from sub-optimal conditions that result from the animal's reduced options to respond to habitat variables such as diet offered, stocking density, and the thermal environment. These variables are all much more restricted in captivity than in the wild habitat. Decreased immune competence and increased susceptibility to disease is frequently observed in captive reptiles (Schumacher 2003; Harvey et al. 2010). They are also likely to be exposed to

higher loads of the fungus as they are in a closed environment. It is likely that environmental temperature, fungal load and immune compromise may all play a part in the development of this disease in tuatara.

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## CHAPTER FOUR

### General discussion



#### 4.1 Overview of Research Aims and Conclusions

*Paranannizziopsis australasiensis* was identified as a key threatening disease process to captive and wild tuatara and other endemic terrestrial reptiles in New Zealand, following its diagnosis in captive tuatara involved in a breed for release programme. The perceived risk of *P. australasiensis*, was based on its close association with the reptile pathogens *Ophidiomyces ophiodiicola* and *Nannizziopsis guarroi* and the assumption that this was an emerging pathogenic fungal organism entering a naïve reptile population. The impacts of virulent pathogens emerging in naïve wildlife populations have been well documented. Diseases such as white nose syndrome in bats (Blehert et al. 2009), chytridiomycosis in amphibians (Skerratt et al. 2007), and sarcoptic mange in wombats (Martin et al. 2018) have all caused significant declines and in some cases extinctions in populations where these diseases have been introduced. The consequences of *P. australasiensis* release into wild reptile populations were previously unknown and suspected to be significant.

Invasive pathogens can have a significant effect on the population size of naïve hosts and may lead to localised extinctions (Skerratt et al. 2007, Allender, Bunick, et al. 2015; Martin et al. 2018). In contrast endemic pathogens, rarely produce high mortality rates and usually persist at low prevalence and low virulence. This occurs through at least one of four mechanisms, reduced pathogen transmission, resistance (reduced pathogen growth by host defences) tolerance (reduced damage to host by host defences without reducing pathogen growth), and / or lower environmentally driven growth rates (Hoyt et al. 2016). For example, white nose syndrome (WNS) in bat populations in Asia, where it is endemic, has significantly less impact on populations in comparison to WNS in bat populations in North America, where it is currently emerging. In this case the cause is thought to be through the mechanism of higher resistance to pathogen growth (Hoyt et al. 2016). With a highly fragmented population that is primarily limited to offshore islands, ecosanctuaries and captivity, the tuatara could easily be threatened by an emergent fungal pathogen.

The aims of this study were to determine if 1) *P. australasiensis* was present in wild populations of tuatara 2) To determine if *P. australasiensis* was present at other captive facilities within New Zealand. 3) to investigate if *P. australasiensis* could infect

other species of endemic New Zealand reptiles both in captivity and in the wild. And 4) To describe the pathology of *P. australasiensis* in tuatara. Determining if *P. australasiensis* was present on two of the Mercury Islands, (Stanley and Cuvier Islands) which have been used as release sites for captive bred tuatara for over 20 years is of particular importance. The hypothesis for this research was that *P. australasiensis* had been introduced into New Zealand captive facilities through the international reptile trade, and subsequently released onto these islands through the release of captive-bred tuatara.

#### **4.1.1 Prevalence of *Paranannizziopsis australasiensis* in wild and captive tuatara**

This is the first study of the prevalence of *P. australasiensis* in New Zealand. In the study described in Chapter 2, seven populations of wild tuatara, located on five remote islands and two ecosanctuaries, and nine populations of captive tuatara were tested for *P. australasiensis*. *Paranannizziopsis australasiensis* was not detected by culture in any wild tuatara. In captive tuatara two individuals from two separate facilities, were positive for *P. australasiensis* on culture, and the involvement of this organism with superficial dermatitis was supported by histology. In contrast to the culture results, *P. australasiensis* was detected using Loop mediated isothermal amplification (LAMP) in tuatara from three out of four islands, one out of two ecosanctuaries and four out of seven captive facilities, at prevalence's varying between 6.1% – 44.4%. Similar prevalence levels have been reported for *O. ophiodiicola* in multiple wild snake populations, in Great Britain (8.6%) and eastern North America (38% and 41%) (Guthrie et al. 2015; Lorch et al. 2016; Franklinos et al. 2017) however, unlike the research presented in this thesis, these studies frequently have not tested reptiles without identified lesions, and this may result in an underestimation of pathogen prevalence within populations by focussing on diseased animals.

