Aspects of the pharmacokinetics of itraconazole and voriconazole in the tuatara (*Sphenodon punctatus*) and application in the treatment of an emerging fungal disease

Dr Sarah Alexander BSc BVMS PGCert (Cons Med)

A dissertation presented to Murdoch University in partial fulfilment of the requirements for a Doctor of Veterinary Medical Science

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“DON’T PANIC”

- Douglas Adams

Declaration of originality

This professional doctorate thesis was completed as part of the requirements for the Doctor of Veterinary Medical Science degree at Murdoch University, in conjunction with undertaking postgraduate coursework units and clinical training in wildlife and zoo medicine at Auckland Zoo. I declare that this thesis is my own account of my research and contains as its main content, work that has not been previously submitted for a degree at any tertiary education institution.

Dr Sarah Alexander BSc BVMS PGCert (Cons med)

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Abstract

Tuatara (*Sphenodon punctatus*) are unique, cold-adapted reptiles endemic to New Zealand. Recently, captive tuatara have been found to be affected by an emerging fungal pathogen, *Paranannizziopsis australasiensis*. *P. australasiensis* causes dermatitis in tuatara, and has caused fatal systemic mycosis in a bearded dragon (*Pogona vitticeps*), and in aquatic file snakes (*Acrochordus* spp). The discovery of *P. australasiensis* infections has prevented the release of tuatara from several captive institutions to offshore islands, and has negative implications for the long-term health and welfare of the animals.

A review of the literature revealed that infections caused by organisms related to *P. australasiensis* are being recognised worldwide as emerging pathogens of reptiles. Little is known about the epidemiology of these often-fatal infections, and treatment with a range of antifungals has met with varying success. There has been little research on antifungal use in reptiles, and none on how environmental temperature affects the pharmacokinetics of antifungals.

This study investigated the microbiological characteristics of *P. australasiensis*, primarily the growth rate of the fungus at different temperatures, and the Minimum Inhibitory Concentration (MIC) of various antifungal agents for *P. australasiensis*. It was determined that the optimal growth temperature for *P. australasiensis* encompasses the range from 20^{o}C-30^{o}C, with scant growth at 12^{o}C, moderate growth at 15^{o}C, and no growth at 37^{o}C. The MICs of antifungals were tested at room temperature and at 37^{o}C, and were not found to be significantly different. MICs of itraconazole and voriconazole

for three isolates of *P. australasiensis* were found to be low, at 0.12mg/L for itraconazole and <0.008mg/L for voriconazole.

The single and multiple dose pharmacokinetics of itraconazole and voriconazole in tuatara were investigated at 12 and 20^{o}C; these are the high and low ends of the tuatara's preferred optimal temperature zone (POTZ). Results showed statistically significant differences in antifungal elimination half-life between temperatures. With the aid of population pharmacokinetic modelling, optimal dosing regimes for both antifungals were developed for tuatara of different weights. It was established that tuatara should be treated at 20^{o}C, at the high end of POTZ, to facilitate rapid attainment of therapeutic antifungal concentrations, improve clinical outcomes and reduce the risk of adverse effects.

While itraconazole demonstrated more predictable pharmacokinetics than voriconazole in tuatara, itraconazole treatment was associated with significant adverse effects. These included elevated bile acids and uric acid concentrations, and weight loss. While voriconazole appears to be safer, its pharmacokinetics are less predictable, with high inter-individual variability in tuatara administered the same dose rate (a phenomenon also observed in humans). While voriconazole may be a useful antifungal in clinically affected tuatara where dosage can be adjusted based on the response to treatment, its use in an asymptomatic quarantine setting may be limited. The use of higher voriconazole doses may increase the likelihood of maintaining therapeutic concentrations in all treated animals, however the risk of adverse effects increases concomitantly. Furthermore, there are currently no published reports of successful treatment of *P. australasiensis* in tuatara with voriconazole.

This study also established haematologic and biochemical reference ranges in a group of tuatara. These demonstrated variability in several parameters based on sex and season, and will be a useful tool for assessing health and disease in these and other tuatara.

Publications from or relating to this thesis

Peer reviewed:

Masters N*, **Alexander S***, Jackson B et al. 2016. ´´Dermatomycosis caused by *Paranannizziopsis australasiensis* in five tuatara (*Sphenodon punctatus*) and a coastal bearded dragon *(Pogona barbata*) in a zoological collection in New Zealand.´´ *New Zealand Veterinary Journal* 64(5): 301-307. * Joint first authors

Humphrey S, **Alexander S**, Ha HJ. 2016. ´´Detection of *Paranannizziopsis australasiensis* in tuatara (*Sphenodon punctatus*) using fungal culture and a generic fungal PCR.´´ New Zealand Veterinary Journal 64(5): 298-300.

Alexander S. 2018 (in press). ´´Tuatara Biology and Husbandry.´´ In: *Mader´s Reptile Medicine and Surgery 3rd edition*, edited by Steve Divers and Scott Stahl. Elsevier.

Alexander S. 2018 (in press). ´´Tuatara Taxonomy, Anatomy, Physiology and Behavior.´´ In: *Mader´s Reptile Medicine and Surgery 3rd edition*, edited by Steve Divers and Scott Stahl. Elsevier.

Media:

Auckland Zoo. 2016. Tuatara treatment with resident vet Sarah Alexander. Produced by Auckland Zoo. http://www.aucklandzoo.co.nz/sites/news/media-releases/Tuatara-research

Conferences and presentations:

Alexander S, Holford N, Paterson S, et al. 2015. ´´A multi-disciplinary approach to investigation of the emerging fungal pathogen *Paranannizziopsis australasiensis* in Tuatara (*Sphenodon punctatus*)´´. Paper presented at the Wildlife Disease Association international Conference, Maroochydore, Australia July 26-30.

Alexander S. 2014. ´´Fungal dermatitis caused by *Paranannizziopsis australiensis* in tuatara (*Sphenodon punctatus*).´´ Paper presented at the Combined Exotics and Avian Conference, UEP/ARAV/AAVAC, Cairns, Australia April 22-25.

Alexander S, Holford N, Paterson S, et al. 2016. ´´Pharmacokinetics of voriconazole in tuatara (*Sphenodon punctatus*).´´ Poster presented at the AAZV/EAZWV/IZWV Conference, Atlanta, Georgia, USA July 16-22.

Alexander S. 2016. "Antifungal pharmacokinetics and sleepless nights with tuatara." Paper presented at the combined New Zealand Wildlife Society and Wildlife Disease Association Australasia Conference, Christchurch, New Zealand, November 26-31.

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Glossary of abbreviations

ALP = Alkaline phosphatase

ALT = Alanine aminotransferase

AST = Aspartate aminotransferase

CANV = *Chrysosporium* anamorph of *Nannizziopsis vriesii*

CHL = Canterbury Health Laboratories

CL = clearance

CLSI = Clinical and Laboratory Standards Institute

CK = Creatine kinase

Css = steady-state concentration

DNA = Deoxyribonucleic acid

DRA = Disease Risk Analysis

h = hour

IM = intramuscular

IV = intravenous

Km = Michaelis-Menten constant

L = litres

L/h = litres per hour

MIC = Minimum Inhibitory Concentration

MPI = Ministry of Primary Industries

mg = milligrams

mg/L*h = milligrams per litre per hour

mL = millilitres

mol/L = moles per litre

NONMEM = Nonlinear mixed effects modelling

NZVP = New Zealand Veterinary Pathology

kg = kilogram

Km = a measure of the affinity of a substrate (drug) for an enzyme. Traditionally it is the drug concentration at half of Vmax

OH-itra = hydroxy-itraconazole

PA = *Paranannizziopsis australasiensis*

PCR = Polymerase Chain Reaction

PO = per os., to be taken orally

POTZ = Preferred Optimal Temperature Zone

RSE = Relative standard error

RUV = random unexplained variability

SD = standard deviation

SDA = Sabouraud Dextrose Agar

SFD = Snake Fungal Disease

SID = once daily

Tmax = time to maximal plasma concentration

$T_{1/2}$ = elimination half-life

µg = micrograms

V = volume of distribution

Vmax = maximum rate of drug metabolism, usually related to enzymatic saturation

Glossary of definitions

Absorption half-life

The time taken for half the administered drug dose to be absorbed into the bloodstream.

Acrodont

Teeth that are attached to the jawbone without sockets.

Area under the curve

The area under the graphical curve in a linear drug concentration vs time plot. Is a measure of drug exposure over time.

Bioavailability (oral)

The percentage of orally administered drug that is absorbed through the gastrointestinal system, as compared to the same amount of drug administered intravenously.

Bootstrap estimates

A method of reliably estimating confidence intervals in a population using resampling from a population subset.

Chrysosporium anamorph of *Nannizziopsis vriesii* (CANV) complex

Group of primary reptile fungal pathogens comprised of organisms from the *Nannizziopsis*, *Paranannizziopsis* and *Ophidiomyces* spp.

Elimination half-life ($t_{1/2}$)

The amount of time taken for the concentration of drug in the bloodstream to halve.

First-order absorption

When a constant proportion of drug is absorbed per unit time (cf zero order absorption).

First-order elimination

When a constant proportion of drug is eliminated per unit time (cf zero order elimination). Elimination mechanisms are non-saturable.

Fomite

An inanimate object or material on which disease-producing agents may be conveyed.

Gastrointestinal transit time

The time from ingestion of food to the defecation of its digested remains.

Isolate/isolates (noun)

Specimen of microbiological material from one species of organism, in this context primarily fungal.

iwi

The New Zealand Māori word for a set of people bound together by descent from a common ancestor or ancestors. Modern meaning: tribe. Is not capitalised.

Km (Michaelis-Menten constant)

a measure of the affinity of a substrate (drug) for an enzyme. Traditionally it is the drug concentration at half of Vmax.

Linear pharmacokinetics

Kinetics where there is a linear relationship between drug dose and plasma drug concentration. There is no saturable mechanism involved.

Mass-specific metabolic rate

The resting energy expenditure of an organism per unit of body mass per day, often expressed in kcal/kg/day.

McFarland

A measurement of cell concentration in solution by optical density.

Minimum Inhibitory Concentration

The lowest concentration of an antimicrobial substance that inhibits growth of the target organism.

Mixed order elimination

When first order (concentration-dependent, non-saturable) elimination is followed by zero order (saturable) elimination.

Nonlinear mixed effect model (NONMEM)

Pharmacokinetic modelling method that accounts for both fixed and random effects, allowing population predictions to be made from sparse data. Also known as population pharmacokinetic modelling.

Non-linear pharmacokinetics

Kinetics resulting from saturable drug transfer (usually protein binding, hepatic metabolic enzymes or active renal transport), where with increasing drug dose past the capacity of the enzymes to metabolise, plasma drug concentration increases by a factor higher than the increase in administered drug dose. The relationship between drug dose and plasma drug concentration is non-linear.

One-compartment distribution

A standard PK modelling approach, where the body is treated as a single homogenous compartment into which drugs are distributed.

Paranannizziopsis australasiensis

A primary dermal fungal pathogen of reptiles, found in tuatara and aquatic file snakes.

Pharmacokinetics

The study of absorption, distribution, metabolism and elimination of medications.

Preferred Optimal Temperature Zone (POTZ)

The temperature range at which an ectothermic species that allows for optimal biological function. This range is different for each species.

Random unexplained variability (RUV)

A measure of random error, which is always present in modelling and is unpredictable. Quantifying RUV gives a measure of how reliable model-based estimates are, and using the correct structural model decreases RUV.

Relative standard error (RSE)

Mathematical tool for assessing reliability of an estimate. The higher an RSE, the less reliable the estimate (some sources quote an RSE of over 30% as unreliable).

Residual error

The difference between an observation and the model prediction of the observation. Used to describe what is leftover after all other sources of variability (such as between subject variability) have been accounted for.

Snout-vent length

The length of an animal when straight, from the tip of the snout to the cloaca.

Steady-state concentration (Css)

Drug concentration where the rate of drug input is equal to the rate of drug elimination.

Time to maximal drug concentration (Tmax)

The time taken for an orally-administered drug to reach its maximal plasma concentration.

Trough concentration

The lowest concentration of a drug when multiple doses are administered; typically found immediately prior to administration of the next dose.

Vmax

Maximum rate of drug metabolism, usually related to enzymatic saturation. Even if drug dosage is increased, the rate that the drug is metabolised cannot increase further and so drug accumulates.

Zero order elimination

When a constant amount of drug is eliminated per unit time (cf first order elimination). Elimination mechanisms are saturable.

Chapter 1: Introduction and literature review

1.1 Tuatara

1.1.1 Taxonomy, distribution and ecology

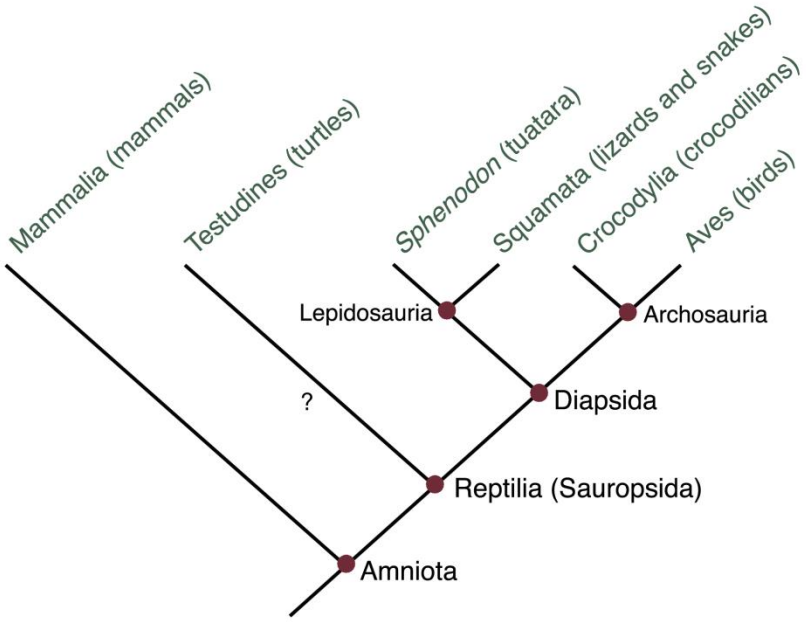
Tuatara (*Sphenodon punctatus*) are the only extant members of the order Rhynchocephalia, a once widespread and moderately diverse group (Cree 2014). They are members of the superorder Lepidosauria, which includes modern lizards and snakes. The ancestors of tuatara probably diverged from snakes and lizards in the early Triassic, an estimated 240-250 million years ago (Evans and Jones 2010) (Figure 1.1). *Sphenodon punctatus* is currently the only recognised species of tuatara, although the Brothers Island subpopulation was briefly classified as a different species (*S. guntheri*) in the 1990s on the basis of variation in blood proteins (Daugherty et al. 1990). Subsequent molecular studies have confirmed these animals all belong to the single species *S. punctatus* (Hay et al. 2010), and they are currently managed as a single species with distinct geographic variants.

The name 'tuatara' is derived from the Maori language, where 'tua' means back or far side, and 'tara' means spike or spine. This is translated as 'peaks on the back', a reference to the tuatara's spiny dorsal crest. The scientific name *Sphenodon* is derived from the Greek words for wedge (sphen) and tooth (odous), describing the wedge-shaped acrodont teeth of tuatara. The species name *punctatus* refers to the large number of spots on the tuatara's body.

Tuatara superficially resemble terrestrial lizards but, as mentioned previously, belong to their own order. Size varies with location, with animals on larger islands consistently being bigger than their counterparts on smaller islands (Cree 2014). Males in the

northerly Poor Knights Islands have been found to weigh up to 1.1kg, with a snout-vent length of 311mm, while males on the cooler, southerly North Brothers Island reached 655g and 256mm in snout-vent length in the same period (Cree 2014). Males are larger than females. The dorsal scales of tuatara are small, granular and usually an olive-brown colour, though green, orange and grey variants exist. The ventral scales are larger and pale grey coloured. The dorsal surface of the animal is covered in white-yellow spots, and there is a spiny crest that runs from head to tail, which is more prominent in males. Tuatara have acrodont dentition, with two parallel rows of teeth in the upper jaw interlocking with one row in the lower jaw.

Figure 1.1 A diagram summarising recent views about the phylogenetic relationships of living amniotes. The branching indicates relative, not absolute, time scale. The placement of turtles is debated, with some recent molecular analyses suggesting a placement within Diapsida. Reproduced with permission (Cree 2014 p.39).

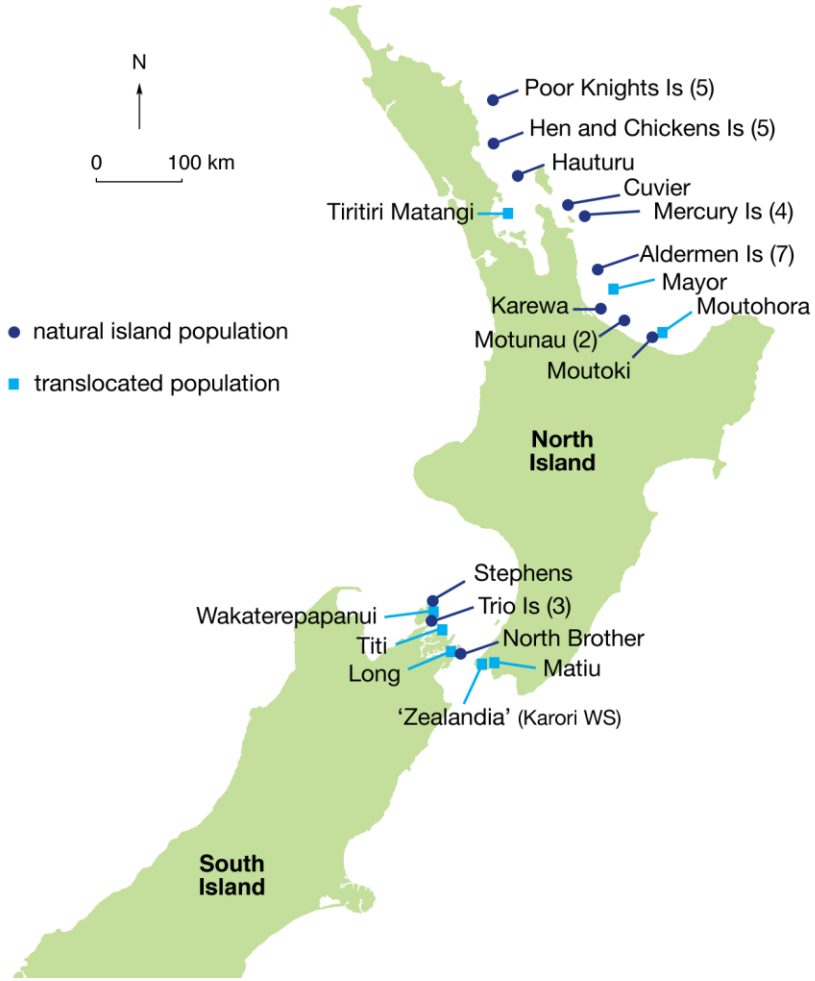


Prior to the arrival of Polynesians and rats, tuatara were widely distributed on both the North and South Islands of New Zealand. Today they are restricted to an estimated 32 offshore islands and several mainland sanctuaries, totalling approximately 0.5% of their pre-human arrival distribution (Cree and Butler 1993). The majority of these islands are in northern New Zealand, with only five in the Cook Strait (between the North and South Islands) and no naturally occurring populations further south. Islands where tuatara are naturally present tend to be cliff-bound, temperate and largely or completely frost-free (Cree 2014). Mean annual temperatures on corresponding mainland latitudes range from 14.1-16.1^{o}C in the north, and 12.1-14.0^{o}C in Cook Strait (National Institute of Water and Atmospheric Research 2015). Larger islands are forested, but smaller islands may only have low, wind-shorn plants. Tuatara populations have survived in sheep pastures on Stephens Island (albeit at lower densities), including under dwellings and lighthouses (Newman 1986, Cree 2014). There are no tuatara on islands where Norway rats (*Rattus norvegicus*) or ship rats (*Rattus rattus*) have been introduced, though pacific rats (*Rattus exulans*) have co-existed with tuatara on nine islands prior to their eradication from all but one island (Cree 2014). Predation by pacific rats is thought to have played a large part in tuatara population declines (Worthy and Holdaway 1995, Towns et al. 2007).

Tuatara are primarily nocturnal, but do emerge during the day to bask. Juvenile tuatara are diurnal for the first few months of life (Whitworth 2006). In forest environments tuatara show little activity in the morning, with a slight increase in the afternoon, and highest activity levels between 6pm and midnight (Gillingham and Miller 1991). Nocturnal emergence is highest when conditions are warm, calm and misty or raining, and appears to coincide with the time of highest density of invertebrate prey (Walls 1983). Stephens Island tuatara are primarily insectivorous, eating mainly beetles with a

mix of other invertebrates. Other dietary items depend on the tuatara's size, habitat and food availability, but include kawakawa (*Macropiper excelsum*) berries, frogs, skinks, geckos, fairy prion eggs and chicks, fluttering shearwater chicks, sparrows and even juvenile tuatara (Newman 1977, Walls 1981, Moore and Godfrey 2006, Bredwig and Nelson 2010).

Figure 1.2 Distribution of free-living tuatara in 2011. Natural populations exist on 32 islands (dark blue circles; the number of islands with tuatara in each major group is indicated). In addition, between 1995 and 2011 tuatara were translocated to seven islands and one large mainland sanctuary (light blue squares). Reproduced with permission (Cree 2014 p.217).



1.1.2 Thermal physiology and metabolic rate

Tuatara are nocturnal ectotherms, relying on environmental heat sources to raise their body temperature above that of their surroundings. Behaviours such as basking and burrowing are the most common methods of thermal regulation. Tuatara can raise their body temperatures by 9°C or more above the surrounding air temperature by basking, and at night may experience temperatures of 7°C or lower without adverse effects (Cree 2014). Tuatara have a region of relative thermal independence between the temperatures of 12- 20°C, and within this temperature range metabolic rate does not increase in a linear fashion with temperature, and is lower than would otherwise be predicted (Cartland and Grimmond 1994).

Wild tuatara have been observed to be nocturnally active when their body temperatures are between 5.5-20.5°C, but this can increase to 30°C when basking (Cree 2014). Cloacal temperature varies seasonally and by time of day, with temperatures found to average between 7.6-10.7°C in winter, and 11.5-23.9°C in summer in a wild population on Stephens Island (Cree et al. 1990). Recommended temperature ranges for captive animals range between 4-15°C in winter and 10-25°C in summer (Boardman and Blanchard 2006). Several studies have evaluated temperature preferences of captive tuatara placed in a thermal gradient, with mean selected temperatures of 20-22°C (Cree 2014). In one study using juvenile tuatara, no significant differences were observed in the time it took animals to catch prey between 12 °C and 20°C, though it was significantly decreased at 5°C (Besson and Cree 2010). Gastrointestinal transit time was significantly slower at the lower temperatures, and results suggested there was no digestion of prey items at 5°C (Besson and Cree 2010). Studies on a wild population of tuatara on Stephens

Island report that gastric emptying time was 20-24 hours during summer, and up to 48 hours in spring (Fraser 1993). No data on gastric emptying time in winter was available.