The overall prevalence for *P. australasiensis* in all tuatara (captive and wild) tested by culture was 0.86%, and by LAMP was 14.9%. If the LAMP is correctly identifying the presence of *P. australasiensis*, then this result represents a large increase in the sensitivity of the testing which has important implications for our understanding of whether this organism is a primary or opportunistic pathogen. The addition of the LAMP assay to our testing procedure has enabled an increased detection rate of *P.*

*australasiensis* presence over culture alone. LAMP is a relatively new molecular diagnostic test, that has increased sensitivity over both culture and PCR (Senarath et al. 2014; Suleman, Mtshali, and Lane 2016), but in some cases false positives have been reported as a result of cross reaction of primers with environmental organisms (Senarath et al. 2014; Suleman, Mtshali, and Lane 2016). The specific LAMP assay used in this study was developed for this purpose and is currently in the process of validation. Based on the accurate sequencing of LAMP positive results that have currently been undertaken (R. Winkworth 2017 pers. comm.), this discussion will assume that the LAMP positive results are truly representative of *P. australasiensis* presence in the tested samples.

#### **4.1.2 Pathology of *Paranannizziopsis australasiensis* in tuatara**

*Paranannizziopsis australasiensis* was found to cause negligible pathology in most tuatara in this study. The majority (75%) of tuatara positive for *P. australasiensis* on LAMP had no detectable dermatitis lesions present. Body condition and haematological parameters were unaffected by *P. australasiensis* presence on the dermis. This suggests that this fungal pathogen can exist at a moderate prevalence, in tuatara populations without causing significant disease. This has similarly been described for *O. ophiodiicola* in some wild snake populations (Guthrie et al. 2015). The potential for reptilian hosts to act as asymptomatic carriers in the transmission of pathogens has not been investigated for any species of *Nannizziopsis*, *Paranannizziopsis* or *Ophidiomyces*. Only two previous studies have detected the related pathogen, *O. ophiodiicola*, on the dermis of snakes in the absence of lesions, and in these studies detection rates have been low (0.6% and 6%) (Paré et al. 2003; Allender, Bunick, et al. 2015). Our findings suggest that disease relates to more than just the simple presence of this organism, and that other factors such as environmental conditions, fungal load or host compromise may be a factor in development of disease. Further, asymptomatic carrier animals are likely to play a role in disease transmission between individuals and, historically, between populations via translocations.

*Paranannizziopsis australasiensis* was found to be the aetiology of multifocal mycogranulomas causing myositis in two captive tuatara in this study. One of these cases also developed an osteomyelitis of the right front foot, and the disease was ultimately fatal. These cases are the first described cases of deep tissue invasion by

*P. australasiensis* in tuatara, and the first described mortality associated with this disease. Previously described cases of *P. australasiensis* have all been superficial mycoses which respond well to topical and systemic treatment (Masters et al. 2016). Both cases had identifiable concurrent disease present, which likely resulted in immunocompromise and increased susceptibility to *P. australasiensis* infection.

*Paranannizziopsis australasiensis* was implicated in an ulcerative lesion from a third captive tuatara, however the failure to identify fungal hyphae on histology or isolate *P. australasiensis* from culture in this case meant the fungi was of questionable significance. Mild dermatitis lesions were seen on 20.3% of *P. australasiensis* positive tuatara. However, there was no significant association found between dermatitis lesions and a positive LAMP result which suggests that there are other important causes of dermatitis in the reptiles in this study. The significance of *P. australasiensis* in the observed lesions can only be inferred. I conclude, that given the high prevalence of *P. australasiensis* in asymptomatic tuatara, confirmation of the involvement of *P. australasiensis* in dermatitis lesions on reptiles, requires biopsies of the lesions for fungal culture, LAMP testing and histology.