Tuatara in cool conditions have been observed to survive for months without food, and studies of metabolic rates suggest that ectothermic reptiles' energy demands may be one-thirtieth that of a similar sized endotherm on an annual basis (Werner and Whitaker 1978). Metabolic rate in tuatara, as measured by oxygen consumption, varied depending on body mass, activity, and temperature, with juveniles having higher mass-specific metabolic rates than adults (Cartland and Grimmond 1994). Tuatara are active at lower metabolic rates than many lizards that are not native to New Zealand, whose activity temperatures often range between 20 ^{o}C and 45^{o}C (Thompson and Daugherty 1998). Their metabolic rate at 13 ^{o}C is similar to that of lizards and some turtles at the same temperature, the difference being that tuatara are active at these temperatures, while many other reptiles are not (Cartland and Grimmond 1994, Thompson and Daugherty 1998).

1.1.3 History of dermatitis in tuatara at Auckland Zoo

Tuatara were first acquired by Auckland Zoo in 1922. A captive breeding program was subsequently initiated, with the first releases occurring in 1996, and animals were repatriated to offshore islands to bolster wild populations. After the first diagnosis of mycotic dermatitis caused by *Paranannizziopsis australasiensis* in 2010, tuatara releases from the zoo were halted to prevent transmission of this organism to wild populations. *P. australasiensis* dermatitis has not been found in any wild populations of tuatara or other New Zealand reptiles to date, though surveillance has been limited.

Dermatitis in tuatara at Auckland Zoo has been a regular presentation in captive animals. Lesions are typically minor and present as yellow-brown discolourations of individual or small groups of scales, commonly on the ventrum and flanks (Figure 1.3). These can progress to erosions and ulcerations in more severe cases (Figure 1.4). Historical medical records covering a 20 year period showed that 57% of tuatara at Auckland Zoo were diagnosed with dermatitis at least once during their time at the zoo, with a higher number of cases in winter than in summer (Jakob-Hoff 2014). In the period 2001-2009 there were a total of 72 cases of dermatitis in tuatara, though it is unknown what proportion of these were actually caused by *P. australasiensis*. This gives an incidence rate of dermatitis of 0.27 cases per tuatara-year at risk for this nine-year period.

Figure 1.3 Confirmed fungal skin lesions (arrowheads) on the ventrolateral gular region of an adult female tuatara.

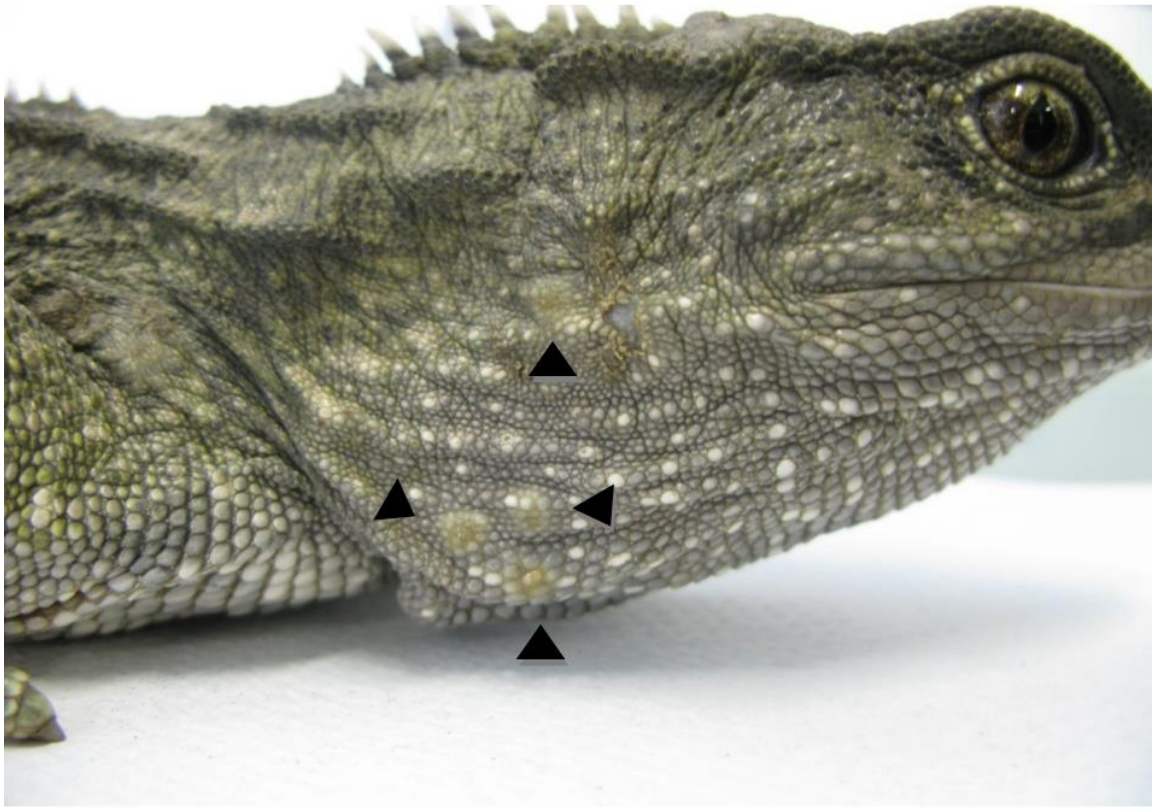


Figure 1.4 Brown discolouration typical of tuatara dermatitis cases, with progression to erosion and ulceration in some areas.

Investigation of these conditions was often not undertaken due to the minor nature of the skin lesions, however when biopsies and cultures were performed, a range of bacteria and fungi, presumed to be commensals or secondary invaders, were isolated. It was not until 2010 that the causative fungus was isolated and identified from biopsy specimens via specialised culture methods, with the aid of collaborators at the University of Alberta Microfungus Collection and Herbarium in Canada. Since then, more cases have been diagnosed and treated successfully with antifungal medications. Diagnosis of *P. australasiensis* at Auckland Zoo is currently based on histopathological findings, macro-and microscopic appearance of fungi when cultured, and PCR and sequencing of culture products.

Prior to 2010, treatment of dermatitis in tuatara was largely empirical. The majority of cases resolved with topical and/or systemic treatment with a range of medications, commonly topical povidone-iodine with the addition of systemic enrofloxacin in more severe cases. These cases often required prolonged treatment, and relapses occurred. Since the diagnosis of primary fungal dermatitis caused by *P. australasiensis*, treatments have been more targeted with the use of itraconazole at 3-5 mg/kg PO SID for up to six weeks, with or without topical 1% terbinafine cream. Excisional biopsy has occasionally been curative in the absence of systemic treatment.

1.2 Paranannizziopsis australasiensis and related infections

Fungal dermatitis caused by *P. australasiensis* is now recognised as a newly emerging disease detected in tuatara (*Sphenodon punctatus*) at Auckland and Hamilton Zoos. *P. australasiensis* has also caused fatal dermatitis in a bearded dragon (*Pogona barbata*) at Auckland Zoo, and in several file snakes (*Acrochordus* sp.) at Melbourne Zoo (Sigler et al. 2013). Prior to speciation, *P. australasiensis* was known as the *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV), of the family Onygenaceae and order Onygenales (Sigler et al. 2013). It is now known that there are many organisms from several genera that were previously classified under the CANV umbrella, and these organisms share many characteristics. This review examines characteristics of, and infections caused by, organisms previously known as CANV in reptiles.

1.2.1 Molecular characteristics, host specificity and pathogenicity

Members of the CANV complex are keratinophilic, ascomycetous fungi (Bowman et al. 2007). Sigler et al. (2013) conducted genetic sequencing of fungal isolates that were part

of the CANV complex, and have reclassified them into three genera – *Nannizziopsis*, *Paranannizzopsis*, and *Ophidiomyces*, in the family Onygenales. This was based on DNA sequences from the internal transcribed spacer (ITS) and small subunit (SSU) regions of the nuclear ribosomal RNA (rRNA) gene (Sigler et al. 2013).

Following sequencing, it was noted that each genus appears to display a level of host specificity (Sigler et al. 2013, Paré and Sigler 2016). *Nannizziopsis* species were isolated from lizards and crocodiles, but not snakes, while *Ophidiomyces* species were found only in snakes. *Paranannizziopsis* species showed less host specificity, and while members of this species have been isolated largely from highly aquatic snakes, *P. australasiensis* has been isolated from species as diverse as tuatara, bearded dragons, and file snakes (Sigler et al. 2013).

Ophidiomyces ophiodiicola has been identified as the cause of disease in colubrids, elapids, boids, pythonids and acrochordids, and has been predominantly found in recently caught captive animals (Sigler et al. 2013). *O. ophiodiicola* has been identified as the cause of 'snake fungal disease' in wild crotalid snakes in Illinois, Virginia and New England (Allender et al. 2011, Sleeman 2013, Smith et al. 2013, Guthrie et al. 2016). *O. ophiodiicola* is currently the only described member of the genus *Ophidiomyces*.

The number of cases of fungal dermatitis in reptiles caused by the CANV complex is high, compared to the number of cases reported to be caused by fungi that are commonly found on reptilian skin, such as *Aspergillus* and *Penicillium* species (Paré et al. 2006).

Three studies have evaluated members of the CANV complex for their pathogenicity. *Nannizziopsis dermatitidis* was determined to be a primary pathogen in veiled

chameleons (*Chamaeleo calyptratus*), while *Ophidiomyces ophiodiicola* was experimentally determined to be the cause of snake fungal disease (SFD) in cottonmouths (*Agkistrodon piscivorous*) and corn snakes (*Pantheropus guttatus*) (Paré et al. 2006, Allender et al. 2015b, Lorch et al. 2015). All three studies concluded that while a breach in skin integrity increased the prevalence and severity of infection, it was not necessary for the development of disease.

The study by Pare et al (2006) also evaluated environmental exposure to *N. dermatitidis* as a transmission route. A single unexposed control animal, and one exposed via a hanging gauze strip in the cage, developed *N. dermatitidis* infection. In this study, three cultures from cage top filters and one from a settle plate in the room also cultured *N. dermatitidis*, indicating the potential for aerosol spread and dissemination in the captive environment (Paré et al. 2006). It was considered that spread by this method was the likely cause of the case found in the unexposed control animal. The authors concluded that CANV complex infections are contagious, a view supported by other authors (Bowman et al. 2007), and can spread via indirect contact or fomites (Paré et al. 2006). This is consistent with a report of CANV (subsequently confirmed to be *Ophidiomyces ophiodiicola*) in brown tree snakes (*Boiga irregularis**)***, where four animals out of a group of 12 were affected, but there was no direct contact between several of the affected animals (Nichols et al. 1999).

CANV complex organisms have not been commonly detected in humans. Human isolates tested by Sigler et al. (2013) were from the *Nannizziopsis* genus, and the majority were from HIV positive individuals. None of the species recovered from humans were

detected in reptiles, which may indicate there is little zoonotic risk associated with handling affected animals (Sigler et al. 2013).

1.2.2 Epidemiology

Little data has been compiled on the epidemiology of infections caused by the CANV complex, with the majority of information coming from case reports rather than a systematic review of a large number of cases.

1.2.2.1 Prevalence

The majority of cutaneous mycoses in squamates have been attributed to a wide variety of fungi of soil origin (Paré et al. 1997). Demonstrating a pathogenic role for these fungi is often difficult, as they are frequently isolated as contaminants (Paré et al. 1997, Nichols et al. 1999, Thomas et al. 2002).

Infections caused by CANV complex organisms were not reported widely in the literature until very recently, however it has been postulated by several authors that infections are likely to be misdiagnosed or poorly recognised for a variety of reasons (Paré et al. 1997, Nichols et al. 1999, Sigler et al. 2013). These include difficulty in culturing the organism, historical lack of availability of advanced molecular diagnostics, reluctance of many pet owners to pursue disease investigation, and the commonly-held belief that fungal infections in reptiles are secondary to underlying illness, injury or suboptimal husbandry (Abarca et al. 2008, Mitchell and Walden 2013). Bowman et al. (2007) suggest that CANV complex infections are common in pet bearded dragons, and have been colloquially known as ‘yellow fungus disease’. It has been suggested that CANV complex-related infections may be more common than currently thought in crocodiles, but

cultures are often overgrown by environmental fungi such as *Fusarium* species and *Purpureocillium lilacinum* (Thomas et al. 2002, Sigler et al. 2013).

Infections have been documented in squamates, tuatara and crocodilians, but not chelonians. The majority of cases in the literature have involved bearded dragons, however this may be due to the popularity of this species as a pet rather than a true species-specific susceptibility. More recently, infections involving wild snakes have become more widely reported, likely as more resources are directed to investigating this emerging fungal pathogen.

Paré et al. (2003) solicited samples of shed squamate skin from zoological institutions and private veterinarians to determine if CANV organisms were part of the normal skin flora. All samples were from animals free of cutaneous lesions or other obvious disease and in good body condition. Samples were plated out and incubated at 28°C for five weeks, after which fungi were identified based on colony and microscopic characteristics. A total of 742 fungal isolates from 50 genera were obtained from 127 reptiles, of which there was only a single isolate of a CANV complex fungus, giving a prevalence of 0.8%. This was from the shed of an African rock python (*Python sebae*) kept on newspaper substrate. The animal appeared healthy but failed to adapt to captivity, and subsequently died of malnutrition, with no evidence of fungal infection on post-mortem (Paré et al.2003). This was the first report of a CANV complex fungus isolated from an animal without visible cutaneous lesions, and was subsequently determined to be *O. ophiodiicola* (Sigler et al. 2013). With the development of more sensitive diagnostic methods, *O. ophiodiicola* has been detected in several asymptomatic corn snakes (Lorch et al. 2015) (Section 1.2.3.2).

Other findings of Paré et al.'s (2003) study included a discernible but weak trend for skin samples from the same institution to grow similar fungi, and it was thought that this may have been due to similarities in substrate and enclosure furniture. The authors further speculated that reptile fungal microflora is likely to reflect the fungal composition of their substrate. Although there has been very limited ecological and epidemiological data on CANV complex organisms published, several reports suggest the fungus is of environmental origin (Bowman et al. 2007, Thomas et al. 2002, Allender et al. 2011, Allender et al. 2015a). Sigler et al. (2013) report one strain of *Nannizziopsis vriesii* isolated from soil in California, however no published studies have further evaluated the source of infections.

Paré et al. (2003) conclude that members of the CANV complex do not appear to be common constituents of normal healthy squamate cutaneous mycoflora. Samples for this study were submitted via mail, and other authors speculate it is possible that competition with other microflora, or temperature and humidity changes experienced during storage and transport, may have resulted in a lower detection rate of CANV complex organisms than would otherwise have occurred (Mitchell and Walden 2013). Culture is also a relatively insensitive diagnostic method for asymptomatic CANV infections. A later study on the pathogenicity of CANV in veiled chameleons revealed that the CANV (later speciated as *N. dermatitidis*) was isolated only twice from shed skin during the study, despite 14 animals being affected (Paré et al. 2006, Sigler et al. 2013). It is not clear in this study how many samples of shed skin were collected, and how many were from animals with confirmed infection.

A study by White et al. (2010) evaluated the prevalence of CANV complex infections in reptiles with dermatological lesions. Lesions from 301 reptiles, from two institutions over a period of 16 years were reviewed. Of these lesions, four were suspected to be CANV complex infections, but only one was confirmed. The confirmed case was in a bearded dragon, and was diagnosed via histopathology, culture and PCR. This animal's cage mate was diagnosed circumstantially. In the same study, another bearded dragon was diagnosed based on histopathology but cultured a *Trichophyton* species, and an Oustallet's chameleon (*Furcifer oustaleti*) was diagnosed based on histopathology, but did not culture a CANV complex organism (White et al. 2010). None of the 82 snakes or 36 chelonians in this review had CANV complex infections. The overall prevalence of CANV complex infections in this study was 1.3% for all animals, but increased to 4.5% for lizards.

Ophidiomyces ophiodiicola has recently been found in wild populations of wild massasauga rattlesnakes (*Sistrurus catenatus*) (Allender et al. 2011). Disease was initially detected as part of an ongoing monitoring program in its ninth year. Prevalence in surveyed snakes from 2000-2007 was 0%, in 2008 was 4.4% (95% CI 1.1-13.2), and in 2010 was 1.8% (95% CI 0-11.1%). A study conducted in Virginia reported a prevalence of 26.7% of *O. ophiodiicola* in the 30 wild snakes captured for the study, however study sites were selected based on anecdotal reports of SFD-like lesions, and random representative sampling was not conducted (Guthrie et al. 2016).

1.2.2.2 Risk factors

Although both *N. dermatitidis* and *O. ophiodiicola* have been established as primary pathogens in veiled chameleons and snakes respectively (Paré et al. 2006, Allender et

al. 2015b, Lorch et al. 2015), these studies indicated that while a breach in skin integrity is not necessary for infection, it is associated with more severe disease.. Similar findings were made in a study of experimental *Ophidiomyces ophiodiicola* infection in corn snakes (*Pantherophis guttatus*), where lesions were detected more frequently in sites subject to abrasion (Lorch et al. 2015). It has not been experimentally determined whether all other CANV complex organisms are primary pathogens. Several authors suggest that pre-existing lesions or wounds may lead to subsequent infection by members of the CANV complex (Allender et al. 2011, Johnson et al. 2011).

It has been proposed that suboptimal husbandry may contribute to the development or severity of infection with CANV complex organisms (Thomas et al. 2002, Bertelsen et al. 2005, Johnson et al. 2011, Toplon et al. 2012). In particular, fungal infections are reported to be triggered by suboptimal temperatures (Fromtling et al. 1979), and in multiple cases a sudden decrease in environmental temperature was reported prior to infection becoming apparent (Thomas et al. 2002, Johnson et al. 2011). In an outbreak involving four of a group of 12 brown tree snakes, it was proposed that warm, humid conditions and access to water may have softened the ventral scales of the animals, making them less resistant to infection (Nichols et al. 1999), and unusually high rainfall was suggested as a contributing factor in an outbreak involving timber rattlesnakes (Clark et al. 2011). Cases have also been recorded when the animals appeared to be in good body condition, with adequate husbandry and no evidence of underlying disease or stressors (Bowman et al. 2007, Hellebuyck et al. 2010).

Several authors have suggested that stress may be a contributing factor in acquiring CANV complex infections (Thomas et al. 2002, Bertelsen et al. 2005, Toplon et al. 2012).

In a case involving four tentacled snakes, the pH of the water was more alkaline than recommended for this species, and the authors speculate this may have predisposed them to infection (Bertelsen et al. 2005). Overcrowding may also cause stress and contribute to enhanced disease transmission via direct contact (Thomas et al. 2002, Toplon et al. 2012). In a commercial breeding colony of leopard geckos, it was noted that colour morphs were initially affected, and it has been suggested that these animals may have a less competent immune system than wild-type geckos, predisposing them to infection (Toplon et al. 2012). Several outbreaks were noted to involve recently caught wild animals (Thomas et al. 2002, Toplon et al. 2012). In these cases it is unknown whether infection was present subclinically when the animals were captured (and the stress of capture and transport may have increased susceptibility to disease), or if infection was acquired in captivity. One author suggested an asymptomatic carrier state may exist in bearded dragons, however no research has been done to support this hypothesis (Bowman et al. 2007).

Cases in the same institution have occurred without direct contact with a clinically affected animal (Nichols et al. 1999, Thomas et al. 2002). While there were several outbreaks in which all in-contact animals were affected (Bertelsen et al. 2005, Hedley et al. 2010, Hellebuyck et al. 2010, Van Waeyenberghe 2010), in other cases in-contact animals were considered to be free of infection (Bowman et al. 2007, Johnson et al. 2011).

1.2.2.3 Fungal source

The source of fungi in CANV complex infections is unknown, but several authors propose an environmental origin (Thomas et al. 2002, Bowman et al. 2007, Allender et al. 2011).

This is supported by the occurrence of infection in wild massasauga rattlesnakes in different locations from two discontiguous sites, occurring in different years (Allender et al. 2011), and by multiple snake species being affected in a single site in Virginia (Guthrie et al. 2016). One isolate of *Nannizziopsis vriesii* has been detected in soil in California (Sigler et al. 2013), however there are no reports of further investigation of fungal origin in the literature. Transmission of infection is suggested to occur by direct contact with affected animals, contaminated substrate or fomites, and by airborne dissemination (Nichols et al. 1999, Paré et al. 2006, Allender et al. 2011). A study by Allender et al. (2015a) concludes that *O. ophiodiicola* is likely to occur as an environmental saprobe (see below), and that it infects snakes opportunistically. While this study appears robust and the conclusions sound, care must be taken in extrapolating findings from one fungal species to another.

Fungal ecology

Very few published studies have been conducted to describe the ecology of CANV infections. The only in-depth study was conducted on *O. ophiodiicola*, under laboratory conditions (Allender et al. 2015a). *In vitro*, *O. ophiodiicola* demonstrated robust growth on a variety of dead materials and possessed a broad complement of enzymes allowing it to saprophytically utilise multiple complex carbon and nitrogen sources. Sparse fungal growth occurred on demineralised-deproteinated shrimp exoskeletons, leading to the conclusion that a protein source is necessary for prolific fungal growth. The fungus grew optimally when incubated at 25^{o}C, with significant growth reduction at 14^{o}C and 35^{o}C, and complete growth inhibition at 7^{o}C. Laboratory experiments demonstrated *O. ophiodiicola's* ability to tolerate pH variation and most naturally occurring sulphur compounds, and to tolerate low matric potentials (water stress) occurring in soil

(Allender et al. 2015a). The data also suggest that the environmental presence of ammonium may be beneficial for the growth of *O.ophiodiicola*, suggesting that incomplete removal of animal waste products, primarily urine and urates, may contribute to fungal growth.

1.2.3 Diagnosis

Diagnosis of CANV complex infection often relies on a degree of clinical suspicion on behalf of the veterinarian. If a fungal culture and/or PCR is not performed, the cause of disease cannot be confirmed. Even when fungal cultures are obtained, in the absence of PCR they are often misdiagnosed as other fungal species, and this is discussed further below.

1.2.3.1 Clinical signs and causative organisms

Infections caused by CANV complex organisms are often aggressive and rapidly progressing, in contrast to those in tuatara which are not as severe (Masters et al. 2016). Lesions are characterised by hyperkeratosis, necrosis, vesicles, ulcers, and crusting, and often progress to fatal systemic disease (Sigler et al. 2013). Infections are contagious, and typically affect multiple animals housed in the same environment (Sigler et al. 2013).

Symptoms of infection vary between species affected, fungus involved, and outbreak conditions. The causative fungi of several of the cases in this literature review have undergone speciation via DNA analysis by Sigler et al. (2013) since the original articles were first published, and this information is included where available. Clinical signs have been grouped into species categories to aid in interpretation.

Lesion characterisation

Lizards

Clinical signs of CANV complex infection in lizards range from focal skin thickening and retention of squames, to vesicular lesions, black skin discolouration, ulcerative dermatitis, focal swelling and bone necrosis (Paré et al. 1997, Martel et al. 2006, Abarca et al. 2008, Johnson et al. 2011). The majority of reported lesions have consisted of an ulcerative dermatitis, with varying degrees of crusting, swelling and necrosis. Lesion appearance, anatomical location and causative organism (where known) are detailed in Table 1.1.

A literature review of infections in lizards shows that almost all lesions in these animals have been rapidly progressive, and have been noted to spread to distant anatomical sites as well as locally (Paré et al. 1997, Bowman et al. 2007, Johnson et al. 2011). Infection in lizards in the cases where fungal species information was available were all caused by members of the *Nannizziopsis* genus, except for one case in a coastal bearded dragon (*Pogona barbata*) caused by *P. australasiensis* (Sigler et al. 2013).

Lesions in lizards are typically located on the limbs, ventrum or rostral head area. This may be because these areas are more frequently in contact with the substrate, and several authors speculate that CANV organisms originate from an environmental source (Thomas et al. 2002, Allender et al. 2011). It is possible that these anatomical locations are more prone to abrasion, facilitating entry of the fungus into the skin, or that the more humid microenvironment typically experienced on skin in closer contact with substrate may facilitate fungal propagation (Allender et al. 2011).

Snakes

Lesions in snakes are primarily found on the head and ventral scales, although they can occur over the entire body. Infection tends to be rapidly progressive, and is variably characterised by subcutaneous swelling, crusting, retained shed, vesicles and plaque-like lesions (Nichols et al. 1999, Eatwell 2010, McLelland et al. 2010, Allender et al. 2011, Sigler et al. 2013). Fungi can invade muscle and bone, and cases of pneumonia, ocular infection and systemic fungal dissemination attributed to *O. ophiodiicola* have been documented in colubrids in the USA (Rajeev et al. 2009, Sleeman 2013, Dolinski et al. 2014). The nasolabial pits are a common site of infection in crotalid snakes, and have been proposed as a possible entrance site for systemic infection (Allender et al. 2015b).

Infection in terrestrial snakes appears to be universally associated with *Ophidiomyces ophiodiicola*. This has previously been referred to as CANV or, in some cases, as various *Chrysosporium* species, prior to more accurate speciation through DNA analysis (Sigler et al. 2013). A recent paper by Pare and Sigler (2016) details historical infections caused by *O. ophiodiicola* in at least 25 snake species in three continents. *Paranannizziopsis* species have been associated with fatal dermatitis in aquatic snakes, and details of these infections are provided in Table 1.1 (Bertelsen et al. 2005, Sigler et al. 2013).

Crocodiles

Only one article has detailed CANV complex infection in saltwater crocodile hatchlings (*Crocodylus porosus*), and this covered multiple outbreaks in animals at the same facility (Thomas et al. 2002). Several affected animals initially had white flaky areas on the underside of the body and caudal thighs. After one month, these animals were found to have creamy, caseous masses on, and occasionally under, scales on the head, back and

feet. Different animals subsequently developed plaque-like lesions, however more details on the appearance and location of these lesions was not reported. A second outbreak at the same facility three years later was also characterised by plaque-like lesions (Thomas et al. 2002). Infection was caused by *Nannizziopsis crocodili* (Sigler et al. 2013).

Although multiple animals were affected in these outbreaks, this is the only report of CANV complex infections involving crocodiles, so care must be taken when interpreting these lesions as being representative of CANV complex infections in this species.

Tuatara

Lesions in tuatara (*Sphenodon punctatus*) have been more subtle and less progressive than in other species (Masters et al. 2016). The most common presentation is as 1-5mm diameter raised, nodular, yellow, crusting dermatitis, usually without ulceration. Lesions have been located on the gular region, ventrum, and ventrolateral tailbase. The causative organism has been diagnosed as *Paranannizziopsis australasiensis* (Sigler et al. 2013).

These lesions have now been diagnosed at multiple institutions in both the north and south islands of New Zealand (unpublished data), however it is possible that other presentations of disease may occur, or fungi may not be recognised or correctly diagnosed as members of the CANV complex.

Other clinical signs

Several authors have reported lethargy, anorexia and weight loss in animals infected by CANV complex organisms. Animals noted with these clinical signs include bearded

dragons and girdled lizards (Edgerton and Griffin 2009, Hellebuyck et al. 2010, Johnson et al. 2011, Masters et al. 2016), however it is possible more animals exhibited these symptoms and went unreported.

Haematological changes

Several affected animals had haematological changes noted. The most common haematologic abnormality reported was a leucocytosis, often due to a lymphocytosis (Paré et al. 1997, Bowman et al. 2007, Hellebuyck et al. 2010, Masters et al. 2016). Other less common abnormal findings included monocytosis, azurophilia, and basophilia (Bowman et al. 2007, Hellebuyck et al. 2010, Masters et al. 2016). The majority of cases in this review did not have haematology results reported.

Isolation of the fungus from lesions via culture, and histopathological evidence of fungal invasion of the tissues, have been used as criteria to establish a member of the CANV complex as the causative agent of fungal dermatitis in some affected animals (Paré et al. 1997, Nichols et al. 1999, Bowman et al. 2007, Abarca et al. 2008, Hedley et al. 2010, Hellebuyck et al. 2010, Toplon et al. 2012). This has also been used to rule out a role for other fungi cultured from the same lesions, when the histologic appearance has not matched that of the cultured organism (Toplon et al. 2012).

Table 1.1 Clinical signs, lesion location and causative organism in reviewed CANV cases.

Species affected	Lesion description	Lesion location	Causative organism	Citation
Parson's chameleon (*Calumma parsonii*)	Grey vesicular lesions containing clear fluid. Small brown crusted lesions.	Left stifle and right elbow, progressing to all limbs, flanks and tail	*Nannizziopsis dermatitidis*	Paré et al. 1997
Jewelled chameleon (*Furcifer campani*)	Localised areas of black discolouration with white exfoliative squames	Right upper lip at mucocutaneous junction, and left hindfoot	*Nannizziopsis dermatitidis*	Paré et al. 1997
Jackson's chameleon (*Chamaeleo jacksonii*)	2cm scab	Adjacent to left tailbase, progressing to ventral tailbase and causing hemipenal prolapse	*Nannizziopsis dermatitidis*	Paré et al. 1997
Green iguana (*Iguana iguana*)	Focal thickening of skin with retention of squames	Distal aspect of left tarsus	*Nannizziopsis guarroi*	Abarca et al. 2008
Green iguana (*Iguana iguana*)	Deep, severe, crusting ulcerative dermatitis	Dorsal anterior right thigh, extending from proximal thigh to mid tibia	*Nannizziopsis guarroi*	Abarca et al. 2008
Bearded dragon (*Pogona barbata*)	Swelling and ulceration, gingival recession and facial oedema	Digit on left hindlimb, progressing to jaw and facial lesions	Not speciated	Johnson et al. 2011
Bearded dragon (*Pogona barbata*)	Superficial, crusty cheilitis, nodular lesions	Lip, progressing to rostral mandible with bony involvement and non-union fracture of mandibular symphysis	Not speciated	Johnson et al. 2011
Bearded dragon (*Pogona barbata*)	Deep, necrotic dermatitis with gingival recession	Rostral mandible, progressing to right tarsus, left carpus, digits and cloaca	Not speciated	Johnson et al. 2011
Bearded dragon (*Pogona barbata*)	Deep, necrotic dermatitis	Right rostral mandible, plantar aspect of D3 on the right hindleg and stump of previously amputated left forelimb	Not speciated	Johnson et al. 2011

Bearded dragon (*Pogona barbata*)	Raised, nodular yellow lesions	Beard, chin, ventral abdomen, right tarsus	*Paranannizziopsis australasiensis*	Masters et al. 2016
Bearded dragon (*Pogona barbata*)	Crusty dermatitis	Left mandible	*Nannizziopsis barbata*	Johnson et al. 2011
Bearded dragon (*Pogona vitticeps*)	Yellow-black thickened skin, osteolysis	Right side of beard, progressing to face and encircling tail	Not speciated	Edgerton and Griffin 2009
Bearded dragons (*Pogona vitticeps*)	Undescribed dermatitis	Head, hindlimbs and ventrum	*Nannizziopsis vriesii*	Van Waeyenberghe et al. 2010
Bearded dragon (*Pogona vitticeps*)	Crusting and periocular swelling, yellow discolouration and necrosis	Periocular area, inguinal region	Not speciated	Hedley et al. 2010
Bearded dragon (*Pogona vitticeps*)	10mm subcutaneous mass	Right flank	Not speciated	Hedley et al. 2010
Bearded dragon (*Pogona vitticeps*)	Focal swelling with raised, crusty skin lesion and painful maxillary gingival swelling	Ventral to right eye, including maxillary gingiva	*Nannizziopsis guarroi*	Bowman et al. 2007
Bearded dragon (*Pogona vitticeps*)	Discoloured, necrotic skin	Left caudoventral abdomen, extending to perineum and tailbase	*Nannizziopsis guarroi*	Bowman et al. 2007
Bearded dragon (*Pogona vitticeps*)	Marked swelling with thickened and creviced skin with serosanguinous exudate. Non-weight-bearing. Osteolysis.	Right forelimb, distal to elbow. Osteolysis of distal radius, ulna and carpus	*Nannizziopsis guarroi*	Bowman et al. 2007
Leopard geckos (*Eublepharis macularius*)	Punctate to 4mm diameter slightly raised, frequently dark red ulcerated nodules. Retained shed	Ventral skin	*Nannizziopsis dermatitidis*	Toplon et al. 2012

Girdled lizards (*Cordylus giganteus*)	Severe ulcerative dermatitis with localised loss of scales, eyelids adhered together with exudate	Periocular region, side of face	*Nannizziopsis vriesii*	Hellebuyck et al. 2010
Ameiva lizard (*Ameiva chaitzami*)	1.5 x 1cm2 hard, smooth, non-ulcerated mass covered by intact skin. Concurrent metabolic bone disease	Left half of the skull, originated from nasal cavity or skull	*Nannizziopsis vriesii*	Martel et al. 2006
Broad-headed snake (*Hoplocephalus bungaroides*)	Crust with retained shed, progressing to severe ulceration	Adjacent to cloaca, progressing to tail tip	*Ophidiomyces ophiodiicola*	McLelland et al. 2010
Tentacled snakes (*Erpeton tentaculatum*)	Small, pale yellow-white dermal lesions. Improved every 14 days after each shed, then recurred	Head and dorsum, tips of, or entire, scales	*Paranannizziopsis crustacea* AND *P. californiensis*	Bertelsen et al. 2005
Brown tree snakes (*Boiga irregularis*)	Erythema and oedema of scales, vesicles containing clear to cloudy serous fluid, rupturing to form raised, brown caseous plaques. Underlying skin haemorrhagic, dry and necrotic. White granular to powdery material between scales.	Ventral scales, beginning where scales overlapped, progressing to cover 50% of animal's ventrum	*Ophidiomyces ophiodiicola*	Nichols et al. 1999
African rock python (*Python sebae*)	None	Normal shed	*Ophidiomyces ophiodiicola*	Paré et al. 2003
Aquatic file snakes (*Acrochordus sp*.)	Multifocal necrotising skin lesions	Not reported	*Paranannizziopsis australasiensis*	Sigler et al. 2013

Massasauga rattlesnakes (*Sistrurus catenatus*)	Severe unilateral subcutaneous swelling, ulceration and crusting, deforming normal anatomy	Head, obstructing nasolabial pits and occasionally extending to cranial orbit and maxillary fang	*Ophidiomyces ophiodiicola*	Allender et al. 2011
Boa constrictor (*Constrictor constrictor*)	Necrotic dermatitis	Mandible, maxilla, dorsal spine	Not speciated	Eatwell 2010
Garter snake (*Thamnophis* sp.)	1.3 x 2cm grayish-white cutaneous nodule. Swollen hemipenis with necrotic foci	Ventrum, cranial to cloaca	*Ophidiomyces ophiodiicola*	Vissiennon et al. 1999
Timber rattlesnake (*Crotalus horridis*)	Yellow to brown crusted skin lesions 1 to >7mm diameter	Primarily head, also lateral and ventral body	*Ophidiomycies ophiodiicola*	McBride et al. 2015
Saltwater crocodiles (*Crocodylus porosus*)	White, flaky skin, progressing to creamy, caseous masses on and under scales, and leathery plaque-like lesions	Underside of body, caudal thighs, snout, head, back, feet, underside of jaw and tail	*Nannizziopsis crocodilii*	Thomas et al. 2002
Tuatara (*Sphenodon punctatus*)	Small areas of raised, yellow, crusting dermatitis	Gular region, ventral tail, adjacent to cloaca, lateral tailbase	*Paranannizziopsis australasiensis*	Masters et al. 2016

Several authors use a combination of histopathology, fungal culture and PCR to identify the causative organism (Abarca et al. 2008, Hellebuyck et al. 2010, Van Waeyenberghe et al. 2010, Toplon et al. 2012, Masters et al. 2016). PCR testing is usually done on cultured fungus, but has also been applied to DNA extracted directly from tissue (Allender et al. 2011). Using PCR has the added benefit of diagnosing the fungus to species level, as they cannot be reliably identified based on microbiological characteristics alone (Sigler et al. 2013, Paré and Sigler 2016). Without PCR, some cases may be provisionally identified to species level based on case signalment, for example, the only CANV complex organism reported to have infected terrestrial snakes has been *Ophidiomyces ophiodiicola* (Sigler et al. 2013). Recently, real-time PCR (qPCR) has been used to identify *O. ophiodiicola* from lesion swabs of infected snakes, and this appears to be more sensitive than conventional PCR (Allender et al. 2015c).

Skin scrapings in affected tentacled snakes revealed bacteria and keratinocytes but no fungi (Bertelsen et al. 2005), so this method may not be suitable for ascertaining the presence of a fungal infection. A potassium hydroxide preparation of hyperplastic skin in a green iguana showed fungal hyphae (Abarca et al. 2008), however this does not prove that fungi are the primary cause of the dermatitis. These methods, if positive, may increase clinical suspicion of a fungal aetiology, but if negative, do not exclude mycosis. Several authors suggest that histopathological findings, in combination with clinical presentation, can be used to diagnose infections caused by the CANV complex (Eatwell 2010, Hedley et al. 2010). In contrast to this, other authors emphasise the importance of obtaining good biopsy specimens for both histopathology and culture to confirm diagnosis (Bertelsen et al. 2005, Abarca et al. 2008). Identification of the fungus to species level may be important in investigating the epidemiology of the disease (Sigler

et al. 2013, Paré and Sigler 2016). Using a laboratory familiar with reptile mycoses for fungal identification may reduce the risk of mis-identification, and is recommended by several authors (Hedley et al. 2010, Toplon et al. 2012, Sigler et al. 2013). If this is not available, it is suggested that samples be retained for further specialist identification (Johnson et al. 2011, Paré and Sigler 2016).

Diagnosis of *P. australasiensis* at Auckland Zoo is currently based on a combination of histopathological findings, culture and PCR of culture products from full thickness skin biopsies, and, more recently, from partially shed scales removed with forceps.

1.2.3.2 Culture and microbiological characteristics

CANV complex organisms can be isolated by routine fungal culture, but are often misdiagnosed as other fungal infections. CANV complex organisms have been misdiagnosed as several *Trichophyton* species, *Trichosporon*, *Geotrichum*, or geophilic and keratinophilic *Chrysosporium* or *Malbranchea* species (Paré et al. 1997, Nichols et al. 1999, Thomas et al. 2002, Bowman et al. 2007, Abarca et al. 2008, Hedley et al. 2010, White et al. 2010, Johnson et al. 2011). The sessile conidia of *N. vriesii* appear very similar to microconidia of various *Trichophyton* species, and this may explain the common mislabelling of CANV complex isolates as *Trichophyton* spp. (Paré et al. 1997).

Bacteria have also been seen on histopathology in cases caused by members of the CANV complex (Thomas et al. 2002, Bertelsen et al. 2005, Paré et al. 2006), and in the absence of a fungal culture, bacteria may be implicated as the cause of the lesions. Bacterial contamination and growth has occurred on several cultures in cases of CANV

complex infection (Thomas et al. 2002, Edgerton and Griffin 2009, Hedley et al. 2010, Johnson et al. 2011).

In an infection challenge conducted by Paré et al. (2006) in veiled chameleons, the fungus was cultured from frozen skin samples in only five out of 14 histopathologically suspicious infections. It is possible that sample handling in other cases may have affected the sensitivity of fungal culture, further leading to infection being under-reported (Mitchell and Walden 2013). Cultures in these reviewed cases have largely been obtained from skin biopsies or post-mortem tissue, however one source reports successful culture from swabbing the skin of an affected girdled lizard (Hellebuyck et al. 2010). By this method, organisms were demonstrated for seven weeks after treatment started. Follow-up swabs at weekly intervals have been recommended, to monitor for the presence of fungi (Van Waeyenberghe et al. 2010). TaqMan real-time PCR appears to be a more sensitive tool than culture for detection of *O. ophiodiicola* (Bohuski et al. 2015). This was not the case when conventional PCR was compared to culture for detection of *P. australasiensis*, possibly due to limitations of the DNA extraction method or the small tissue sample size available for analysis (Humphrey et al. 2016).

Culture has been successful on potato dextrose agar (PDA), phytone yeast extract, Mycosel agar, and Sabouraud dextrose agar (Paré et al. 1997, Thomas et al. 2002, Bertelsen et al. 2005, Abarca et al. 2008, Hellebuyck et al. 2010, Van Waeyenberghe et al. 2010, Sigler et al. 2013), however growth at different temperatures has varied (Abarca et al. 2008, Sigler et al. 2013). Several CANV complex isolates have not grown well at 35^{o}C, and this was the case with *P. australasiensis*, which did not grow at all at

this temperature (Sigler et al. 2013). *P. australasiensis* has been grown successfully at both 25 °C and 30 °C (Alexander et al. 2014).

It is important to recognise that a negative culture does not necessarily rule out infection. In one case, growth was unsuccessful on MacConkey agar, but samples from the same lesions have grown on Sabouraud dextrose agar (Abarca et al. 2008). Frozen thawed skin samples from affected massasauga rattlesnakes did not grow any fungi on Sabouraud agar, however PCR on DNA extracted from these tissues identified *C. ophiodiicola* (now known as *O. ophiodiicola*) (Allender et al. 2011).

Fungal cultures can also grow contaminant fungi such as *Aspergillus* species (Edgerton and Griffin 2009), and without strong clinical suspicion of CANV complex infection, other fungi may be identified as the primary cause of disease. Toplon et al. (2012) recommend using selective media that includes cycloheximide and antibacterial antibiotics to improve the likelihood of culturing CANV complex organisms.

In a study by Sigler et al. (2013), the CANV complex organisms shared several growth characteristics. All were moderately fast growing on potato dextrose agar (PDA) at 30°C. They had yellowish-white, velvety to powdery, dense, and sometimes zonate, colonies with uncoloured to yellowish reverse. All were cycloheximide tolerant, and perforated hairs. All produced aleuroconidia, and these resembled aleuroconidia also observed in some *Chrysosporium* and *Trichophyton* species (Sigler et al. 2013). This may be the cause of the misidentification of several CANV specimens as *Chrysosporium* and *Trichophyton* fungi (Sigler et al. 2013). All reptile isolates tested by Sigler et al. (2013) had urease

activity, hydrolysed milk solids, and had undulate hyphal branches. The majority of isolates were also observed to produce a skunk-like odour.

Importantly, it is noted that morphological and growth characteristics are insufficient to differentiate between and within genera, and that DNA sequencing was required to confirm fungal species (Sigler et al. 2013, Allender et al. 2015a). Almost all CANV complex species produce arthroconidia in culture as well as in infected cutaneous tissues, and these are considered to be the primary mode of transmission of infections between reptiles (Sigler et al. 2013).

Several isolates have been noted to produce aleuroconidia when cultured, and these have been found to be pyriform (tear-drop shaped) or clavate (club-shaped), usually single- but occasionally two-celled. They are borne sessile (formed directly on sides of hyphae, not on stalks) or at the end of branched, fertile hyphae (Paré et al. 1997, Nichols et al. 1999, Abarca et al. 2008).

1.2.3.3 Histopathological findings

Post-mortems were not conducted in all animals that died or were euthanased, however histological findings of both biopsies and post-mortems are similar between species.

The majority of animals that underwent post mortem exhibited localised tissue destruction and fungal invasion, though there were three confirmed cases of systemic disease in two bearded dragons and a jewelled chameleon (Paré et al. 1997, Bowman et al. 2007, Johnson et al. 2011). One bearded dragon had a hepatic granuloma with intralesional hyphae (Bowman et al. 2007), while another had both granulomatous

hepatitis and myocarditis with intralesional fungal elements (Johnson et al. 2011). A jewelled chameleon had a granulomatous mass involving the caudal lung, kidney and coelomic cavity wall, with intralesional hyphae (Paré et al. 1997). It is unknown whether the lesions in the jewelled chameleon were a result of systemic spread, or inhalation of fungal spores.

Cutaneous lesions are often characterised by epidermal hyperplasia or necrosis, with heterophilic or granulomatous inflammation (Nichols et al. 1999, Bertelsen et al. 2005, Paré et al. 2006, Bowman et al. 2007, Hedley et al. 2010, Hellebuyck et al. 2010, Allender et al. 2011, Johnson et al. 2011, Toplon et al. 2012). Lesions frequently penetrated the epidermis and dermis, with occasional involvement of deeper tissues including skeletal muscle and bone (Paré et al. 1997, Nichols et al. 1999, Paré et al. 2006, Allender et al. 2011, Bowman et al. 2011). It is proposed that fungal proliferation in the dermis and deeper tissues may lead to dissemination of fungi and systemic infection (Paré et al. 2006). Histopathology is an important component of disease investigation, as it distinguishes disease from mere presence of the fungal pathogen, as CANV complex organisms can occasionally be detected in asymptomatic animals (Paré et al. 2003, Bohuski et al. 2015).

Animals in the *N. dermatitidis* experimental challenge involving veiled chameleons were euthanased sequentially to observe the gross and histological stages of infection (Paré et al. 2006). Animals that were euthanased earlier in the study had less severe lesions than those that completed the 42-day study. Fungal hyphae initially proliferated in the superficial layers of keratin, then penetrated downwards through the epidermis, dermis

and into skeletal muscle as infection progressed (Paré et al. 2006). It was noted that the animals with the most severe lesions were from the group of animals with scarified skin.

Fungal hyphae have been observed both in granulomas and in the surrounding tissue, and are described as parallel-walled, branching and septate, with a diameter between 2 and 6µm (Nichols et al. 1999, Thomas et al. 2002, Bertelsen et al. 2005, Bowman et al. 2007, Hellebuyck et al. 2010, Paré et al. 2006, Toplon et al. 2012, Masters et al. 2016). Hyphae were usually numerous and visible on standard haematoxylin and eosin (H&E) staining, and in most reviewed cases Periodic Acid Schiff (PAS) or methenamine silver staining were used to better visualise fungal elements. Arthroconidia were observed histopathologically in several cases, indicative of active fungal proliferation (Nichols et al. 1999, Thomas et al. 2002, Paré et al. 2006, Toplon et al. 2012). These conidia were occasionally observed on the surface of lesions, providing a potential means of fungal transmission to in-contact animals. In lesions observed in brown tree snakes, arthroconidia were correlated with white, powdery areas that were seen grossly between the ventral scales (Nichols et al. 1999).

1.2.4 Susceptibility testing

Several papers have reported results of susceptibility testing for CANV complex organisms. Hellebuyck et al. (2010) reported low Minimum Inhibitory Concentrations (MICs) for itraconazole (0.5 mg/L), voriconazole (0.25 mg/L), amphotericin B (1 mg/L) and terbinafine (0.5 mg/L) against an isolate of *Nannizziopsis vriesii* using the broth microdilution method. Another study, using different testing methods, reported broad zones of inhibition around itraconazole, ketoconazole and terbinafine for an unspecified

CANV complex organism (Abarca et al. 2008). A recent study reported terbinafine MICs of 0.015 mg/L against two isolates of *Ophidiomyces ophiodiicola*, with an unspecified MIC testing method (Kane et al. 2017).