#### **4.1.3 *Paranannizziopsis australasiensis* in endemic geckos and skinks**

*Paranannizziopsis australasiensis* was detected by LAMP in 18.8% of endemic skinks and geckos from captive, ecosanctuary and wild sources. *Paranannizziopsis australasiensis* was detected in twelve species where it has not previously been reported. No positive geckos or skink had any skin lesions present or identifiable health compromise. There were no positive results on culture for geckos or skinks. There have been no previous studies performed on identifying possible reservoir species involved in the transmission of other similar fungal pathogens. *Paranannizziopsis australasiensis* appears to have very little effect on native New Zealand reptiles. I conclude that *P. australasiensis* can act as a commensal organism in these species and their involvement as reservoir species in the transmission of *P. australasiensis* is likely.

#### **4.1.4 *Paranannizziopsis australasiensis* in soil samples**

*Paranannizziopsis australasiensis* was detected by LAMP in three out of eighty soil samples collected from tuatara burrows and high use areas of reptile enclosures, as

described in Chapter 2. Positive samples were collected from both captive (n=2) and wild (n=1) sites. The captive samples came from enclosures both with and without *P. australasiensis* positive reptiles. The detection of *P. australasiensis* DNA in the soil could be explained by recent contamination by an infected host, movement of fomites by keepers, or a false positive on LAMP from a cross reaction with a closely related fungal species. However, prolonged environmental survival of these types of fungal pathogens has been postulated by several authors and must be considered for *P. australasiensis*. While there have been few studies investigating the source of these fungi, current evidence suggests that *O. ophiodiicola* can persist both as an environmental saprophyte and a keratophilic pathogen of reptilian hosts (Allender, Baker, et al. 2015). Disease in tuatara caused by *P. australasiensis* has been observed to occur seasonally in captivity and re-infections following treatment are common (Masters et al. 2016; Pas 2017 pers. comm.). The detection of *P. australasiensis* DNA from soil in this study suggests that survival of this organism as an environmental saprophyte is likely and may be an important part of the transmission of this disease in captive and wild populations.

#### **4.1.5 *Paranannizziopsis australasiensis* on Stanley and Cuvier Island**

*Paranannizziopsis australasiensis* was found on both Stanley and Cuvier islands. However, *P. australasiensis* was not detected in any tuatara on Cuvier Island and tuatara were unable to be located on Stanley Island. *Paranannizziopsis australasiensis* was detected in Pacific Geckos (*D. pacificus*) and Common Geckos (*W. maculatus*), from Cuvier Island at a prevalence of 81.8% and 7.4% respectively, and an overall prevalence for all species tested (including tuatara) of 20.7%. On Stanley Island *P. australasiensis* was detected in Duvaucel's Geckos (*H. duvaucelii*) and Shore Skinks (*O. smithi*) at a prevalence of 3.8% and 7.4% respectively, and with an overall prevalence of 5.4%. *Paranannizziopsis australasiensis* was also detected in a single soil sample from Stanley Island. No pathology was detected in any positive reptiles from these two islands. From these findings I conclude that the release of captive bred tuatara, that have recovered from dermatitis associated with *P. australasiensis* or are carrying *P. australasiensis* on the dermis, in the absence of clinical disease, is likely to be of negligible risk to existing reptile populations on Stanley and Cuvier Islands. Resuming the breed for release programmes of healthy tuatara from Auckland and Hamilton Zoos is recommended.

The detection of *P. australasiensis* on these islands indicates that either this organism has been previously released there or naturally occurs there. The hypothesis that *P. australasiensis* had been introduced into New Zealand captive facilities through the international reptile trade, and subsequently released onto these islands through a single release event of affected captive-bred tuatara now seems unlikely due to the wide spread detection of *P. australasiensis* by LAMP in other wild sites in New Zealand. Translocations of reptiles have been an important conservation management tool for decades in New Zealand, and there have been numerous movements of tuatara and other reptile populations. There are no reports of tuatara or other reptiles being translocated from Cuvier Island directly to other wild sites. However, multiple translocations of Duvaucel's geckos directly from Stanley Island to three islands in the Hauraki Gulf, occurred between 2012 and 2014 providing an opportunity for the movement of *P. australasiensis* to other wild sites. It is therefore remotely possible that *P. australasiensis* may have been introduced to the wild from the release of captive bred tuatara. However, given these translocations have occurred relatively recently, it seems unlikely that the wide geographical distribution could be attributed entirely to a single release event. It is possible that *P. australasiensis* has been introduced into multiple wild populations through either multiple release events, or more likely that this is an endemic fungal species that originates from sources within New Zealand.