A study involving 32 isolates of members of the CANV complex, of which three were from green iguanas, one from a giant girdled lizard, and 28 from bearded dragons, reported predominantly low MICs of itraconazole and voriconazole (Van Waeyenberghe et al. 2010) using the broth microdilution method. The MIC_{50}, where 50% of isolates were inhibited, was 0.0313 mg/L for both itraconazole and voriconazole, while the MIC_{90} was 0.25 mg/L for itraconazole, and 0.0625 mg/LL for voriconazole. One isolate in this study showed acquired resistance to itraconazole (Van Waeyenberghe et al. 2010).

Three cases of infection with members of the CANV complex have used miconazole or clotrimazole topically (Bowman et al. 2007, Hellebuyck et al. 2010, Hedley et al. 2012), and another used a topical formulation containing nystatin, however there have been no susceptibility studies published for these drugs and CANV complex organisms.

1.2.5 Treatment and outcome

Treatment of CANV complex infections is often unsuccessful, as disease can progress rapidly and be slow to respond to therapy. Frequently there is a delay between observing clinical signs and initiating appropriate treatment, and this can be due to a low clinical suspicion of fungal disease, or inability to grow the organism from biopsy. Several cases in the literature were treated with antibiotics rather than antifungal

agents, and definitive diagnosis was obtained post-mortem (Nicholson et al. 1999, Bertelsen et al. 2005, Johnson et al. 2011).

In this review, itraconazole was found to be the most commonly used systemic antifungal for treatment of infections caused by members of the CANV complex. The majority of animals treated with this drug were lizards, and dose rates ranged from 2.5 to 10 mg/kg given orally once daily (Paré et al. 1997, Bowman et al. 2007, Edgerton and Griffin 2009, Eatwell 2010, Hedley et al. 2010, Van Waeyenberghe et al. 2010, White et al. 2010, Johnson et al. 2011, Masters et al. 2016). Of the 37 cases that used itraconazole, 18 died or were euthanased, 17 recovered, one was receiving ongoing treatment at the time of writing, and one was lost to follow-up. Of the animals that died, itraconazole-related hepatotoxicity is suggested as a cause in five animals (Van Waeyenberghe et al. 2010). Several of the successfully treated cases received adjunctive treatment with topical antifungals including terbinafine and chlorhexidine, and multiple cases resolved with itraconazole and excisional biopsy or amputation of the affected limb.

One author recommended pulse dosing of itraconazole, as this is commonly used in infections in humans, and may have less systemic side effects (Bowman et al. 2007). Edgerton and Griffin (2009) describe the use of pulse treatment with itraconazole, in combination with weekly lesion debridement and the application of topical antifungal agents. The affected bearded dragon had osteolytic lesions associated with fungal infection, requiring tail amputation. This case was ongoing at the time of publication, with no resolution but continuing treatment one year post-diagnosis. The maintenance itraconazole regime used was 5 mg/kg once daily with two weeks on, one week off, one week on, one week off, repeating up until the time of writing (Edgerton and Griffin

2009). It is important to note that a compounded itraconazole formulation was used in this case. A study in black-footed penguins (*Spheniscus demersus*) showed compounded itraconazole to have significantly decreased absorption and lower plasma concentrations than the commercially available formulation when given at the same dose rate (Smith et al. 2010).

Voriconazole was used in a total of eight lizards, one of which died (Hellebuyck et al. 2010, Van Waeyenberghe et al. 2010). This is a lower fatality rate than itraconazole-treated animals, suggesting that voriconazole may be a safer, effective treatment, although confounding factors and differences in case severity may have skewed results. This hypothesis was tested by Van Waeyenberghe et al. (2010) in a study that treated 14 similarly-affected CANV complex cases in bearded dragons, with seven animals administered itraconazole at 5 mg/kg PO SID and seven treated with voriconazole at 10 mg/kg PO SID. Of these, five animals in the itraconazole treatment group died, and one in the voriconazole treatment group died. Of these six deaths, the animal in the voriconazole treatment group had disseminated fungal infection, while no fungus was seen histologically on the internal organs of the itraconazole-treated animals, and drug-induced hepatotoxicity was the speculated cause of death based on increased AST levels in serum (Van Waeyenberghe et al. 2010).

Ketoconazole was used successfully in the treatment of two green iguanas, in conjunction with topical terbinafine and chlorhexidine (Abarca et al. 2008). The only other ketoconazole-treated case in this review was lost to follow-up. Ketoconazole is not commonly used in reptiles, with the more selective and less hepatotoxic azoles used preferentially (Mitchell 2006). It is interesting to note that there were no recorded

fatalities with ketoconazole treatment, however the sample size is too small to evaluate this with any confidence.

The pharmacokinetics of terbinafine as a nebulised single dose and a sustained subcutaneous implant have been evaluated in cottonmouths (*Agkistrodon piscivorous*) for the treatment of *O. ophiodiicola* (Kane et al. 2007). This study showed that both treatment modalities resulted in therapeutic plasma terbinafine concentrations against O. ophiodiicola (>0.015 mg/L), however there are currently no published reports on the successful application of these treatments to clinical cases of snake fungal disease.

Topical terbinafine, chlorhexidine, clotrimazole and silver sulfadiazine have been used in conjunction with systemic treatments (Bowman et al. 2007, Abarca et al. 2008, Edgerton and Griffin 2009, Hedley et al. 2010, Masters et al. 2016). It is unclear what contribution these agents may have made to recovery, though several animals treated with topical iodine, as the only antifungal, did not recover (Nichols et al. 1999, Bertelsen et al. 2005, Johnson et al. 2011). A group of four tentacled snakes survived with disease for four months prior to death with topical iodine as the sole antifungal treatment, however these animals shed every 14 days, and the authors suggest this may have contributed to prolonging their survival by reducing fungal load (Bertelsen et al. 2005).

Excisional biopsy has been clinically successful as a sole treatment in tuatara (Masters et al. 2016), and amputation of the affected limb in conjunction with a two week course of systemic itraconazole resulted in recovery in a bearded dragon (Bowman et al. 2007). However, many lesions are too large for excisional biopsy when presented, and others are in anatomical sites not amenable to surgical treatment, such as the head or cloaca.

In-water treatments have been attempted for several aquatic animals. In an outbreak involving saltwater crocodile hatchlings, saltwater baths did not improve the condition, and appeared to irritate the animals (Thomas et al. 2002). In the same outbreak, clinical success was achieved with a combination of plaque removal, topical iodine and temporary dry-docking. Formalin was then added to the water twice daily at a final concentration of 0.013% (Thomas et al. 2002). Using this treatment regime, ten animals died, and an unreported number of affected animals survived. Another author reports that acidification of exhibit water has led to resolution of infection in some, but not all, cases of infection in unspecified species of aquatic snakes (Sigler et al. 2013). The method and concentration of water acidification is not stated. Bertelsen et al. (2005) also speculate that failure to maintain an acidic environment was a contributing factor in fatal *Paranannizziopsis crustacea* infection in tentacled snakes.

Several cases in the literature report infection without treatment, and the vast majority of these animals died or were euthanased (Thomas et al. 2002, Martel et al. 2006, Allender et al. 2011). All captive animals that did not receive systemic antifungals died, including those animals that received topical antifungals with or without systemic antibiotics (Nichols et al. 1999, Bertelsen et al. 2005, Johnson et al. 2011). There is a single report of a wild timber rattlesnake (*Crotalus horridus*) apparently recovering from dermatitis caused by *O. ophiodiicola* infection after several cycles of ecdysis with no other treatment (Smith et al. 2013).

Van Waeyenberghe et al. (2010) emphasise the importance of pharmacokinetic studies to determine optimal treatment regimens using antifungals, and several authors recommend continuing treatment past the resolution of clinical signs (Bowman et al.

2007, Hellebuyck et al. 2010). Full details of the treatment and outcome of each reviewed case that received antifungal treatment are presented in Table 1.2. Medication was given orally unless otherwise stated. Those animals that did not receive antifungal treatment are not included in the table.

Tuatara have lower metabolic rates than the other reptile species in the reviewed CANV complex cases. I hypothesise that tuatara's lower metabolic rate translates to slower drug metabolism and elimination, meaning lower dose rates of antifungal agents will be required in their treatment.

Table 1.2 Treatment and outcome in reviewed cases of infection by members of the CANV complex.

Species affected	Treatment	Outcome	Citation
Parson's chameleon (*Calumma parsonii*)	Itraconazole 10 mg/kg SID for 21 days	Decreased appetite, condition loss, elevated serum CK and AST levels. Recovered	Paré et al. 1997
Jewelled chameleon (*Furcifer campani*)	Itraconazole 10 mg/kg SID	Died after 6 days, widespread systemic disease	Paré et al. 1997
Jackson's chameleon (*Chamaeleo jacksonii*)	Ketoconazole 25 mg/kg q48h	Lost to follow-up	Paré et al. 1997
Green iguana (*Iguana iguana*) – two animals	Ketoconazole 20 mg/kg SID Chlorhexidine 2% topically SID Terbinafine (dose rate not specified) topically SID	Both recovered	Abarca et al. 2008
Bearded dragon (*Pogona barbata*)	Itraconazole 5 mg/kg SID	Died	Johnson et al. 2011
Bearded dragon (*Pogona barbata*)	Topical iodine	Euthanased within 4 months of onset	Johnson et al. 2011
Bearded dragon (*Pogona barbata*)	Itraconazole 10 mg/kg SID	Died after 3 weeks	Masters et al. 2016
Bearded dragon (*Pogona vitticeps*)	Compounded itraconazole 5 mg/kg pulse dosing (see text for details). Silver sulfadiazine 1%, chlorhexidine 2% and terbinafine 1% topically (frequency not specified). Weekly lesion debridement	Ongoing treatment after one year	Edgerton and Griffin 2009

Bearded dragons (*Pogona vitticeps*)	Itraconazole 5 mg/kg SID	5/7 died, increased AST in 4 animals, suggested itraconazole induced hepatotoxicity	Van Waeyenberghe et al. 2010
Bearded dragons (*Pogona vitticeps)*	Voriconazole 10 mg/kg SID	1/7 died, increased AST in 3/7 animals, suggested voriconazole-induced hepatotoxicity	Van Waeyenberghe et al. 2010
Bearded dragons (*Pogona vitticeps*)	Itraconazole 10 mg/kg SID for 6 weeks Topical clotrimazole 1% BID F10 vivarium disinfection	7/13 recovered, others euthanased	Hedley et al. 2010
Bearded dragon (*Pogona vitticeps*)	Itraconazole 10 mg/kg SID for 6wks Topical chlorhexidine 0.125% (frequency not specified) Itraconazole 10 mg/kg SID Topical miconazole 2% SID	Reduced appetite, apparent recovery, recurrence after 6 months (see below). Anorexia and weight loss, died.	Bowman et al. 2007
Bearded dragon (*Pogona vitticeps*)	Itraconazole 10 mg/kg SID Topical iodine SID	Lesion improvement, weight loss and anorexia. Died.	Bowman et al. 2007
Bearded dragon (*Pogona vitticeps*)	Amputation of affected limb Itraconazole 5 mg/kg q48h for 14 days	Recovered	Bowman et al. 2007
Girdled lizard (*Cordylus giganteus*)	Topical miconazole, nebulised amphotericin B (dose reate and frequency not specified for either medication) Voriconazole 10 mg/kg SID for 10 weeks	No improvement Resolved	Hellebuyck et al. 2010
Three lizards of unspecified species	Itraconazole, unspecified dosing regimen and duration	One recovered One euthanased due to disease One lost to follow-up	White et al. 2010

Brown tree snakes (*Boiga irregularis*)	Topical iodine	Died 3-14 days after onset	Nichols et al. 1999
Boa constrictor (*Constrictor constrictor*)	Itraconazole 5 mg/kg SID Topical silver sulfadiazine 1% BID	Died after 3 weeks	Eatwell 2010
Tentacled snakes (*Erpeton tentaculatum*)	Topical iodine. Shed every 14 days.	Died after 4 months	Bertelsen et al. 2005
Garter snake (*Thamnophis* sp)	Itraconazole, unspecified dose rate and interval	Died	Vissiennon et al. 1999
Saltwater crocodile (*Crocodylus porosus*)	Saltwater baths Plaque removal, topical iodine, temporary dry-docking Add formalin 0.013% to water BID	No improvement 10 died, unknown number of affected animals survived	Thomas et al. 2002
Tuatara (*Sphenodon punctatus*)	Itraconazole 5 mg/kg SID for 4 weeks Topical terbinafine 1% SID for 3 weeks	Resolved	Masters et al. 2016
Tuatara (*Sphenodon punctatus*)	Excisional biopsy Itraconazole 5 mg/kg SID for 4 weeks Topical terbinafine 1% SID for 4 weeks	Resolved	Masters et al. 2016
Tuatara (*Sphenodon punctatus*)	Excisional biopsy Itraconazole 5 mg/kg SID for 2 weeks, then 2.5mg/kg for 2 weeks	Resolved	Masters et al. 2016
Tuatara (*Sphenodon punctatus*)	Itraconazole 3 mg/kg SID for 6 weeks	Resolved	Masters et al. 2016
Tuatara (*Sphenodon punctatus*)	Excisional biopsy only	Resolved	Masters et al. 2016

1.3 Impact of temperature on the pharmacokinetics of drugs in reptiles

There are few published studies that explore impact of temperature variation on the pharmacokinetics of drugs and metabolites in reptiles, and none involving antifungal drugs. Most reptile drug formularies do not state at what temperature the dose rate and interval were determined, despite several studies showing significant variation in drug metabolism and elimination at different temperatures. When reptiles are unwell it is advisable to treat them at the higher end of their preferred optimal temperature zone (POTZ) (Cooper 2006), however this is not always possible. The effect of temperature on drug pharmacokinetics is also beginning to be explored in human medicine, as therapeutic hypothermia becomes more commonly used in various brain and cardiac injury management situations.

The environmental temperature reptiles experience affects processes linked to metabolic rate, including gastrointestinal motility, enzyme activity, immune function and distribution and absorption of antibiotics (Mader et al. 1985, Hodge 1987, Caligiuri et al. 1990). Oxygen consumption and heart rate have been shown experimentally to increase at higher temperatures in tuatara and two species of snake, and it is reasonable to assume this occurs in other reptile species (Jacobson and Whitford 1970, Wilson and Lee 1970).

1.3.1 Antibiotic studies in reptiles

Several studies used the aminoglycoside antibiotic amikacin to test temperature-related variation in drug pharmacokinetics in snakes and tortoises (Mader et al. 1985, Caligiuri et al. 1990, Johnson et al. 1997). The aminoglycosides are predominantly eliminated

renally, so pharmacokinetics are likely to be affected by metabolic rate and renal perfusion and, therefore, temperature. A study using amikacin in gopher snakes (*Pituophis melanoleucus catenifer*) found significant differences in amikacin clearance and volume of distribution, but not half-life, at 25°C and 37°C (Mader et al. 1985). The larger volume of distribution at 37°C indicates the drug was distributed more widely to tissues, so was theoretically more effective in treating systemic infections. The same study also found the bacteria being targeted was twice as sensitive to amikacin *in vitro* at the higher temperature (Mader et al. 1985). A similar study in ball pythons (*Python regius*) showed no significant differences in amikacin pharmacokinetics at 25°C and 37°C (Johnson et al. 1997).

In gopher tortoises (*Gopherus polyphemus*), amikacin clearance was slower and elimination half-life was longer at 20°C than 30°C, while volume of distribution remained the same at both temperatures (Caligiuri et al. 1990). Metabolic rate and oxygen consumption was lower in gopher tortoises housed at lower temperatures by a ratio of 2:1, and this resulted in longer mean residence time and slower clearance of amikacin from the body at a ratio of almost 2:1 (Caligiuri et al. 1990), indicating that metabolic rate directly influenced pharmacokinetics in this study.

These studies show some variation in outcome and conclusions, indicating that even using the same drug, the differences in pharmacokinetics with temperature in reptiles are uncertain.

1.3.2 Therapeutic hypothermia and drug pharmacokinetics in humans

In humans, therapeutic hypothermia is employed as a tool to limit brain and myocardial ischaemic injury while stabilisation or repairs are performed. Hypothermia involves cooling until the core body temperature decreases from the normal 37^{o}C to 32-34^{o}C (Zhou and Poloyac 2011). In general, this mild hypothermia was found to significantly decrease clearance, but not protein binding, of a variety of drugs, resulting in an increase in drug and metabolite plasma concentrations. Hypothermia may increase the duration of effect of medications, and increase the likelihood of toxic effects if dosage is not adjusted appropriately (van den Broek et al. 2010, Zhou and Poloyac 2011). Specific alterations in drug pharmacokinetics during hypothermia may be metabolism and elimination route specific, and drugs that are highly metabolised and with a high clearance are more likely to be affected by hypothermia (van den Broek et al. 2010, Zhou and Poloyac 2010). Therapeutic drug monitoring is recommended in these situations, particularly when using drugs with a low therapeutic index, active metabolites, or high clearance (van den Broek et al. 2010)

1.3.3 Metabolic rate and toxicity

A study by Hodge (1978) showed that metabolic rate of the Florida banded water snake can be controlled by altering the ambient temperature, and that this affected the metabolism, clearance and maximum plasma concentration (Cmax) of intramuscularly-administered gentamicin (Hodge 1978). Lower temperatures resulted in lower metabolic rate, higher Cmax and slower clearance of gentamicin. Lower metabolic rate resulted in decreased production of urates in both control and gentamicin groups, slower active transport of gentamicin and therefore lower intracellular gentamicin

concentrations (Hodge 1978). These lower concentrations meant that despite the higher plasma gentamicin concentrations and the longer time the drug spent in the body, there was less potential for nephrotoxic effects (Hodge 1978). This is important as it illustrates that the intuitive assumption that higher plasma concentrations of a drug result in increased potential for toxicity may not always be correct, and that toxicity is dependent on drug pharmacokinetics.

1.4 Itraconazole

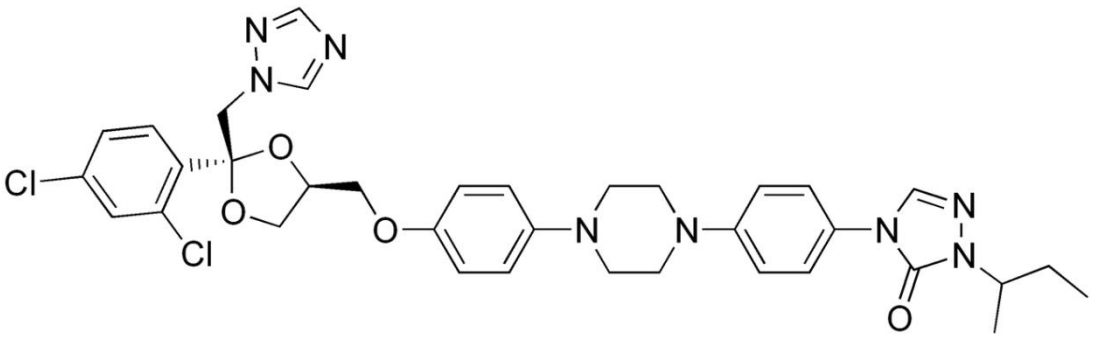
1.4.1 Indications in humans and other species

Itraconazole is a triazole antifungal that was first synthesized in 1980 (Hardin et al. 1988). It has the molecular formula $C_{35}H_{38}Cl_2N_8O_4$, and its structure is shown in Figure 1.5. Itraconazole has a broad spectrum of antifungal activity and is most commonly used in the treatment of aspergillosis, candidiasis, fungal dermatitis and nail bed infections in humans and animals (Vantrubova et al. 2010). Itraconazole has been used with variable success in the treatment of CANV complex infections in several reptile species at dose rates of 5-10 mg/kg orally once daily (Paré et al. 1997, Bowman et al. 2007, van Waeyenberghe et al. 2010, Johnson et al. 2011). Itraconazole has also been used in lizards at lower, allometrically scaled dosages ranging from 0.4 mg/kg orally every 20 hours to 1.7 mg/kg every 83 hours (Girling et al. 2009). These lower dose rates resulted in apparent resolution of Aspergillus infection, however itraconazole concentrations were not measured, and there were concomitant changes in husbandry which may have contributed to resolution.

1.4.2 Mechanism of action

Itraconazole binds to fungal but not mammalian cytochrome P450 enzymes (Mundell 1990). The nitrogen atoms in itraconazole interact with fungal cytochrome P450 (CYP)3A, inhibiting function of the enzyme lanosine 14-α-demethylase. This enzyme is essential in the conversion of lanosterol to ergosterol, an important compound in the fungal cell membrane and the equivalent of cholesterol in mammalian cells (Prentice and Glasmacher 2005, Vantrubova et al. 2010). This renders the fungi unable to multiply and accounts for itraconazole's fungistatic activity. Itraconazole administration may also result in the intracellular accumulation of ergosterol precursors, potentially contributing to eventual cell rupture and the fungicidal activity of the drug (Mundell 1990).

Figure 1.5 Molecular structure of itraconazole (Tarnacka et al. 2013).



1.4.3 Formulation and absorption

Itraconazole was initially available only in capsule form, and this is soluble only under very acidic conditions such as those in gastric secretions (Hardin et al. 1988, Orosz et al. 1996, Prentice and Glasmacher 2005). Absorption of the capsule formulation improved

when administered with food and was dose-dependent (Barone et al. 1993, Prentice and Glasmacher 2005). Itraconazole is highly lipophilic and a weak base, and absorption of solid drug, as in capsules, is enhanced by administration with a fatty meal (Heykants et al. 1989, Orosz et al. 1996). It has also been suggested that differences in the anatomy and physiology of gastrointestinal tracts between species may affect the rate and extent of itraconazole absorption (Orosz et al. 1996).

This difficulty in obtaining good absorption through oral administration of itraconazole led to practitioners using various methods in an attempt to enhance absorption. This has included use of commercial suspending vehicles, compounded vehicles, and administration of the drug dissolved in a combination of orange juice and hydrochloric acid, then mixed with gruel (Orosz et al. 1996, Smith et al. 2010). These methods do not result in absorption as efficient as that of the commercially available oral solution (Smith et al. 2010).

The oral solution of itraconazole was developed in the late 1980s (Heykants et al. 1989) and is marketed as Sporanox® (Janssen-Cilag Pharmaceuticals, Auckland, New Zealand). The solution combined itraconazole with hydroxypropyl-β-cyclodextrin, a ring of substituted glucose molecules (Willems et al. 2001). When both the oral solution and the capsule formulation were administered with food, bioavailability of the oral solution was up to 37% higher than the capsule formulation. Administration in a fasted state increased bioavailability by a further 30% when compared to dosing with food (Willems et al. 2001). The oral solution was also noted to improve predictability of itraconazole blood concentrations compared with the oral capsules (Davis et al. 2005, Prentice and Glasmacher 2005). A new itraconazole formulation with higher bioavailability has

recently been developed, and initial pharmacokinetic studies are still being conducted on this product (Abuhelwa et al. 2015).