## 4.2 Dermatitis in Tuatara

There was no detection of *P. australasiensis* in 86.9% of tuatara with dermatitis. In the tuatara that had both mild dermatitis lesions and *P. australasiensis* detected by LAMP, causation can only be inferred, as biopsies were not performed on these lesions, and culture of all but two cases failed to detect *P. australasiensis*. The aetiology of these lesions were not investigated further, but I conclude there are other causes of dermatitis in tuatara that are more common than primary infection with *P. australasiensis*. Anecdotal reports of brown spots dating back several decades are therefore unlikely to represent a long history of undiagnosed *P. australasiensis* in tuatara in New Zealand. To investigate this hypothesis further, a review of 'Huia' the

New Zealand wildlife post mortem data base, and analysis by LAMP of archived mycotic dermatitis and osteomyelitis samples from multiple captive facilities around New Zealand is currently being conducted by the author and other researchers.

From this study superficial dermatitis lesions in tuatara were found to be common in captive, ecosanctuary and wild populations of tuatara, with an increased frequency of lesions in captive and ecosanctuary tuatara and geckos. Dermatitis is common in many species of captive reptiles. Decreased immune competence resulting from sub-optimal husbandry conditions and high environmental loads of saprophytes are well documented predisposing factors of dermatitis in reptiles (Schumacher 2003; Harvey et al. 2010). The finding of high levels of dermatitis in captive reptiles across New Zealand, with no association to the pathogen *P. australasiensis*, suggests that captive husbandry needs to be refined to better simulate the natural environment for these animals.

#### **4.3 Limitations of the study**

As this was a cross sectional survey, the results represent only one time point for each tuatara population with sampling spread over a period of two years. The prevalence of *P. australasiensis* has been observed to vary with season, with a higher incidence of clinical disease observed over the winter months in one captive population of tuatara (Jakob-Hoff 2014; Masters et al. 2016). Sample collection from wild tuatara during winter is not feasible due to behavioural torpor of tuatara and unsuitable weather conditions for field work. A single sample point does not provide detailed information of the pathogenesis of *P. australasiensis* positive tuatara over the course of an infection. The time of sampling may have coincided with recovery from, or the beginnings of clinical disease, and may therefore underestimate the virulence of this pathogen in tuatara.

The quality of samples able to be collected in the field is also a limiting factor in this study. Skin swabs are likely to collect only a low quantity of DNA, from the host and any possible pathogens present, whilst also being contaminated with environmental organisms. Equally difficult is maintaining the viability of these samples for culture

during prolonged periods in field conditions. Culture requires large quantities of viable organisms for growth (Bohuski et al. 2015; Paré and Sigler 2016), and thus sample collection and storage under field conditions may decrease the sensitivity of this test further. The utilisation of LAMP in this study helped to alleviate this limitation as this test is able to amplify very small amounts of DNA (Notomi et al. 2000), but this is tempered by a lack of detailed information on the specificity of the LAMP test. Sequencing of a subsample of positive LAMP results suggests that this test has a high degree of specificity for *P. australasiensis* (Winkworth 2017 pers. comm.) but further quantification of this would be ideal for future studies.

#### 4.4 Conservation Management Implications

These findings of wide spread occurrence and low virulence of disease caused by *P. australasiensis* in tuatara, endemic geckos and skinks have been used to inform the conservation management plan for tuatara. The Department of Conservation (DOC), in consultation with the Ministry for Primary Industries (MPI) and an advisory group (the *Paranannizziopsis australasiensis* Network for Tuatara Surveillance: (PaNTS)) decided to lift the quarantine on tuatara at affected captive facilities and the movement of tuatara between captive populations, ecosanctuaries and wild populations is no longer restricted based on *P. australasiensis* status. However, tuatara with dermatitis remain under quarantine and movement restrictions.