1.4.4 Metabolism

Itraconazole is metabolised by the liver and is eliminated in gallbladder secretions and (up to 40%) in the urine (Prentice and Glasmacher 2005, Vantrubova et al. 2010). Hepatic metabolism is dose-dependent (Hardin et al. 1988, Barone et al. 1993) resulting in non-linear pharmacokinetics. This means that if the dose is increased, the proportion of drug eliminated may change due to the drug saturating the metabolic pathways.

Itraconazole has a large number of metabolites in humans, with the main metabolite being hydroxy-itraconazole (Heykants et al. 1989). Hydroxy-itraconazole has significant antifungal activity of a very similar spectrum of action to the parent compound, but may not be as potent *in vivo* (Heykants et al. 1989, Prentice and Glasmacher 2005). This raises the overall absolute oral bioavailability of itraconazole to at least 80% (Heykants et al. 1989, Prentice and Glasmacher 2005). Human studies have shown hydroxy-itraconazole to attain approximately two times the plasma concentration of itraconazole (Heykants et al. 1989, Manire et al. 2003), however a study in Kemp's ridley sea turtles (*Lepidochelys kempii*) showed hydroxy-itraconazole concentrations to be only 6% of itraconazole concentrations (Manire et al. 2003). It was postulated this could be a result of differences in drug metabolism due to lower metabolic rate or differences in cytochrome P-450 isoforms in sea turtle liver (Manire et al. 2003). Other studies in humans have found hydroxy-itraconazole concentrations to be between zero and 10 times those of itraconazole, again demonstrating significant inter-patient variability

(Orosz and Frazier 1995, Manire et al. 2003). Human studies have shown that itraconazole does not induce its own metabolism (Heykants et al. 1989).

1.4.5 Known pharmacokinetic profile in humans and selected other species

Steady-state concentrations in healthy human volunteers were reached after 10-15 days of itraconazole therapy in several studies (Hardin et al. 1988, Heykants et al. 1989, Barone et al. 1993, Orosz et al. 1996), and after 7 days in another study (Cauwenbergh et al. 1988). Itraconazole displays non-linear pharmacokinetics with saturation of drug metabolism in humans at therapeutic doses (Prentice and Glasmacher 2005).

Itraconazole has excellent tissue penetration, resulting in good bioavailability at the site of infection (Gamble et al. 1997, Heykants et al. 1989, Jones et al. 2000, Prentice and Glasmacher 2005). It also appears to be preferentially deposited in exudates such as pus, and can potentiate phagocytosis by leukocytes (Gamble et al. 1997, Heykants et al. 1989). Itraconazole is relatively poorly distributed in the cerebrospinal fluid (CSF); however clinical success has been reported with use of itraconazole in central nervous system (CNS) disease. It has been hypothesized that the concentration of itraconazole in CSF does not reflect the concentration in brain parenchyma (Kethireddy and Andes 2007).

Itraconazole was found to accumulate in Amazon parrots at dosages of 10 mg/kg, suggesting saturable metabolism in this species. However, there was no accumulation at dosages of 5 mg/kg (Orosz et al. 1996), illustrating linear pharmacokinetics at lower doses. Studies in humans have determined itraconazole kinetics were non-linear, i.e. they show a disproportionately higher increase in plasma concentration when dose rate

was increased. It is thought this is due to saturation of first-pass sites for metabolism in the gut mucosa and liver (Heykants et al. 1989, Prentice and Glasmacher 2005).

Significant inter-individual variation in metabolism has been shown in many species (Manire et al. 2003, Orosz and Frazier 1995). Itraconazole has been shown to accumulate in plasma in several species including bearded dragons, illustrated by an increase in trough concentrations over time (van Waeyenberghe et al. 2010). This may increase the risk of toxicity over prolonged periods of treatment.

Disposition in skin

The pharmacokinetic profile of itraconazole in the skin is unique and can result in significantly higher concentrations than those found in plasma (Cauwenbergh et al. 1988, Willems et al. 2001, Vantrubova et al. 2010). Itraconazole is delivered to the skin via sebum, sweat and passive uptake by keratinocytes (Cauwenbergh et al. 1988). Although penetration into skin and nails is slow, it does not diffuse back into the plasma, and is removed only by exfoliation and nail regrowth (Cauwenbergh et al. 1988, Heykants et al. 1989, Orosz and Frazier 1995, Willems et al. 2001). Itraconazole concentrations in human skin differed depending on the region sampled, with lowest concentrations in palmar stratum corneum, moderate concentrations in the back stratum corneum and high concentrations and longer persistence in the beard region (Cauwenbergh et al. 1988). Itraconazole concentrations in sweat were low, but concentrations in the sebum were 5 to 10 times higher than plasma concentrations during treatment with itraconazole, and were still at therapeutic concentrations one week after stopping drug administration. Low concentrations of itraconazole were still present in the palmar stratum corneum when concentrations in sweat and sebum were

undetectable, indicating that low concentrations were incorporated directly into keratinocytes in the basal layer.

It is thought that the main mechanism of itraconazole distribution to the skin is via sebaceous glands, followed by direct incorporation into keratinocytes, and a small amount via sweat (Cauwenbergh et al. 1988). A difference in thickness of the stratum corneum and in distribution of sweat and sebaceous glands in various parts of the body could therefore explain the differences in uptake on various body surfaces (Cauwenbergh et al. 1988, Heykants et al. 1989). High concentrations are also seen in human nails, and it is speculated this is as a result of drug diffusing via the nail bed (Cauwenbergh et al. 1988).

Reptiles do not have sebaceous glands in their skin, so the assumption that itraconazole concentrations remain high in the skin even after dosing has ceased may not be applicable, although there may be some incorporated into the epidermis and scales. Lesions caused by members of the CANV complex often have a heterophilic serocellular crust (C. Harvey 2014 pers. comm.), and it is possible this exudate may contain significant amounts of itraconazole during treatment. Several authors state that concentration in the target tissues will more closely reflect clinical efficacy than will plasma concentrations (Cauwenbergh et al. 1988, Heykants et al. 1989, Gamble et al. 1997), however it is not always possible to conduct studies to evaluate tissue drug concentrations, so plasma concentrations are used as a guide.

Plasma protein binding

The proportion of plasma protein binding of a drug affects its distribution and the amount of unbound drug available for use in the body. The “free drug hypothesis” states

that it is only the unbound proportion of drug that is available for antimicrobial activity (Theuretzbacher et al. 2006, Holford 2009). Plasma protein binding of itraconazole in humans is high at 99.8%, and is mainly bound to albumin (Heykants et al. 1989, Prentice and Glasmacher 2005, Willems et al. 2001). The lipophilic nature of itraconazole results in high tissue concentrations, including 10 times plasma concentrations in the skin, three times plasma in liver and 17 times plasma in fat (Jones et al. 2000, Willems et al. 2001). This also results in tissue concentrations remaining higher than plasma concentrations for some time after administration ceases (Willems et al. 2001). Particularly high concentrations are found in fluid containing organic material, such as pus and sputum (Heykants et al. 1989). Concentrations in tissues mostly composed of fluid, such as the aqueous humour, saliva and CSF, are negligible (Heykants et al. 1989).

In rats and dogs, itraconazole concentrations in the brain tissue were significantly higher than the negligible concentrations found in CSF (Heykants et al. 1989). Several authors assert that this disproves the generally-held tenet that free drug concentration (i.e. the 0.2% of itraconazole unbound to plasma proteins) alone accounts for antimicrobial activity, and that availability of drug to the infection site is related instead to tissue drug concentration (Heykants et al. 1989, Schäfer-Korting et al. 1995).

It is very difficult to measure unbound drug in tissue as it involves measuring drug concentrations in intracellular spaces, and this is rarely done. The currently accepted practice then is to measure drug concentrations in tissue as a whole, which of course includes capillaries, blood present in those vessels, intracellular fluid as well as the tissue itself (N. Holford 2014 pers comm.). It remains to be seen whether this is an accurate method of assessing drug availability for antimicrobial action, and it is beyond the scope

of this review to explore this debate further. Total (bound and unbound) plasma concentration of itraconazole, coupled with clinical response, are the most commonly used and accepted methods to monitor the efficacy of itraconazole therapy (Davis et al. 2005).

Half-life

The elimination half-life of a drug is the amount of time it takes for plasma drug concentration to decrease by fifty percent (Sharma and McNeill 2009). The half-life of itraconazole in humans has been reported as 15-21 hours after a single dose, and 30-35 hours following multiple doses of the capsule formulation (Heykants et al. 1989, Orosz et al. 1996). Chronic dosing of itraconazole in humans revealed significant accumulation, as evidenced by increasing trough concentrations throughout a 15 day study (Hardin et al. 1988).

The half-life of itraconazole in Amazon parrots has been shown to be between 3.7 and 7.2 hours, with no significant differences between days 1 and 14 of administration, or between dosing with 5 mg/kg or 10 mg/kg itraconazole (Orosz et al. 1996). In pigeons the half-life was 13.3 hours at an average dose rate of 10.3 mg/kg itraconazole (Lumeij et al. 1995), 5.8 hours in black-footed penguins (Smith et al. 2010), and 9.1 hours in Humboldt penguins (*Spheniscus humboldti*) (Bunting et al. 2009). Itraconazole's half-life is significantly longer in the few reptile species tested, and in spiny lizards was 48.3 hours (Gamble et al. 1997), and in Kemp's ridley sea turtles was 75 hours (Manire et al. 2003). A similarly long half-life of itraconazole may be expected in tuatara.

Time to maximal drug concentration

Time to maximal drug concentration (Tmax) using capsules in Amazon parrots varied between 3.7 and 6.9 hours (Orosz et al. 1996) when administered with an orange juice, hydrochloric acid and gruel mix, and in fasted domestic pigeons was 4 hours (Lumeij et al. 1995). In black-footed penguins given the itraconazole oral solution Tmax was 3.7 hours, and in humans was 5.7 hours (Willems et al. 2001, Smith et al. 2010).

Volume of distribution

Volume of distribution of a drug is the ratio of the amount of drug in the body and the concentration of drug in the plasma (Sharma and McNeill 2009). A low volume of distribution indicates a drug stays largely in the plasma and is not distributed extensively to other tissues. Itraconazole has a high volume of distribution in humans, at 10.7L/kg (Heykants et al. 1989), 17 L/kg in dogs (Heykants et al. 1987) and 6.3 L/kg in horses (Davis et al. 2005)

1.4.6 Adverse effects

Itraconazole has a lack of endocrine side effects when compared to its predecessor ketoconazole, and is less toxic in humans than amphotericin B (Hardin et al. 1988, Orosz et al. 1996). The most common side effects in humans are abdominal pain, nausea, vomiting and dyspepsia (Manire et al. 2003).

Itraconazole can cause an elevation in liver enzyme activity in serum, and these should be monitored during therapy. Liver damage may be severe enough to cause or contribute to mortality (van Waeyenberghe et al. 2010). In one clinical study on the treatment of infections caused by the CANV complex, five out of the seven bearded

dragons administered itraconazole at 5 mg/kg once daily died (van Waeyenberghe et al. 2010). Lesions suggestive of itraconazole toxicity were not demonstrated on histological examination, however the authors speculate that this was the cause of death (L. van Waeyenberghe 2014 pers. comm.). Serum AST levels were raised in four out of seven of these animals, however haemogram, bile acids and creatine kinase measurement were not undertaken in this study to help differentiate between hepatic and non-hepatic causes of AST elevation. A bearded dragon in a study conducted by Bowman et al. (2007) was given itraconazole at 10 mg/kg once daily for 21 days and showed a small decrease in appetite, but normal biochemistry. In the same study a different bearded dragon on the same dosing regime showed severe anorexia and weight loss, however on post-mortem there was no evidence of toxicity (Bowman et al. 2007). These results clinically illustrate the significant inter-individual variability noted in other studies of itraconazole pharmacokinetics. In other cases of infection caused by the CANV complex, itraconazole was linked to decreased appetite and condition loss, an unexplained increase in CK and AST activity, and a decreased PCV (Section 1.2.5).

In rats, itraconazole has been reported to cause hepatotoxicity, with dose-dependent hepatocellular necrosis, bile duct hyperplasia and biliary cirrhosis (Somchit et al. 2004). An analysis of toxicity data in humans has shown that plasma itraconazole concentrations above 17.1 mg/L have a high probability of toxicity (Lestner et al. 2009), however this was measured using bioassay rather than conventional high-performance liquid chromatography (HPLC) techniques. The authors suggest that this concentration using bioassay approximates 2. 5mg/L as measured by HPLC.

As a result of studies suggestive of itraconazole toxicity in reptiles, pulse dosing has been recommended (Bowman et al. 2007). A bearded dragon with a chronic infection caused by a member of the CANV complex was treated with itraconazole in a pulse fashion, and this failed to completely resolve the lesions (Edgerton and Griffin 2009). This may be because pulse therapy is ineffective, or because the authors used compounded itraconazole, which studies in birds show is poorly absorbed (Smith et al. 2010). Although pulse therapy has been shown to be successful in other animals and humans (De Doncker et al. 1997), particularly for onychomycosis, the mechanism of diffusion of itraconazole into reptile skin may be sufficiently different as to make such dosing regimens unsuccessful. Ideally studies that describe the concentration of itraconazole in reptile skin would first be conducted to determine how long and at what concentrations it persists there following oral dosing.

It may be prudent to monitor or decrease concentrations of certain drugs when given concurrently with itraconazole, as administration with itraconazole has been shown to modify plasma concentrations of drugs metabolised by cytochrome P450. (Manire et al. 2003, Vantrubova et al. 2010). There is a long list of drugs that fall into this category, some of the more commonly used ones include ivermectin, corticosteroids, benzodiazepines, rifampin and amphotericin B (Plumb 2011). In a study on itraconazole pharmacokinetics in Kemp's ridley sea turtles (Manire et al. 2003), rifampin was administered concurrently with itraconazole in two subjects, and it is possible this resulted in lower serum itraconazole concentrations than would otherwise have been observed (Drayton et al. 1994, Plumb 2011).

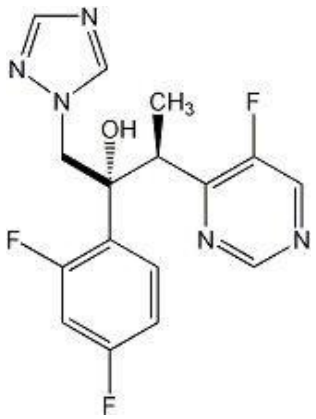
1.5 Voriconazole

1.5.1 Indications in humans and other species

Voriconazole is a second-generation triazole antifungal and is synthetically derived from fluconazole by modification of its chemical structure (Roffey et al. 2003, Theuretzbacher et al. 2006, Beernaert et al. 2009). It has the chemical formula $C_{16}H_{14}F_3N_5O$, and the structure is illustrated below (Figure 1.6).

Voriconazole has potent activity against a broad range of clinically significant fungi including *Aspergillus*, *Candida*, *Cryptococcus* and, importantly, has been shown to be effective in treating infections caused by members of the CANV complex (Roffey et al. 2003, Scope et al. 2005, Theuretzbacher et al. 2006, Van Waeyenberghe et al. 2010). It is commonly used in humans to treat invasive aspergillosis and Candida infections in immunocompromised hosts.

Figure 1.6 Chemical structure of voriconazole (Roerig 2008).



There have been only three published studies of voriconazole use in reptiles. Those are in bearded dragons (Van Waeyenberghe et al. 2010), girdled lizards (Hellebuyck et al. 2010), and red-eared sliders (Innis et al. 2008). This review includes data from studies in

humans and other animals in an attempt to provide a comprehensive overview of voriconazole characteristics and pharmacokinetics, the assumptions made in these studies and how the data is of relevance to developing a treatment protocol in tuatara.

1.5.2 Mechanism of action

Voriconazole acts by inhibiting cytochrome P450-dependent 14-α-lanosterol demethylase, an enzyme required for the synthesis of ergosterol. This results in the replacement of ergosterols in fungal cell membranes by 14-α-methylated sterols, causing disruption of membrane structure and function and inhibiting fungal growth (Beernaert et al. 2009). Compared to ketoconazole and itraconazole, voriconazole has a greater selectivity for fungal than rat cytochrome P450 liver enzymes (Roffey et al. 2003). Antimycotics do not eliminate infection completely as they are very rarely fungicidal, but the inhibition of fungal growth creates conditions allowing recovery from infection with the aid of the animals' immune system (Vantrubova et al.2010). It is reported that the *in vitro* activity of voriconazole against filamentous fungi exceeds that of itraconazole (Diekema et al. 2003).

1.5.3 Formulation and absorption

Voriconazole is available in tablet, oral solution and injectable intravenous formulations and is commercially known as VFEND® (Pfizer, Auckland, New Zealand). The majority of animal studies have used oral formulations, as intravenous use is often impractical over long periods. A study in red-eared sliders used the intravenous formulation subcutaneously at a dose rate of 5 mg/kg and reported that serum concentrations only

exceeded the target concentration of 1µg/mL for the first two hours, making this dosage and route unsuitable for use in these animals (Innis et al. 2008).

Several studies have used tablets crushed and suspended in water, orange juice and dilute hydrochloric acid, or a commercial suspending agent in an attempt to provide precise dosing and enhance drug absorption from the gastrointestinal tract (Burhenne et al. 2008, Flammer et al. 2008, Sanchez-Migallon Guzman 2010). One study in African Grey parrots found significant differences in the maximal plasma concentration and area under the curve when the same dosage was given with water versus a commercial suspending agent (Flammer et al. 2008). The commercial suspending agent may have provided a more uniform suspension, altered gastrointestinal transit time or increased drug solubility (Flammer et al. 2008). The studies reported in this thesis used the oral suspension of voriconazole that comes as a powder to be reconstituted, as this provided more consistent and accurate dosing than crushing and suspending tablets prior to administration.

Following oral dosing using tablets in humans, voriconazole is rapidly absorbed within two hours, and has a bioavailability exceeding 90% that is not affected by gastric pH. The presence of food in the stomach significantly delays its absorption and decreases bioavailability, but does not affect trough voriconazole concentrations (Purkins et al. 2003, Theuretzbacher et al. 2006). In humans it is recommended that voriconazole be administered more than one hour before or one hour after a meal (Purkins et al. 2003). In chickens, bioavailability was less than 20% following oral administration to non-fasted animals at a dose rate of 10 mg/kg. In this study on chickens the drug was administered by crushing tablets and suspending them in water (Burhenne et al. 2008). Administering

the same dosage and formulation to fasted racing pigeons showed a voriconazole bioavailability of 43.7%. It is possible the presence of food in the gastrointestinal tract of the chickens may have slowed absorption and decreased bioavailability of voriconazole, as in humans. When laboratory-synthesised voriconazole of 98% purity was suspended in two different solutions and administered orally, bioavailability was in excess of 75% in mice, rats, rabbits, guinea pigs and dogs (Roffey et al. 2003). These animals had ad-lib access to food throughout the studies, so the effect of food in the gastrointestinal tract was not assessed.

1.5.4 Metabolism

Voriconazole is eliminated predominantly after biotransformation, and undergoes extensive hepatic metabolism by cytochrome P450 enzymes in the liver in all species investigated (Roffey et al. 2003, Theuretzbacher et al. 2006, Beernaert et al. 2009). In humans it is the CYP2C19 enzyme that is predominantly involved in voriconazole's metabolism, with a lesser contribution from CYP3A4 (Roffey et al. 2003). Levels of CYP2C19 can vary significantly between individuals, thus there is significant inter-individual variation in plasma concentrations of voriconazole even when given at the same dosage. This high level of variation in plasma concentration has also been reported in other animals including chickens, Amazon parrots and bearded dragons (Burhenne et al. 2008, Sanchez-Migallon Guzman et al. 2010, Van Waeyenberghe et al. 2010). This variability suggests that higher-end doses may be required to ensure voriconazole trough concentrations do not fall below the concentration required to inhibit growth of the fungus in all patients.

Several animal studies have reported a decline in maximal concentration, minimum concentration and elimination half-life over time when giving the same amount of voriconazole (Roffey et al. 2003, Beernaert et al. 2009). This effect has been noted in mice, rats, dogs, bearded dragons and African Grey parrots but not in humans, girdled lizards, guinea pigs, rabbits or chickens (Roffey et al. 2003, Burhenne et al. 2008, Beernaert et al. 2009, Van Waeyenberghe et al. 2010). This is thought to be due to voriconazole inducing its own metabolism, a hypothesis supported by the findings of dose-related increases in hepatic cytochrome P450 content and relative liver weight in both rats and dogs (Roffey et al. 2003).

This autoinduction indicates that while an administered dosage may stay above minimum inhibitory concentration (MIC) at the start of the study, at the end of a six week course this may not be the case. This may require the use of higher doses throughout treatment, or increasing dosage in line with monitored voriconazole concentrations in the plasma (Flammer et al. 2008). Autoinduction is not seen in humans; this may be because of a species-specific difference or because lower dosages are used in people (Roffey et al. 2003). It should be noted that the study in girdled lizards only sampled plasma drug concentrations for the first three days of treatment, which may not have been sufficient for autoinduction to occur (Hellebuyck et al. 2010).

Both liver weight and cytochrome P450 levels returned to normal in rats one month after cessation of treatment (Roffey et al. 2003). If tuatara are similar to rats, this would indicate that the time between multiple dose voriconazole studies at different temperatures would need to be at least one month to ensure liver enzyme levels have returned to normal and will not affect the pharmacokinetics of subsequent studies.

In humans, voriconazole is eliminated mainly by hepatic metabolism; very little is excreted unchanged in urine and faeces (Theuretzbacher et al. 2006). Voriconazole's major metabolite is N-oxide UK-121,265 and shows no significant antifungal activity (Flammer et al. 2008, Roffey et al. 2003).

1.5.5 Pharmacokinetic profile in humans and selected other species

In most cases, voriconazole exhibits nonlinear pharmacokinetics, meaning that the steady-state concentration of drug in the blood does not increase proportionally with dose, so if the amount of drug administered is doubled, the steady state concentration in plasma increases by a factor of more than two (Roffey et al. 2003, Theuretzbacher et al. 2006, Burhenne et al. 2008, Beernaert et al. 2009). In voriconazole this is thought to be a result of capacity-limited metabolism due to saturation of the enzymes required to metabolise the drug (Beernaert et al. 2009). This means pharmacokinetic data cannot be extrapolated from one dosage to another (Flammer et al. 2008), so it is not possible to make an assumption that decreasing the oral dose by a particular amount will result in plasma concentration staying above a certain level.

In children under twelve years of age voriconazole has been shown to have linear pharmacokinetics (Theuretzbacher et al. 2006), and this has also been shown to occur in Amazon parrots, with a doubling of dose from 12 to 24 mg/kg resulting in a doubling of maximal plasma concentration and area under the curve (Sanchez-Migallon Guzman et al. 2010). It is possible non-linear pharmacokinetics may exist in Amazon parrots at different doses; however this has not been investigated. There do not appear to be any studies in the scientific literature on the pharmacokinetic behaviour of voriconazole in

juvenile animals. This was a contributing factor in the decision to limit the animals in my study to those that were considered to be in the adult weight range, in an effort to reduce potential age-related variability in drug metabolism.