Disease caused by *P. australasiensis* in reptiles exotic to New Zealand has been reported to be rapid and fatal. Disease has been reported from captive central bearded dragons (*Pogona vitticeps*), Australasian water dragons (*Intellagama lesueurii*) and aquatic file snakes (*Acrochordus sp.*) (Sigler, Hambleton, and Paré 2013; Masters et al. 2016; Pas 2017 pers. comm.). Fatalities resulting from systemic spread and mycotic hepatitis has been reported in bearded dragons. Increased virulence of *P. australasiensis* in these species is suspected to be related to the hosts higher preferred body temperature, and environmental conditions, in comparison to tuatara and other New Zealand reptiles. Temperature requirements for these species fall within the optimal growth range of *P. australasiensis* of 25°C to 30°C (Masters et al. 2016). Where institutes house both exotic and native reptiles, vigilance in quarantine

screening and separation of housing, servicing personnel and equipment should be applied to prevent the transmission of *P. australasiensis* from native reptiles to other species where disease is likely to be fatal.

The hypothesis that this organism was introduced into New Zealand through the importation of exotic reptiles now seems unlikely. The high virulence of disease seen in exotic reptile species compared to the low virulence detected in tuatara and other endemic reptiles, makes it probable that this organism has spread from New Zealand reptiles to captive exotic reptiles. The wide spread detection of *P. australasiensis* across multiple endemic reptile populations in New Zealand further supports this theory. Wild populations sampled in this study have all had historical translocations of reptiles documented (Wilson 2010; Cree, 2014; Romijn and Hartley 2016), with the exception of Hen Island. The detection of *P. australasiensis* in tuatara of Hen island at a prevalence of 25.8% is further evidence to support this being an organism originating from sources in New Zealand. However, Hen island has previously suffered from pacific rat (*Rattus exulans*) incursions, and subsequent pest elimination activities, as well as being used as site for translocations of invertebrates and Little Spotted Kiwi (*Apteryx owenii*) (Jolly, 1991), and intermittent monitoring of tuatara populations (Cree, 2014). The historical activity on this island confounds interpretation of the presence of *P. australasiensis*, as spread may have occurred through fomites on contaminated equipment or personnel. To conclusively disprove the theory that exotic reptiles imported this disease into New Zealand, testing of an island population where no previous translocations have occurred, and little human presence has been reported would be required. Alternatively, the testing of skin samples from reptile specimens in museums that predate the importation of exotic reptiles into New Zealand could be performed.

#### **4.5 Areas of further research**

Koch's postulates have been fulfilled for *O. ophioidiicola* and *N. dermatitis* as primary pathogens (Paré et al. 2006; Allender, Baker, et al. 2015; Lorch et al. 2015) and this information has been extrapolated to include similar organisms such as *P. australasiensis* (Sigler, Hambleton, and Paré 2013). However the results of my study

suggest that *P. australasiensis* is not a primary pathogen in native New Zealand reptiles. Studies that further investigate the causal relationship of *P. australasiensis* and dermatitis lesions in tuatara are warranted. In future studies biopsies and histology of lesions where *P. australasiensis* has been detected by molecular tools would be useful to establish the role of this fungus in the observed lesions. Experimental infection studies would also be of use but are unlikely to be approved by ethics committees for this iconic species. My study suggests that there are other causes of superficial dermatitis in tuatara in New Zealand and it may be of more value to the conservation management of the species to investigate the role of other organisms and environmental and husbandry factors in the development of the relatively common dermatitis observed.

Towards the conclusion of this study a second *Onygenales* fungal species, *Paranannizziopsis californiensis*, was detected in tuatara at Auckland Zoo. Dermatitis lesions associated with this fungal organism, in tuatara appear clinically indistinguishable from lesions caused by *P. australasiensis*. Diagnosis of this organism has been made based on histology, culture, sequencing and LAMP (Ha pers. 2017 comm.; Pas 2017 pers. comm.; Winkworth 2017 pers. comm.). Both *P. australasiensis*, and *P. californiensis* have been sequenced and are available from GenBank (*P. australasiensis* ITS KF477218 IGS KP691520 SSU KF466866 and *P. californiensis* ITS KF477224 IGS KP691521 SSU 446867) (Sigler, Hambleton, and Paré 2013; Bohuski, 2015). A specific LAMP has been developed to enable accurate detection of *P. californiensis*. The LAMP assay uses primers that target a section of the intergenic spacer which differs in nucleotide length in comparison to *P. australasiensis*, and *P. crustacea*. No cross reaction has been detected between the *P. australasiensis* LAMP primers, and *P. californiensis* LAMP primers (Winkworth 2017 unpubl.).

The distribution of *P. californiensis* in New Zealand has not been studied. In our study *P. californiensis* would have been detected on culture of lesion samples if large numbers of viable organism were present. However due to the low specificity of culture these infections may have been missed. The LAMP developed for *P. australasiensis* would not detect *P. californiensis* as this test is specific for *P. australasiensis* only.

Given the high occurrence of dermatitis lesions in tuatara, the poor association with dermatitis and *P. australasiensis*, and the long history of lesions in captivity, further work on the prevalence of *P. californiensis* in New Zealand is warranted.

The mode of transmission of *P. australasiensis* has not yet been established, and current recommendations are frequently made by extrapolating information from associated fungal species. The detection of *P. australasiensis* by LAMP in soil samples, suggests that this pathogen may survive as an environmental saprophyte. Studies determining the specific ability of *P. australasiensis* to survive and multiply in varying environmental conditions are required, to enable greater understanding of transmission, and therefore disease mitigation.

#### 4.6 Conclusions

*Paranannizziopsis australasiensis* is widespread in captive and wild populations of native New Zealand reptiles. Superficial dermatitis is also very common among these reptiles, with a higher frequency of dermatitis in captive animals. There was no significant association of *P. australasiensis* presence and dermatitis in this study suggesting that *P. australasiensis* is not the primary cause of dermatitis in most of the reptiles examined. In tuatara with both dermatitis and molecular detection of *P. australasiensis*, there was a low virulence of disease, with many LAMP positive tuatara being asymptomatic. Increased severity of disease with strong evidence of *P. australasiensis* involvement was seen in two captive tuatara, where concurrent disease was present. I conclude that *P. australasiensis* is not a threat to wild populations of tuatara or other endemic reptiles, and that disease caused by *P. australasiensis* is more severe when the hosts immune system is compromised by disease or other stressors occurring in captivity. This suggests that unlike other members of this fungal group, *P. australasiensis*, in New Zealand reptiles, is not a primary pathogen, but may be better described as an opportunistic keratophilic pathogen, and in most cases as a saprophytic fungus. In other reptile hosts where *P. australasiensis* was detected, no disease was identified, and it is hypothesised that *P. australasiensis* occurs as a commensal organism on these species, serving as a reservoir for transmission.

The wide spread occurrence of *P. australasiensis* at a low level of virulence reported in this study leads to the probability that this is an endemic organism from New Zealand, rather than an invasive pathogen. While there are no similar populations outside of New Zealand for comparison, based on the characteristics of the New Zealand environment and the growth of *P. australasiensis* in vitro, I propose that lower environmentally driven growth rates are the main mechanism by which *P. australasiensis* maintains moderate levels of low virulence in tuatara populations in New Zealand.

The hypothesis for this study was that *P. australasiensis* had been released into isolated wild populations with the translocation of captive bred tuatara. While I have not conclusively disproven this hypothesis, based on my findings of the wide geographical occurrence and low virulence of *P. australasiensis* in New Zealand reptiles, this organism is unlikely to have been introduced to New Zealand from a single release event. The origins of *P. australasiensis* in wild populations may instead be from multiple release events, and translocations, or more likely this organism may have originated in the New Zealand environment or herpetofauna.

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