Studies by Hellebuyck et al. (2010) and Van Waeyenberghe et al. (2010) have described the pharmacokinetics of voriconazole in bearded dragons and girdled lizards respectively. The administered dosages of 10 mg/kg resulted in trough concentrations at least ten times greater than the minimum inhibitory concentrations for the isolates of the CANV complex fungi in question. However due to voriconazole's nonlinear pharmacokinetics it is not possible to predict that one tenth or even one fifth of the dose would result in trough concentrations still above the required MIC of the fungal isolates, and it is not possible to predict pharmacokinetic parameters across species (Beernaert et al. 2009). Pharmacokinetics in the rat were gender-dependent with males having lower serum voriconazole concentrations than females, however this is a phenomenon often observed in rats but not in other species (Roffey et al. 2003).
In humans steady-state plasma concentrations were achieved after five to seven days of oral dosing (Theuretzbacher et al. 2006).

Disposition in skin

There is limited pharmacokinetic data available on the disposition of voriconazole in skin. Clinically, voriconazole has been used successfully in humans with severe, invasive fungal dermatitis (Troke et al. 2008). Voriconazole has been studied in guinea pigs in an experimental model of dermatophytosis, and after 12 days of administration of 20 mg/kg orally once daily, maximal plasma concentration was 6.3 mg/L, total skin concentration was 18.3 µg/g, and unbound skin concentration was 1.4 µg/mL (Saunte

et al. 2007). The authors concluded that there was accumulation of the drug in skin that could not solely be explained by protein binding.

Plasma protein binding

Binding of voriconazole to plasma proteins was reported to be 58% in humans (Roffey et al. 2003), in African Grey parrots it varied between 38 and 67% (Flammer et al. 2008), and was 67% in mice, 66% in rats, 60% in rabbits, 45% in guinea pigs and 51% in dogs (Roffey et al. 2003). Unbound drug concentration in the plasma is shown to have a better correlation with clinical outcome than total concentration, however unbound drug concentration is rarely measured (Theuretzbacher et al. 2006).

Half-life

Elimination half-life of voriconazole in humans is approximately six hours, however due to the drug's non-linear pharmacokinetics this is dose-dependent (Theuretzbacher et al. 2006, Flammer et al. 2008). In racing pigeons dosed at 10 mg/kg using crushed tablets suspended in water the elimination half-life was ten hours (Beernaert et al. 2009), however in chickens dosed using the same method and dose rate, half-life was less than two hours (Burhenne et al. 2008, Beernaert et al. 2009). The racing pigeons were fasted and the chickens were not; this would be expected to cause variation in drug absorption and time to maximal concentration, but not in drug metabolism and elimination. In horses, voriconazole half-life after a 10 mg/kg oral dose was 7.8-12.9 hours (Colitz et al 2007). It is likely that significant species differences in metabolism, possibly in CYP2C19 enzymes levels, are the cause of such different half-lives. In African Grey parrots the half-life of voriconazole was 1.1 hours at 6 mg/kg, and 1.6 hours at 12 mg/kg, again demonstrating that pharmacokinetics of voriconazole change with dose rate, making

extrapolation of pharmacokinetic parameters difficult or impossible at different dosages, even within the same species (Flammer et al. 2008, Roffey et al. 2003).

Time to maximal plasma concentration

The time to peak plasma concentration (Tmax) is influenced by bioavailability, rate of absorption and sometimes dosage of the drug. In humans, voriconazole's Tmax was less than two hours following oral dosing (Theuretzbacher et al. 2006). In Amazon parrots it was one hour at 12 mg/kg and two hours at 24 mg/kg voriconazole (Sanchez-Migallon Guzman et al. 2010), in racing pigeons it was just over two hours at 10 mg/kg voriconazole (Beernaert et al. 2009), and in African Grey parrots it was two and four hours for 6 and 12 mg/kg respectively (Flammer et al. 2008). Voriconazole Tmax varies greatly between mammal species and is reported as two hours in mice (10 mg/kg), six hours in male rats (30 mg/kg), one hour in female rats (30 mg/kg), one hour in rabbits (10 mg/kg), eight hours in guinea pigs (10 mg/kg), 7.8-12.9 hours in horses (10 mg/kg) and three hours in dogs (6 mg/kg), where the numbers in parentheses indicate the oral dosages administered (Roffey et al. 2003, Colitz et al. 2007). Tmax is important in determining the number of time points used in sampling, as it may affect calculation of half-life and determination of dosing interval for multiple dose studies.

The two studies conducted in reptiles by Hellebuyck et al. (2010) and Van Waeyenberghe et al. (2010) did not measure voriconazole Tmax. Van Waeyenberghe et al. (2010) assumed it to be two hours, presumably extrapolated from the human Tmax, and Hellebuyck et al. (2010) assumed a Tmax of three hours. Given the observed species differences in other studies it is possible that samples taken at these times do not represent the true maximal drug concentrations of voriconazole. These measurements

may have led to incorrect calculation of other pharmacokinetic parameters; however the two studies in question described treatment of clinical cases and did not include the calculation of other values such as half-life and area under the curve.

Maximal drug concentrations in humans were 83% higher in young females than young males, however no dosage adjustment is currently recommended to compensate for this (Theuretzbacher et al. 2006).

Volume of distribution

The volume of distribution of voriconazole in humans is high, at 2.0-4.6L/kg (Theuretzbacher et al. 2006). In chickens this was lower at 1.68L/kg (Burhenne et al. 2008), and in horses was 1.6L/kg (Colitz et al. 2007). The lower tissue distribution of voriconazole in chickens would contribute to the shorter half-life observed in this species

1.5.6 Adverse effects

Approximately 30% of humans experience transient visual disturbances such as hallucinations during voriconazole therapy that resolve with cessation of treatment. Other side effects include liver function test abnormalities (13%) and dermatological reactions such as rashes and photosensitivity (6%) (Theuretzbacher et al. 2006). Some studies suggest that visual adverse events and elevations in aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin (but not alanine aminotransferase (ALT)) are more likely to occur with higher plasma voriconazole concentrations, but there is no statistically significant association between voriconazole trough concentrations and hepatotoxicity (Pascual et al. 2008, Tan et al. 2006). Trough plasma voriconazole

concentrations above 5.5 mg/L were associated with toxic encephalopathy in 31% of patients, and trough concentrations above 8 mg/L had an estimated 90% probability of resulting in neurotoxicity (Pascual et al. 2008). Co-medication with omeprazole, which is metabolised by the same hepatic enzymes as voriconazole, resulted in higher drug concentrations and increased probability of neurotoxicity (Pascual et al. 2008, Shirasaka et al. 2013). Symptoms of toxicity resolved with cessation of voriconazole treatment.

In racing pigeons, 20 mg/kg of crushed and suspended tablets administered twice daily caused clinically relevant hepatotoxicity, as evidenced by behavioural indicators of illness, increased serum AST activity and histological changes including hepatocellular vacuolisation, and portal heterophilic and lymphocytic infiltration. Pigeons in this study had voriconazole plasma concentrations ranging from 5.85 (± 3.12) to 15.88 (± 7.99) mg/L throughout the study (Beernaert et al. 2009). There were no adverse effects reported in chickens administered 10 mg/kg of voriconazole suspension of crushed tablets once daily (Burhenne et al. 2008), however low oral bioavailability and low plasma concentrations were reported in this study. It is possible the same dose rate in other animals under fasting conditions or with different capacities to metabolise voriconazole could potentially result in higher plasma concentrations that may cause side effects. It is also difficult to assess visual disturbances in animals, so it is possible side effects may be occurring but are unrecognised. In a retrospective study of voriconazole toxicity in multiple penguin species, clinical signs of toxicity included anorexia, lethargy, ataxia, paresis, apparent vision changes, seizure-like activity and generalised seizures (Hyatt et al. 2015). All penguins showing signs of toxicity had trough plasma voriconazole concentrations above 5.5 mg/L, and those with plasma concentrations above 30 mg/L had moderate to severe neurological signs. There was no

evidence of voriconazole-induced hepatotoxicity in any of the penguins in this study (Hyatt et al. 2015).

A study by Van Waeyenberghe et al. (2010) in bearded dragons administered 10 mg/kg of voriconazole orally every 24 hours showed significant elevations in serum AST activity in three out of seven animals. This was thought to be an indication of liver damage; however it was not determined if this was caused by fungal invasion into the liver or by voriconazole-related toxicity (Van Waeyenberghe et al. 2010). The study did not measure creatine kinase (CK), and it is also possible for muscle damage to cause elevations in AST. One animal in this study died during voriconazole treatment with fungal hyphae found in the liver and lungs, and again it was not clear on histopathology if the mortality was caused by drug hepatotoxicity or consequences of fungal invasion (L. Van Waeyenberghe 2014, pers. comm.).

Due to the nonlinearity and high inter-individual variability in voriconazole metabolism, it is recommended that both single and multiple dose studies be conducted for each species in question when designing a treatment protocol (Beernaert et al. 2009), however this is rarely practical in zoo medicine.

1.6 Rationale and aims of this study

1.6.1 Rationale

The discovery of fungal dermatitis in Auckland Zoo's tuatara has halted plans to release some of these animals back into the wild, and threatens the health of these tuatara. The basis for this study was the findings of a Disease Risk Analysis (DRA) for *P. australasiensis*

in Auckland Zoo's tuatara, which recommended a risk management strategy of treating all tuatara with an antifungal for a minimum of six weeks within a quarantine setting prior to transfer to another site (Auckland Zoo 2013, unpublished). In addition, it was recommended if there was no significant difference in antifungal pharmacokinetics at different temperatures, it would be preferable from a logistical, husbandry and welfare perspective for the animals to be treated in their normal enclosures at ambient temperatures.

Prior to initiating pharmacokinetic studies, it is critical to determine what dose of medication is required *in vitro* to inhibit growth of the target organism. This allows determination of a target concentration for *in vivo* pharmacokinetic studies. Characterising fungal growth at different temperatures provides information that may be used in the medical and husbandry management of affected animals.

While treatment of tuatara with oral itraconazole has been clinically successful, there are no pharmacokinetic studies to support the dose rates and intervals used. Tuatara inhabit cold climates unsuitable for most reptiles, and consequently have very low metabolic rates. This low metabolic rate can be expected to affect drug pharmacokinetics, potentially necessitating the use of different drug dosing protocols than those designed for other reptile species. Several studies in other reptiles have shown that ambient temperature may affect drug pharmacokinetics (Hodge 1978, Mader et al. 1985), however this has not been evaluated using antifungals. There have been few studies on the effectiveness and dosages required for use of antifungals in reptiles, with treatment recommendations often based on data from mammals or birds. Voriconazole is only just starting to be used in veterinary medicine, and it will be useful

to determine if it is an effective and safer alternative to itraconazole for antifungal treatment in reptiles. This study will also provide evidence of *in vitro* efficacy of itraconazole and voriconazole against *P. australasiensis*.

Establishing a safe, effective treatment protocol for *P. australasiensis* infections is critical to the future management of captive tuatara held by Auckland Zoo. The results of this study have broader application to the treatment of reptiles with antifungals within the range of their POTZ, and add to the growing body of knowledge on CANV complex-associated infections. There have been few published antifungal pharmacokinetic studies in reptiles. This study will provide evidence of *in vitro* efficacy of itraconazole and voriconazole against *P. australasiensis*, and facilitate the development of evidence-based treatment protocols which may be able to be applied to other cold-adapted reptiles. Establishing an effective treatment protocol using voriconazole is of particular benefit, as voriconazole shows potential to be a safer alternative to itraconazole in the treatment of mycoses in reptiles.

1.6.2 Study aims

The aims of this study are:

- To characterise the *in vitro* growth of *P. australasiensis* at different temperatures.
- To determine the MICs of itraconazole and voriconazole for *P. australasiensis* isolates sourced from Auckland Zoo's animals.
- To determine single and multiple dose pharmacokinetics of itraconazole and voriconazole in tuatara at the high and low end of the POTZ of tuatara.

- To develop appropriate dosing regimens for itraconazole and voriconazole for the treatment of *P. australasiensis* in tuatara.
- To establish haematological and biochemical reference ranges for captive tuatara, as a basis for monitoring and evaluating the health of these animals throughout the study and into the future.

This study is novel and addresses an issue of significant practical relevance to captive wildlife institutions in New Zealand. It also has broader application to the captive management of reptiles and the growing concern regarding emerging fungal disease in reptiles caused by members of the CANV complex.

Chapter 2: General methods

2.1 Overview of study design

The first step in this study was to determine optimal fungal growth temperatures and Mean Inhibitory Concentrations (MICs) of itraconazole and voriconazole for *P. australasiensis*. Following this, a pharmacokinetic study was conducted to investigate single dose pharmacokinetics of itraconazole and voriconazole in healthy tuatara at 12^{o}C and 20^{o}C, reflecting the lower and upper limits of the tuatara's Preferred Optimal Temperature Zone (POTZ). Healthy animals were divided into itraconazole and voriconazole treatment groups, and these did not change throughout the study. Health screening of the tuatara, including haematology, biochemistry, radiographs and physical examination, was performed prior to the commencement of the single dose studies. The itraconazole group completed the single dose study at 12^{o}C and then at 20^{o}C, while the voriconazole group completed the study at 20^{o}C and then at 12^{o}C. This meant that each animal served as its own control, in a crossover study design. There was a five-week washout period between studies at the two temperatures. Blood samples were taken at predetermined intervals following the drug administration to determine antifungal concentrations, and characterise individual pharmacokinetic profiles for each drug. Following this, pharmacokinetic modelling was performed to estimate pharmacokinetic parameters and simulations were undertaken to explore appropriate dose regimen (dose rate and dosing intervals) for multiple dose studies.

Multiple dose studies were each planned to be conducted over an eight week period, with six weeks of medication administration and two weeks to measure drug elimination. The clinical study used a fixed sequence design with the investigation at 12^{o}C first, and after a fifteen-week washout period, repeated at 20^{o}C. Blood samples were collected at predetermined intervals to determine antifungal concentrations, and

pharmacokinetic modelling was used to analyse the data. This led to alterations in the dosing regimens for the 20°C studies. Health screening was performed for each animal before each multiple dose study commenced, and regularly during the studies to monitor haematologic and biochemical values. These health screening data were used to develop haematologic and plasma biochemical reference ranges for tuatara (see Chapter 6).

2.2 Permits and ethics approval

This research was conducted with approval from Murdoch University's Animal Ethics Committee (permit RW2627/14). Ethics approval was also granted by Auckland Zoo's Animal Ethics Committee. A Wildlife Act Authority application to use tuatara in research was granted from New Zealand's Department of Conservation (permit 38610-FAU), in consultation with iwi.

2.3 Animal selection and health screening

2.3.1 Animal selection and grouping

Healthy tuatara used in the study were adults (>20 years of age), with a minimum weight of 400 g. Ages ranged from 21 years to unknown-aged adult, as many were adults when acquired from wild populations up to 25 years ago. At the time of this study Auckland Zoo held 12 adult tuatara: 4 males and 8 females above 400 g. Three of these animals (one male and two females) were unavailable for the single dose study as they were in a display enclosure in the zoo, while the remaining nine were housed off-show. All nine of these animals underwent health screening, though only eight animals were required

for this part of the study. Health screens comprised a physical exam, dorso-ventral radiograph, and haematological and biochemical testing. All health screening results were within normal limits for this species (see Section 2.3.2 and Chapter 6), but, to retain equal sex ratios for each group, only two of the three males were used for this study. The heaviest male was excluded, as the other two males were of similar body weight, making the groups roughly equal in terms of weight distribution. The eight selected tuatara were divided into two treatment groups: an itraconazole and a voriconazole group, which remained unchanged throughout the study. Each group comprised one male and three females, of comparable size and age.

For the multiple dose studies, all twelve tuatara participated. The four animals that did not participate in the single dose studies were allocated to the itraconazole or voriconazole groups in a way that maintained similar age, sex and weight ratios, so that there were four females and two males in each group. Several animals were unknown-aged adults, and these animals have their ages listed as a 'greater than' value. Population demographics and drug allocation for the study population are displayed in Table 2.1.

There was a four month washout period between multiple dose studies to ensure all previously administered drugs had been eliminated and to allow tuatara time to recover from any physiologic changes related to the study, including blood sampling. One month before commencement of the second multiple dose study, blood samples were taken to confirm the absence of antifungal drug concentrations, including itraconazole's active metabolite hydroxy-itraconazole (OH-itraconazole), in the plasma of all tuatara. No tuatara had detectable antifungal concentrations.

Table 2.1 Population demographics for tuatara studies. Weight and age was at the time of animal allocation, one month prior to the first single dose studies commencing. Minor weight changes occurred throughout the study.

Animal ID	Medication group	Sex	Age (years)	Weight (kg)
101	Itraconazole	F	20	0.486
104	Itraconazole	M	>44	0.789
105	Itraconazole	F	>43	0.472
107	Itraconazole	F	>44	0.542
109	Itraconazole	F	22	0.489
112	Itraconazole	M	>44	1.030
202	Voriconazole	F	20	0.479
203	Voriconazole	M	>44	0.952
206	Voriconazole	F	>44	0.517
208	Voriconazole	F	>44	0.556
210	Voriconazole	M	22	0.912
211	Voriconazole	F	22	0.432

2.3.2 Health screening

All samples were stored refrigerated at 4^{o}C and analysed within 2 hours of collection.

Haematology

Blood was collected from the caudal vein for measurement of haematologic and biochemical values. Blood was collected into lithium heparin tubes, and fresh blood smears were prepared using the wedge technique. Haematology was performed by a single technician at a commercial laboratory (New Zealand Veterinary Pathology, Auckland, NZ), and reported values for haematocrit, haemoglobin, mean corpuscular

haemoglobin concentration, white blood cell count, fibrinogen, and a white blood cell differential count. White blood cell numbers were determined using the chamber count method, except when there was insufficient blood volume, in which case an estimate based on blood smear examination was reported. Haemoglobin (Hb) was measured using an automated optical method (HemoCue Hb 201+, Radiometer Pacific, NZ). Packed cell volume (PCV) was measured by loading capillary tubes with whole blood and centrifuging them at 10,000RPM for 5 minutes, then reading the results from a standard chart. Mean corpuscular haemoglobin concentration (MCHC) was determined using the equation MCHC = Hb/PCV. Fibrinogen was determined using the heat precipitation method (Millar et al. 1971).

Total white blood cell counts were performed manually, using a haemocytometer and 1% ammonium oxalate solution (Samour 2000). Smears for estimated white cell counts and differential counts were stained using Romanowsky stains (Leishman's and May Grunwald stains, Amber Scientific, Australia), and read using published methods (Dacie and Lewis 1995, Samour 2000). For some samples, insufficient blood was available for a white cell count using the haemocytometer, and an estimated count from a smear was performed (see Chapter 7 for haemocytometer and estimated white cell count comparisons). All haematology at NZVP was read and reported by the same operator throughout the study.

Results were evaluated using reference intervals compiled from several sources, including previous testing of healthy zoo animals, and unpublished data from healthy wild tuatara from the Department of Conservation (Jakob-Hoff 1996, Boardman & Blanchard 2006, Gartrell et al 2006). All animals were determined to have blood values

within the reference ranges and less than three standard deviations from the mean, so on this basis and the absence of clinical signs of disease were considered clinically normal and suitable for participation in the study. The same haematology and biochemistry testing was repeated 4-6 weeks prior to each phase of drug administration, to allow for regeneration of any lost blood volume.

Biochemistry

Whole blood (0.1 ml) was used to conduct biochemical assays using the Avian/Reptilian Profile Plus rotors in the Abaxis Vetscan classic chemistry analyser (Abaxis, USA). 100µL of lithium heparin-preserved whole blood was pipetted into the rotor, and samples were run immediately. The following parameters were measured for each animal: aspartate aminotransferase (AST), bile acids (BA), creatine kinase (CK), uric acid (UA), glucose (Glu), calcium (Ca^{2+}), phosphorous (Phos), total plasma protein (TP), albumin (Alb), globulins (Glob), sodium (Na^{+}), potassium (K^{+}) and chloride (Cl^{-}).

Radiography

A dorso-ventral radiograph was taken of each animal while conscious prior to entry into the study using a DR digital radiography system (Sound-Eklin, USA). This allowed evaluation of bone density, organ size, and whether the females were gravid. On radiographs it was possible to detect the presence of large developing ovarian follicles or calcified eggs in a gravid animal, depending on the time of year. All animals had radiographic findings that were within normal limits.

Physical examination and faecal screening

Each animal was given a thorough physical examination, including oral exam, ophthalmologic exam, coelomic auscultation, and visual examination of the whole body and cloaca. Several animals had regenerated tails or scarring from injuries sustained over ten years prior to this study. Physical examination findings were considered normal in all animals. There was no evidence of ocular, nasal, oral or aural discharge. Heart rate and respiratory rate and quality were normal, swelling of the coelom was not palpated, and cloacal appearance was normal. There were no skin lesions, and demeanour and locomotion were assessed as normal. Routine faecal examinations using ZnSO4 floatation were conducted twice yearly for the previous 5 years, and did not detect any faecal parasites.

2.4 Animal housing and care during study

2.4.1 Enclosure design and environmental maintenance

Enclosure design

Animals were housed individually during the study. Tuatara are territorial and do not cohabit in small spaces, so individual housing eliminated the possibility of fighting and unequal food or environmental access. Study enclosures were constructed of untreated pine plywood, and had internal dimensions of 1500 x 800mm, with a height of 600mm. Enclosure floors were lined with corrugated cardboard to provide an easily maintained surface that provided adequate grip for the animals. Substrate such as mulch was not provided, as this would allow animals to thermoregulate.

Animals were provided with a shallow plastic water dish of 300mm diameter, in which they could bathe and pass urates and faeces. This was cleaned and replaced when necessary, at a minimum once weekly. Each tuatara was provided with a hide tunnel constructed of double-walled corrugated high density polyethylene culvert pipe of 225mm diameter, cut into semicylindrical lengths of 600mm (Figure 2.1). Flaps of plastic cut from black garbage bags were taped at one end to provide an entry and exit point while maintaining darkness, the other end was positioned against a wall of the enclosure. This gave the animals a place to hide, and was large enough for them to turn around in.

Temperature control

Studies were conducted at 12^{o}C and 20^{o}C. Reverse-cycle air conditioning units were used to maintain temperature, and dataloggers (Thermochron iButtons, Maxim, USA) were placed at two points in each room to ensure temperatures remained within an acceptable range. These were set to record temperatures every 30 minutes throughout the study period. Temperatures in the 12^{o}C room ranged from 10.5-13.0^{o}C, and in the 20^{o}C room from 19.5-21^{o}C. A thermal imaging camera (Trotec IC080LV infrared camera) was also used periodically to compare the temperature of the animal with the surrounding environment.

Photoperiod

Photoperiod was maintained at 12 hours by use of an automatic timer. The same photoperiod was applied in all parts of the study to minimise variation in metabolic rate associated with season or circadian rhythms. Ultraviolet (UV) lighting was provided using two 39-watt tubes (Reptisun T5 High Output 10.0 UVB lamp, ZooMed, USA) above each

enclosure. These were positioned at one end of the enclosure 600mm above the floor, and set to come on for two three-hour intervals each day. Temperature below the lights was monitored using dataloggers to ensure it was not significantly different from the desired study temperature.

Figure 2.1 Thermal imaging camera image of a tuatara maintained at 20°C during the study.

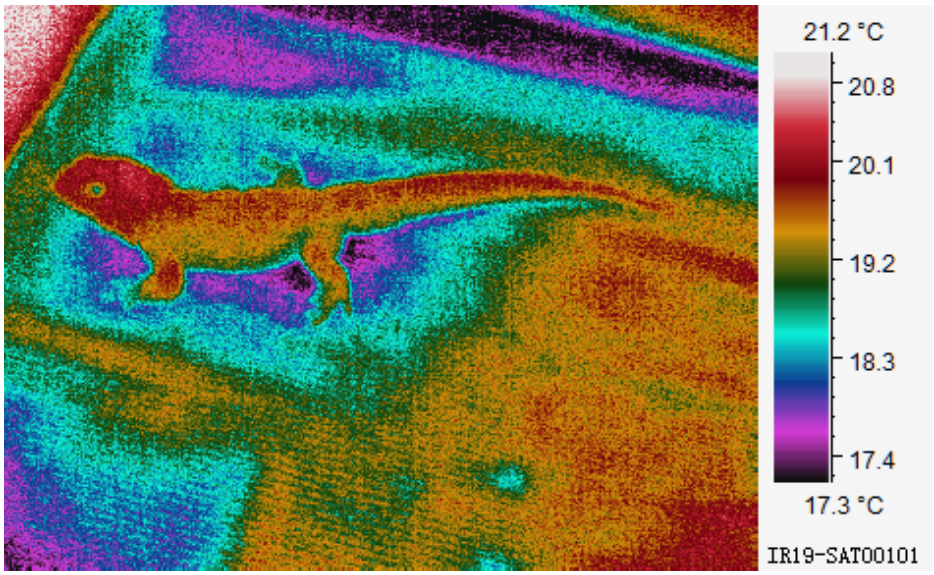
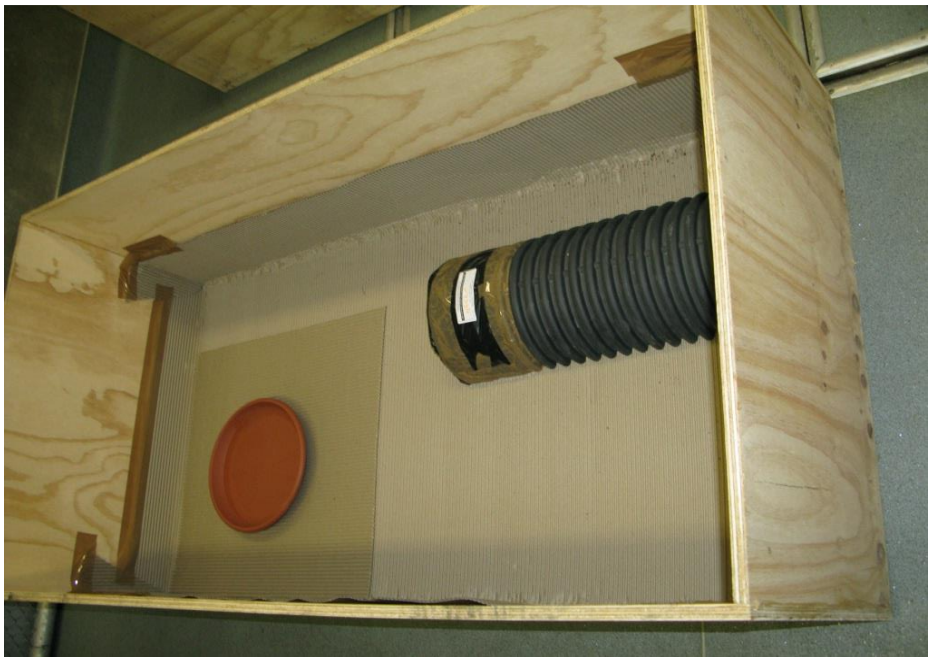


Figure 2.2 Study enclosure set-up.



Acclimatisation

Animals had an acclimatisation period of one week in the study environment prior to experiments beginning. Tuatara have been shown to reflect their environmental temperature 15 minutes after moving into a new environment (Wells et al. 1990), and other temperature-related pharmacokinetic studies in reptiles have used an acclimatisation period of one week (Caligiuri et al. 1990, Johnson et al. 1997) and one month (Mader et al. 1985). The acclimatisation process was designed to ensure tuatara adapted to the experimental environment, and that their internal temperature and metabolic rate had stabilised at the new ambient temperature. Cloacal temperature was measured using a digital thermometer the day prior to medication administration, and was found to reflect that of the environment.

2.4.2 Diet

Animals were fed their usual diet, which is 2-3 locusts (*Locusta migratoria*) or 3-4 field crickets (*Gryllus bimaculatus*) per feed, dusted with a calcium powder (Calcium Plus, Repashy Superfoods, UK). This was supplemented with kawakawa (*Macropiper excselsum*) berries, when in season. Animals held at 20^{o}C were fed twice weekly, and animals at 12^{o}C were fed once every two weeks based on recommendations from tuatara researcher Associate Professor Alison Cree, University of Otago (pers comm June 2014).

2.5 Blood sampling and medication administration

2.5.1 Sampling method

Restraint

For blood collection, tuatara had a head bandage applied prior to sample collection (Figure 2.3). Head bandaging involves placing two cotton wool balls over the eyes and securing them with a moderately tight elastic bandage (Vetrap, 3M, USA). This produces a vaso-vagal response which lowers heart rate and blood pressure, producing a 'trance-like' state (Hernandez-Divers 2006). This reduces animal movement and stress during minor procedures such as blood sampling. The tuatara was initially restrained horizontally, with one hand behind the angle of the jaw and the other over the lumbar area, restricting movement to reduce the risk of the animal moving and injuring itself or the handler. The animal was then moved into an upright position, with the head towards the ceiling, tail towards the floor and the ventrum pointed towards the operator collecting blood. Following sampling, the head bandage was removed and the animal returned to its enclosure.

Volume

A volume of 0.2 mL of blood was required for antifungal testing per sample, to provide an adequate plasma volume for repeat testing if required. An additional 0.2 mL of blood was required for health screening at set intervals during the study. The total blood volume required was 1.2 mL per animal over a period of two days for single dose studies, and 3.4 mL over a period of up to eight weeks for multiple dose studies. This is less than the recommended 1% of body weight that can be taken from a healthy reptile on a single

occasion (Douglas Mader, pers comm January 2014), which would equate to 4 mL in a 400 g animal.

Figure 2.3 Tuatara restrained in horizontal recumbency with head bandage applied.

Location and technique

Blood was taken from the ventral coccygeal vein, approximately 20-30% of the way down the tail. Tuatara do not possess hemipenes (Boardman and Blanchard 2006), so there was no need to sample more distally to avoid these, as would be necessary in lizards. Jugular and cephalic venepuncture sites were investigated on a deceased specimen, and these were found not to be practical options. Placing of a jugular catheter to allow repeat atraumatic sampling would require general anaesthesia, which could alter the metabolism of the animal and potentially influence the pharmacokinetics of the antifungal agents. It was also considered that placement of a jugular catheter would be awkward due to the tuatara's head and neck conformation, and the cranial position of the heart in the thoracic cavity. Use of an indwelling catheter would also necessitate

the removal of additional blood volumes, to ensure circulating venous blood was being sampled, rather than blood or saline sitting in the catheter. The ventral coccygeal vein is the standard site used by researchers and clinicians for obtaining blood samples from tuatara (Boardman and Blanchard 2006) and was selected as the most appropriate for this study.

The ventral coccygeal vein is not visible superficially and is located using anatomical landmarks. The animal was restrained as described above, and the skin overlying the venepuncture site prepared with an alcohol swab. A 25 gauge 1.5" needle (BD PrecisionGlide Needle 0.5 x 38 mm, BD, Singapore) attached to a 1 mL syringe (BD 1 mL tuberculin syringe, BD, Singapore) was inserted on a 45-degree angle between scales on the ventral midline of the tail. Blood was most reliably obtained just before the needle hit the ventral surface of the coccygeal vertebrae though, at times, repositioning was required. No lymph contamination was observed. No more than three attempts to draw blood were made, after which the animal was returned to its enclosure even if a sample was not obtained.

Blood for antifungal plasma concentration measurement was immediately transferred into a small potassium EDTA tube (K_2EDTA Microtainer, Beckton, Dickson and Company, USA), and inverted several times to ensure adequate mixing. Use of serum tubes was investigated, but this resulted in loss of a significant amount of plasma in the clot, so use of tubes containing an anticoagulant were preferred. Potassium EDTA was requested by the testing laboratory in preference to lithium heparin due to their familiarity with the product. In blood collection for health screening, blood was dispensed into lithium heparin gel tubes (Lithium heparin and plasma separator Microtainer, BD, USA), as this

was the preferred anticoagulant for haematologic and biochemical analysis in reptiles (Mader 1999).

2.5.2 Sample processing, storage and transport

Samples for plasma drug concentration

Following blood collection, samples were centrifuged for 10 minutes at approximately 1200 g in a LW Scientific ZipSpin (LW Scientific, USA). Plasma was then harvested using a 100 µL pipette (Piccolo 100 µL Minipette, Abaxis, USA) and transferred to plastic 0.5 mL tubes (Free Standing Screw Tubes 0.5 mL, Scientific Specialties Inc, USA), labelled and frozen at -20^{o}C until transport. Samples were couriered in batches on ice to Canterbury Health Laboratories (CHL) for analysis, and were confirmed as frozen when they arrived. Following receipt, samples were kept frozen by the laboratory at -80^{o}C prior to analysis.

2.5.3 Medication administration

Medication was administered using a 0.3 mL insulin syringe (BD 0.3 mL Ultra-Fine Insulin syringe, Beckton, Dickinson and Company, USA) with the needle and hub removed. This allowed for more accurate dosing than using a 1 mL syringe. The animal was held horizontally on a flat surface by a handler, and the jaws opened using a wooden tongue depressor or the syringe itself. Medication was administered into the caudal oral cavity, the animal was then returned to its study enclosure and observed for 5 minutes afterwards. No regurgitation or loss of medication was noted using this method.

2.6 Itraconazole and voriconazole drug assays

2.6.1 Liquid Chromatography Mass Spectrometry-Mass Spectrometry assays

Antifungal drug concentrations were measured using aLiquid Chromatography Mass Spectrometry-Mass Spectrometry (LCMS/MS) assay. This is a combination of two laboratory techniques: High Performance Liquid Chromatography (HPLC) and tandem Mass Spectrometry (MS/MS). HPLC separates different compounds within a mixture based on their chemical and physical properties such as size, charge and affinity to other molecules. The mixture is known as the mobile phase, and the mobile phase travels through the stationary phase, or column, where analytes are separated. The mobile phase then enters the mass spectrometer.

The mass spectrometer conditions and settings were previously optimised for the compounds of interest and precursor-to-product ion transitions were selected. Data acquisition was performed via selected reaction monitoring (SRM).

Analytes then interact with the electrospray ioniser in the mass spectrometer (Q0), which uses a nebulising gas (usually nitrogen) to create a fine mist of molecules, which are then exposed to an electrical field that ionises them. These ions then enter the first quadrupole (Q1), where the ions of interest are allowed to pass through using selective electron fields. The selected ions from Q1 are then passed through the second quadrupole (Q2), where they are fragmented. Selected fragments then pass through the third quadrupole (Q3) and are detected at the Channel Electron Multiplier (CEM). This is displayed as ion counts per second versus time which creates a series of peaks (chromatogram).

Quantitation of analytes was done using peak area ratios and assessing these against calibration curves of known standard concentrations.

2.6.2 Drug assay procedure

Plasma (30 µL) was mixed with deuterated internal standards of 98-99.4% purity (Toronto Research Chemicals Inc, Canada) in acetonitrile (30 µL) to precipitate protein. After centrifugation, the supernatant was diluted and injected onto the reverse-phase HPLC system. Antifungals and internal standard were detected by LCMS/MS and quantitated. The process for preparing samples and standards, and the drug assay procedure, are detailed in Appendix 3.

Samples were prepared and analysed as duplicates, and the mean of these duplicates provided the final result for each sample. All duplicate samples were within 20% of each other, or were re-run until this acceptable level of agreement was achieved.

2.6.3 LCMS/MS conditions

This assay used a 3200 QTRAP LC/MS/MS system, with hybrid triple quadrupole/ion trap capabilities. The column used was a Kinetex XB-C18 100A reverse-phase silica-based column (50 x 2.1 mm) and particle size of 2.6 µm. LCMS/MS conditions are displayed in Tables 2.2 and 2.3.

Table 2.2 Retention time of analytes (minutes). Q1 = precursor ion mass (analyte mass + 1) of antifungal being measured. Q3 = product ion mass of the two most common fragments of the antifungal being measured.

Analyte	Q1	Q3	Retention time (min, approx.)
Voriconazole 1	350.0	281.0	3.6
Voriconazole 2	350.0	127.0	3.6
Itraconazole 1	705.0	392.0	4.0
Itraconazole 2	705.0	256.0	4.0
Hydroxy-itraconazole 1	721.0	408.0	3.8
Hydroxy-itraconazole 2	721.0	256.0	3.8
D3-Voriconazole	353.0	284.0	3.6
D3-Voriconazole	353.0	127.0	3.6
D5-Itraconazole 1	710.0	397.0	4.0
D5-Itraconazole 2	710.0	256.0	4.0
D5-Hydroxy-itraconazole 1	726.0	413.0	3.8
D5-Hydroxy-itraconazole 2	726.0	256.0	3.8

Table 2.3 Solvent gradient for LCMS/MS.

Time (min)	Flow rate (µL/min)	% eluent A	% eluent B
0	600	95	5
1.0	600	95	5
2.0	600	90	10
3.0	600	10	90
5.0	600	10	90
7.0	600	95	5
8.5	600	95	5

2.6.4 Development of calibration curves

Standards of voriconazole, itraconazole and hydroxy-itraconazole were prepared as described in Appendix 3. Calibration curves were produced by using standards over the concentration range 0-10 mg/L in drug-free tuatara plasma. Results were plotted graphically, and a line of best fit calculated for the results, plotting analyte concentration against analyte peak area ratios. The equation of this line was then used in determining antifungal concentrations.

2.6.5 Assay validation: precision, accuracy and matrix effects

Inter- and intra-run precision was estimated at three concentrations. For the inter-run statistics, measurements were conducted on a six point calibration, over two weeks, with a total of six replicates at each concentration. The intra-run statistics were calculated from six replicates at each concentration on a single day. The inter- and intra-run statistics are recorded in Appendix 4.

The following results were produced by the assay validation process:

Carryover: This was determined by three consecutive injections of the top standard followed by three blank injections. There was no detectable carryover.

Limit of detection: This is the concentration where the signal to noise ratio is <3:1

Limit of quantitation: This is the concentration where the signal to noise ratio is <10:1

Linearity: the method is linear to 10 mg/L

Matrix effects: Matrix effects for all four analytes had a % CV <10. European Medicines Agency guidelines (European Medicines Agency 2011) state matrix effects should be <15%

Recovery: Recovery of hydroxy-itraconazole was 100%. Voriconazole (101%) and itraconazole (103%) appear to have enhanced recovery in plasma, due to their reduced solubility in aqueous media. All standards and QC samples were prepared in drug free human plasma to eliminate this effect. Recovery was calculated at 3 concentrations, using the methods described in Bansal and DeStefano (2007).

2.6.6 Tuatara plasma matrix effects

The antifungal standards prepared in plasma were, by necessity, prepared using human drug-free plasma (DFP). It was not possible to collect sufficient drug-free plasma from tuatara for the purposes of this research, but enough was available to test for matrix effects, and ensure human DFP was comparable to tuatara DFP for the experimental conditions and analytical method. A chromatogram of drug-free tuatara plasma, and a chromatogram of tuatara plasma plus all four deuterated internal standards, did not show any peaks in the tuatara plasma that would interfere with drug assays (Appendix 5).

2.7 Non-compartmental pharmacokinetic analysis

Definitions and equations were derived from Concepts in Clinical Pharmacokinetics 4th Edition (DiPiro et al. 2005)

Elimination rate constant and half-life of antifungal drugs

The terminal elimination rate constant (k_{el}) describes the rate of elimination of the drug from the body. It was determined by calculating the slope of elimination from sequentially decreasing drug concentrations in the body. The points to be included in the equation are those drug concentrations in the terminal portion of the log-

concentration-time curve, or those after the peak concentration has occurred, and where there is a consistent decline in concentration. Determining which data pairs are suitable for inclusion is aided by plotting the points on a natural log graph and observing the slope to ensure the overall trend is one of declining drug concentrations. The terminal elimination rate constant is used in calculating elimination half-life and volume of distribution.

The terminal elimination rate constant was calculated using the SLOPE function in Microsoft Excel, and expressed in h^{-1}.

The half-life ($t_{½}$) of a drug is the amount of time it takes for the concentration of the drug in the body to halve during the elimination phase. It is expressed in hours, and calculated using the formula:

$t_{½} = \ln(2)/k_{el}$

Area under the curve

Area under the concentration-time curve (AUC) is the area under the plot of drug concentration against time, and represents total exposure to the drug over time. It is calculated using the trapezoidal rule to the last point that drug concentration was measured (AUC_{0-t}) and extrapolated to give AUC to infinity ($AUC_{0-\infty}$) using the following equation, and is expressed in mg/L*h:

$AUC_{0-\infty} = AUC_{0-t} + (C_{last}/k_{el})$

where C_{last} is the last measured concentration.

Clearance

Clearance (CL) is the volume of plasma which is completely cleared of drug per unit time. Clearance is typically hepatic metabolism or excretion, renal excretion, or a combination of these pathways. Clearance is most accurately calculated after an intravenous dose; after an oral dose and without knowledge of the oral bioavailability data it is termed "apparent clearance" (CL/F). Apparent clearance is generally reported L/h/kg, and calculated using the equation:

$CL/F = (\text{dose (mg)}/AUC_{0-\infty}\text{ (mg/L*h)}) / \text{weight (kg)}$

Volume of distribution

Volume of distribution (V) is the theoretical volume of plasma that would be necessary to account for the total amount of drug in an animal's body, if that drug was present throughout the body at the same concentration as found in the plasma. Volume of distribution is most accurately calculated after an intravenous dose; after an oral dose this is termed "apparent volume of distribution" (V/F). It is usually expressed in L/kg, and is calculated using the equation:

$V/F = (CL/k_{el}) / \text{weight (kg)}$

Loading dose

Where appropriate, use of a loading dose shortens the time taken for a drug to reach the target plasma concentration. The loading dose is expressed in mg, and is calculated using the equation:

LD = V(L) * target concentration (mg/L)

Coefficient of variation

The coefficient of variation (CV) is a measure of the ratio of the standard deviation to the mean. It provides a measure of the variability within a dataset. In pharmacokinetics it is often expressed as a percentage, and is calculated using the following equation:

CV = (standard deviation / mean) * 100

Statistical analysis of pharmacokinetic variation with temperature

Statistical analysis to compare pharmacokinetic parameters was conducted using the paired t-test in GraphPad Prism version 6.07 (GraphPad Software, USA). Data were analysed for normality using the Shapiro-Wilk normality test. Where necessary, data underwent log transformation to produce a Gaussian distribution, allowing the parametric paired t-test to be used on transformed data. Non-parametric tests such as the Wilcoxon matched pairs test were not suitable for use due to the small sample sizes in this study. Significance was assessed at a level of $p<0.05$.

2.8 Model-based pharmacokinetic analysis

Pharmacokinetic modelling enables the prediction of appropriate dosing and dosing intervals for administering multiple doses of a drug to a large population, based on single and/or multiple dose data, and a target drug concentration. Population pharmacokinetic modelling was conducted by Professor Nick Holford (Professor of Clinical Pharmacology, University of Auckland).

A pharmacokinetic (PK) model assuming one compartment distribution, first-order elimination and first-order absorption was used to describe the time course of

concentrations. The model was parameterised in terms of clearance, volume of distribution and absorption half-life.

The effect of lower temperature (12^{o}C) compared with the reference at 20^{o}C was modelled by a factor reflecting the fold decrease in Vmax or clearance at 12^{o}C compared with 20^{o}C. A mixed effects model was used to estimate population parameters assuming log-normal between and within subject variability of PK parameters.

The same structural and random effects model was used for both antifungals, with different parameters estimated for each drug. Because of limited sampling and large between animal variability it was decided to make plausible assumptions about the residual error in the single dose studies rather than attempt to estimate it. Residual error was assumed to be a combination of 10% proportional (coefficient of variation) and 0.3 mg/L additive (standard deviation) components. The residual error parameters were assumed to be the same for both drugs.

Mixed effects modelling was performed using NONMEM v7.3.0 with the Intel Fortran Compiler v11. The control stream used for the model is provided in Appendix 6.

2.9 Limitations

The facility where MIC testing was conducted was not able to monitor overnight room temperature, meaning the results for this part of the experiment could not be linked with a set temperature. It would also have been ideal to conduct MIC testing at lower temperatures, but the capacity to conduct this testing was not available.

The plasma protein binding of the antifungal drugs in tuatara plasma was not investigated, as it was not possible to collect enough drug-free plasma from tuatara for this process. Protein binding of itraconazole is discussed in Section 1.5.5 and voriconazole in Section 1.6.5. Protein binding may reasonably be expected to vary with temperature, and these data would have provided more information to base target concentration and dosage recommendations on.

As intravenous data on itraconazole and voriconazole in tuatara was not available, oral bioavailability could not be calculated. Oral bioavailability of itraconazole in other species is discussed in Section 1.5.3, and voriconazole in Section 1.6.3. Oral bioavailability data is used to more accurately calculate volume of distribution and clearance, so would be used in recommended dosage calculations.

The small sample sizes in this study limited the utility of statistical tests. Larger sample sizes providing more data would allow more accurate statistical comparisons between datasets, however the sample sizes were limited by animal welfare concerns and the availability of suitable subjects. Population pharmacokinetic modelling is specifically designed for use in cases of limited sampling, and the results derived from modelling facilitate interpretation of differences when sample sizes are small.

Haematology and biochemistry reference ranges for a population of healthy tuatara were also established in this study (Chapter 6). Reference ranges were divided based on sex and season where significant differences in parameter values were noted. However, as there were only four male tuatara in the population, sex-based differences in particular should be treated with caution.

Chapter 3: Culture and Minimum Inhibitory Concentration testing

3.1 Introduction

3.1.1 MIC testing of filamentous fungi

Antifungal susceptibility testing is not widely used in veterinary practice, but has been a recognised diagnostic tool in human medicine for over 20 years (Fothergill 2012). *In vitro* susceptibility testing should (1) provide a reliable measure of the relative activities of two or more antifungal agents; (2) correlate with *in vivo* activity and predict the likely outcome of therapy; (3) monitor the development of resistance among a normally susceptible population of organisms; and (4) predict the therapeutic potential of newly discovered agents (Espinell-Ingroff et al. 2009).

3.1.2 Clinical and Laboratory Standards Institute guidelines

The Clinical and Laboratory Standards Institute (CLSI) is a collaborative organisation that develops and implements clinical laboratory standards for microbiological testing. The CLSI has published standard methods for antifungal susceptibility testing for both yeasts and moulds. *P. australasiensis* is a mould, and the relevant CLSI reference for determining the antifungal MIC to inhibit the growth of yeasts and moulds is document M38-A2 (CLSI 2008). This document details the recommended broth microdilution method for determining the antifungal MIC testing of moulds, which stipulates the concentration of fungal conidia, antifungals and other reagents to determine antifungal MICs.

P. australasiensis and related fungi are not specifically discussed in the M38-A2 document, and it has been stated that different assay conditions, often determined through trial and error, may be required to determine the antifungal MICs for unlisted

species of fungi (Fothergill 2012). Moulds are slow growing fungi and can be difficult to test using microtiter methods. This is due to dehydration of the inoculum, and that it may take up to 6 days before fungal growth is detected in the drug-free control well (Espinel-Ingroff et al. 2009, Fothergill 2012). The MIC endpoint for moulds is determined as the lowest concentration that prevents discernible fungal growth, seen as the first optically clear well (CLSI 2008). Fungal isolates are considered susceptible to the antifungals amphotericin B, itraconazole, voriconazole, posaconazole, and caspofungin when the MIC is <1.0 µg/mL, are considered to have intermediate susceptibility with an MIC of 2.0 µg/mL, and are considered resistant with an MIC >4.0 µg/mL (Fothergill 2012).

3.1.3 Colorimetric plates for antifungal susceptibility testing

Colorimetric testing is not covered in the CLSI documents, but is a frequently-used, user-friendly method of determining antifungal MICs. The availability of commercial plates means that the tester does not have to design their own plates, which can be labour and cost-intensive and have quality-control and replication limitations (Wanger 2012). Several studies have established Sensititre YeastOne (TREK Diagnostic Systems, Cleveland, OH) colorimetric testing to be comparable with results obtained using the CLSI guidelines, when comparing MICs of itraconazole and voriconazole for *Aspergillus* species moulds (Martin-Manzuelos et al. 2003, Castro et al. 2004).

Sensititre YeastOne colorimetric testing uses the addition of the colour indicator Alamar Blue to each well (Espinell-Ingroff et al. 1999). This agent changes colour from blue to purple to red through an oxidation-reduction reaction. Fungal growth causes a colour

change, thus the first blue well indicates the minimum concentration of antifungal required to inhibit growth of the organism.

No published accounts on the use of Sensititre plates to determine MICs of CANV organisms could be found, however correspondence with Dr Jean Paré (pers comm) has indicated that Sensititre plates can be used successfully for this purpose, with the use of trial and error in determining appropriate inoculum concentrations and incubation times.

3.2 Methods

3.2.1 Isolate recovery from affected animals

Animals with lesions grossly suspicious for *P. australasiensis* infection underwent biopsy under general anaesthesia. Animals were anaesthetised with isoflurane in 100% oxygen via mask, or with intravenous (IV) alfaxan at 5 mg/kg via the caudal vein. When sufficiently sedated they were intubated and maintained on 2-4% isoflurane in 100% oxygen as required, using a ventilator delivering 3-6 breaths per minute. Gross debris was removed from skin with gentle saline flushing, but no other skin preparation was performed. Lesions were biopsied using a biopsy punch (3-5 mm diameter), and were divided in two using a sterile scalpel blade. Wounds were sutured using 3/0 absorbable braided suture material (Polysyn, Surgical Specialties Corp, USA). All animals received a single meloxicam (Metacam, Boehringer Ingelheim, Germany) injection at 0.2 mg/kg intramuscularly (IM) for pain relief at the end of the procedure.

Half of the lesion was preserved in phosphate-buffered formaldehyde and submitted to a pathology service, New Zealand Veterinary Pathology, Auckland (NZVP), for histopathological examination. The other half of the lesion was placed in a sterile urine collection jar and sent at room temperature for culture at the Ministry of Primary Industries (MPI) laboratory.

3.2.2 Culture of isolates

Culture was performed by staff at the MPI's Investigation and Diagnostic Centre, with methods adapted from information provided by Dr. Lynne Sigler (University of Alberta Microfungus collection and Herbarium, Canada). The method used is detailed in Appendix 1.

Large populations of contaminant fungi and bacteria were associated with some skin samples and overgrew quickly on the Sabourauds Dextrose Agar (SDA) plates without antibiotics (SDA-). The SDA plates with antibiotics (SDA+) and the Mycosel plates grew *P. australasiensis* -suspicious colonies within 2 weeks, and these were then subcultured onto new SDA+ plates.

After 7-10 days lactophenol cotton blue preparations were made, allowing microscopic examination of fungal morphology (Figure 3.5). At this time, fungi were subcultured onto a SDA slope with cycloheximide to allow them to be sent to other institutions. SDA slopes sent to CHL were stored in a -80^{o}C freezer prior to use for MIC testing.

Further growth studies were subsequently conducted at MPI at 12, 15, 20, 22, 30 and 37^{o}C on Potato Dextrose Agar (PDA) when resources became available. Photographs

were taken after 18 days of incubation to compare growth at these temperatures. It was not possible to conduct MIC testing at MPI due to staff and resource constraints.

3.2.3 Preparation of inoculum

Fungal samples sent to CHL were retrieved from the -80°C freezer one week prior to conducting MIC testing. This allowed time to subculture the isolates onto SDA slopes to ensure they were still viable. A total of three samples were available and suitable for testing. Cultures were incubated at 30°C or at room temperature (approximately 23°C), and this allowed assessment of growth at these temperatures.

A working inoculum was prepared from each of these samples. The procedure for this is detailed in Appendix 2. The process was performed in duplicate for each fungal isolate, so that each isolate could be incubated and undergo MIC testing at both 30°C and 23°C. This allowed assessment of any differences in MICs at the two temperatures.

3.2.4 Sensititre plates

Sensititre YeastOne YO2IVD plates contain ascending concentrations of voriconazole, itraconazole, 5-flucytosine, fluconazole and caspofungin. Each plate has two sets of these concentrations, to allow replication if desired. The plate format is illustrated below (Figure 3.1), where the numbers indicate concentration in mg/L.

3.2.5 Reading results

Results were read on the day the control well showed growth, as indicated by a colour change from blue to red. *P. australasiensis* is slow-growing, and results were read after

7 days for the plates incubated at 30°C, and after 13 days at 23°C. Results were read by the same operator for each sample and checked again one day later to ensure there were no further colour changes. The MIC is read as the lowest antifungal concentration that inhibits growth, indicated by a lack of colour change.

Figure 3.1 Sensititre Yeastone YO2IVD plate format (Thermo Scientific Microbiology).

SENSITITRE™ YEASTONE™ PLATE FORMAT

Plate Code: **YO2IVD**

	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12	VOR 0.25	VOR 0.5	VOR 1	VOR 2	VOR 4	IZ 2
B	FC 0.03	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4	FC 8	FC 16	IZ 0.03	IZ 1
C	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32	FZ 64	FC 32	IZ 0.06	IZ 0.5
D	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	IZ 0.12	IZ 0.25
E	POS	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12	VOR 0.25	VOR 0.5	VOR 1	VOR 2	VOR 4	IZ 2
F	FC 0.03	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4	FC 8	FC 16	IZ 0.03	IZ 1
G	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32	FZ 64	FC 32	IZ 0.06	IZ 0.5
H	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	IZ 0.12	IZ 0.25

ANTIMICROBICS	
POS	Positive Control
FC	5-Flucytosine
FZ	Fluconazole
CAS	Caspofungin
VOR	Voriconazole
IZ	Itraconazole

3.3 Results

3.3.1 Culture temperatures

Subjectively, fungal growth was faster and more profuse at 30°C than at room temperature (Figure 3.2). Subsequent growth studies at MPI showed minimal growth at 12°C, moderate growth at 15°C, and good growth at 20, 22 and 30°C, with absence of growth at 37°C (Figure 3.3).

P. australasiensis cultured using this method was creamy-to-yellow, slightly powdery and had a noticeable strong, sweet odour (Figure 3.3). The microscopic appearance was as described by Sigler et al. (2013) (Figure 3.4).

Figure 3.2 Growth of three *P. australasiensis* isolates at 23°C (left) and 30°C (right) after 7 days at CHL on SDA+ slants.

Figure 3.3 Growth of three *P. australasiensis* isolates at 12°C to 37°C after 18 days at MPI on PDA plates.

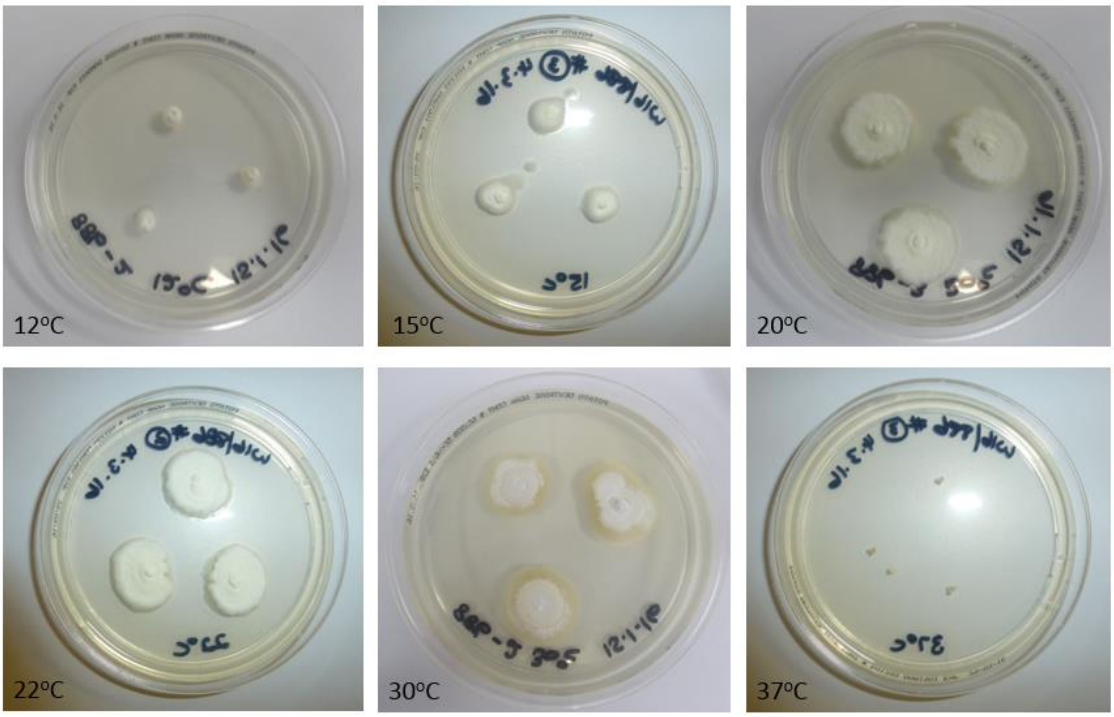
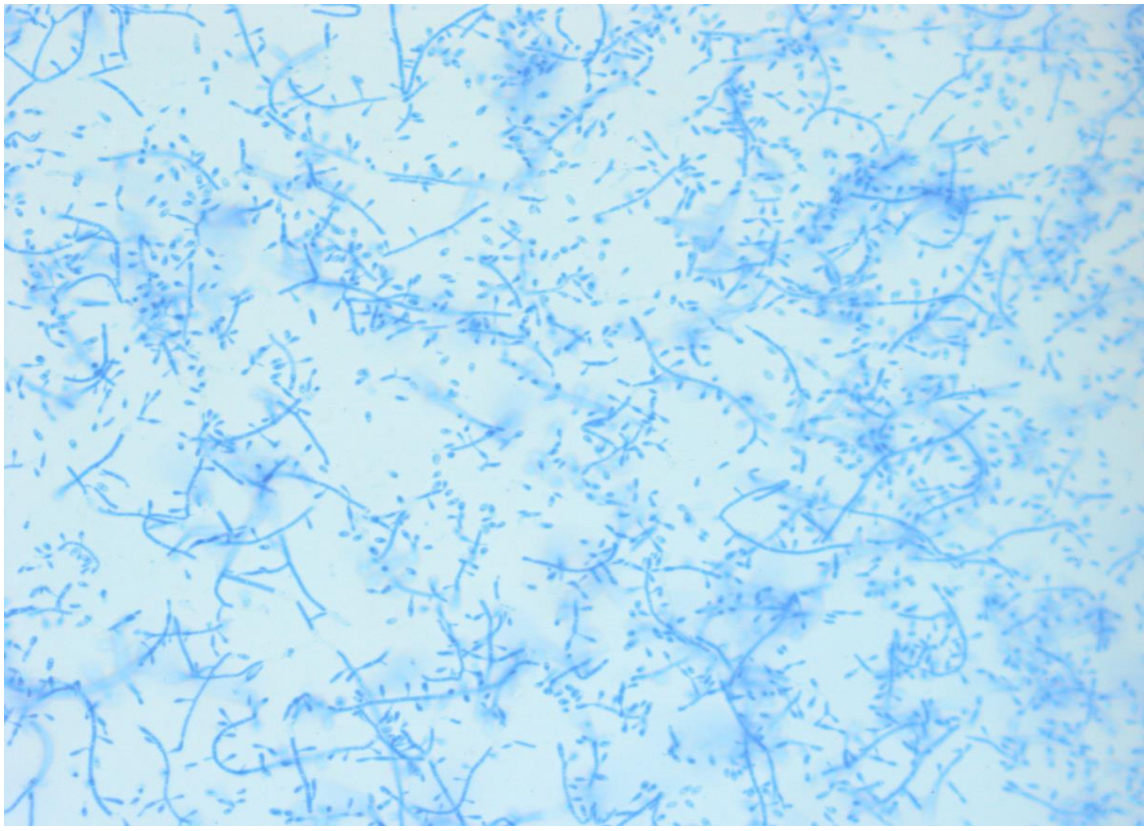


Figure 3.4 Lactophenol Cotton Blue preparation of *P. australasiensis*, 400x magnification (courtesy MPI).



3.3.2 Itraconazole MIC results

Minimum Inhibitory Concentration results were read on the first day that distinct colour change from blue to red was detected in the control well (Table 3.1, Figure 3.5). This was 7 days at 30°C, and 13 days at room temperature.

Table 3.1 Itraconazole MIC results.

Temperature	Isolate 1 MIC (mg/L)	Isolate 2 MIC (mg/L)	Isolate 3 MIC (mg/L)	Read time
Room temp	0.12	0.12	0.12	Day 13
30°C	<0.03	0.12	0.06	Day 7

Figure 3.5 Sensititre YO2IVD plate showing MIC results for five antifungals.

3.3.3 Voriconazole MIC results

Minimum Inhibitory Concentration results were read as for itraconazole results (Section 3.3.2) and are described below (Table 3.2).

Table 3.2 Voriconazole MIC results.

Temperature	Isolate 1 MIC (mg/L)	Isolate 2 MIC (mg/L)	Isolate 3 MIC (mg/L)	Read time
Room temp	0.008	0.015	0.015	Day 13
30°C	<0.008	<0.008	<0.008	Day 7

3.3.4 Other antifungal agent MIC results

Sensititre YO2IVD plates also determine MICs for the antifungals flucytosine, fluconazole and caspofungin. Results are reported here to add to the body of knowledge on *P. australasiensis*, though these antifungals are not part of this pharmacokinetic study.

Minimum Inhibitory Concentration results were read as for itraconazole results (Section 3.3.2) and are described below (Table 3.3).

Table 3.3 Flucytosine, fluconazole and caspofungin MICs for *P. australasiensis.*

Isolate	Temperature	Flucytosine MIC mg/L	Fluconazole MIC mg/L	Caspofungin MIC mg/L	Read time
1	Room temp	>32.0	32.0	2.0	13 days
	30°C	>32.0	16.0	>8.0	7 days
2	Room temp	>32.0	64.0	>8.0	13 days
	30°C	>32.0	16.0	>8.0	7 days
3	Room temp	16.0	64.0	8.0	13 days
	30°C	>32.0	32.0	>8.0	7 days

3.4 Discussion

3.4.1 General discussion

There were differences in fungal growth rates at different temperatures between laboratories. At MPI the growth rates were the same for three isolates at 20, 22 and 30°C, while at CHL growth was slower at room temperature (approximately 23°C) than at 30°C. This could be because room temperature may have dropped to below 20°C at night at CHL, or because different isolates were used at each laboratory, as it is possible that different isolates have different temperature growth profiles. It is unlikely the variation was due to different agars being used at each laboratory (SDA at CHL and PDA at MPI). CHL were not equipped to monitor overnight room temperature remotely at the time the experiments were conducted.

Minimum inhibitory concentrations of voriconazole and itraconazole were similar for all three isolates of *P. australasiensis*. The fact that the MIC endpoints were read almost one week later in the room temperature samples indicates that fungal growth was slower at room temperature than 30°C. The MICs did not differ significantly at different incubation temperatures, however it was recommended to use the MICs obtained at 30°C, as they were faster to grow and possibly more accurate (Ros Podmore, medical laboratory scientist, Canterbury Health Laboratories, pers comm May 2014). These suggest that an MIC of 0.12 mg/L is appropriate for itraconazole, and 0.008 mg/L for voriconazole to inhibit *P. australasiensis*.

Flucytosine, fluconazole and caspofungin are commonly used to treat *Candida* and other yeast infections, and are not considered as effective as itraconazole and voriconazole against ascomycetes (Cuenca-Estrella et al. 2006, Berger 2015). It is therefore unsurprising that the MICs for these antifungals were high compared to those of itraconazole and voriconazole.

Few published studies have determined the MICs of CANV isolates, but those available reported similarly low MICs for itraconazole and voriconazole (Hellebuyck et al. 2010, van Waeyenberghe et al. 2013). A study evaluating the MICs of 32 CANV isolates reported a MIC_{50} of 0.0313 mg/L for both itraconazole and voriconazole, and an MIC_{90} of 0.25 mg/L for itraconazole and 0.0625 mg/L for voriconazole. This study conducted MIC testing at 30°C using the CLSI guidelines published in document M38-A2 (van Waeyenberghe et al. 2013). These MICs were similar to our results reported above.

MICs, in combination with pharmacokinetic data including protein binding and area under the curve, can be used to develop a target concentration for plasma antifungal

drugs. The data obtained here were used to assist in determine dosing rates and intervals for the multiple-dose pharmacokinetic studies.

3.4.2 Target concentrations for multiple dose studies

3.4.2.1 Itraconazole

The MIC range of itraconazole for *P. australasiensis* was <0.03-0.12 mg/L at 30^{o}C. Determining a suitable dose for multiple dose studies ideally requires data on itraconazole protein binding, which was not available as part of this study (see Section 2.7.1). A target of 20 times the higher MIC for steady-state concentration was considered appropriate, to ensure that sufficient free drug was available for distribution and action at target sites. This target was arrived at after extensive consultation with Prof. Nick Holford, in an effort to maximise therapeutic efficacy while minimising the likelihood of toxicity. This results in a steady-state trough target concentration of 2.4 mg/L for itraconazole.

3.4.2.2 Voriconazole

The MIC of voriconazole for *P. australasiensis* was <0.008 mg/L at 30^{o}C. As with itraconazole, data on protein binding was not available, and a target of 20 times the MIC for steady-state concentration was considered appropriate. This results in a steady-state target trough concentration of 0.16 mg/L for voriconazole.

3.5 Conclusions

Growth of *P. australasiensis* varied significantly with incubation temperature, with maximal growth occurring between 20^{o}C and 30^{o}C. It is possible that different isolates may have different growth temperature profiles, and this could be investigated with

further studies. MICs of itraconazole and voriconazole for *P. australasiensis* were low, and were similar to those reported elsewhere (van Waeyenberghe et al. 2013, J. Paré 2014 pers. comm.). It would be ideal to also conduct MIC testing at lower temperatures, however interpretation of an end-point may be difficult due to slow fungal growth. The MICs determined here provide a sound basis for estimation of plasma target concentrations of itraconazole and voriconazole for *in vivo* pharmacokinetic studies.

Chapter 4: Itraconazole pharmacokinetics in tuatara

4.1 Introduction

Itraconazole is a triazole antifungal that has a broad spectrum of antifungal activity, and is commonly used in the treatment of aspergillosis, candidiasis, fungal dermatitis and nail bed infections in humans and animals (Vantrubova et al. 2010). Itraconazole has been used with variable success in the treatment of fungal dermatitis in reptiles, and adverse effects including anorexia and fatal hepatotoxicity have been reported (Bowman et al. 2007, Johnson et al. 2011, Paré et al. 1997, van Waeyenberghe et al. 2013).

Itraconazole undergoes hepatic metabolism, and its main metabolite is hydroxy-itraconazole (Heykants et al. 1989). Hydroxy-itraconazole has significant antifungal activity with a very similar spectrum to the parent compound. In humans, hydroxy-itraconazole concentrations have been observed to range from zero to ten times the concentration of itraconazole, demonstrating significant inter-patient variability (Heykants et al. 1989, Manire et al. 2003, Orosz and Frazier 1995, Prentice and Glasmacher 2005).

Pharmacokinetic studies on itraconazole in reptiles are sparse, with one study on Kemp's ridley sea turtles (Manire et al. 2003), one study on bearded dragons (van Waeyenberghe et al. 2013), and one study on spiny lizards (Gamble et al. 1997). The study in Kemp's ridley sea turtles recommended administration of itraconazole orally (as capsules) at 5 mg/kg once daily (SID) or 15 mg/kg every 3 days (Manire et al. 2003), while in bearded dragons 5 mg/kg PO SID of an unspecified formulation of itraconazole achieved above therapeutic concentrations, but resulted in presumed fatal hepatotoxicity (van Waeyenberghe et al. 2013). In spiny lizards, a dose rate of 23.5

mg/kg SID of the itraconazole capsules (opened and given with food) achieved therapeutic concentrations (Gamble et al. 1997). None of these studies evaluated the effects of temperature on pharmacokinetics.

The aim of this study was to investigate the pharmacokinetics of itraconazole and its active metabolite, hydroxy-itraconazole, after oral administration of a liquid formulation of itraconazole in tuatara. Single and multiple dose studies were conducted at high and low ends of the tuatara's preferred optimal temperature zone (POTZ) to assess variability in itraconazole pharmacokinetics with temperature.

4.2 Methods

Single dose studies were conducted first, and the results of these informed the methods used in the multiple dose studies. For general methods relating to animal selection, environmental conditions, drug assay method and pharmacokinetic analysis, see Chapter 2.

Mixed effects pharmacokinetic modelling was performed using NONMEM v7.3.0 with the Fortran Compiler v11. Modelling for itraconazole concentration-time data in tuatara assumed one compartment pharmacokinetic model, first-order elimination and first-order absorption (see Chapter 2 and Appendix 6).

Animal ethics approvals were obtained from Murdoch University, Auckland Zoo and the Department of Conservation (see Section 2.2).

Plasma antifungal concentrations were determined using Liquid Chromatography and tandem Mass Spectrometry (LCMS/MS). Drug assay methods and HPLC conditions are described in Section 2.6 and Appendices 3 and 4. All reported white blood cell counts were obtained using a haemocytometer.

4.2.1 Single dose studies

Dose determination

Oral itraconazole has been used to treat mycotic infections involving fungi of the CANV group at 5-10 mg/kg SID PO in various reptile species (Paré et al. 1997, Bowman et al. 2007, van Waeyenberghe et al. 2010, Johnson et al. 2011, Masters et al. 2016). At Auckland Zoo, clinical success has been achieved with itraconazole 3-5 mg/kg PO SID, (and in one case, 3 mg/kg PO q48h) in tuatara, with no observable adverse effects. Presumptive itraconazole toxicity has been documented at 5 mg/kg SID in bearded dragons (van Waeyenberghe et al. 2010), and itraconazole-related anorexia was observed in bearded dragons receiving itraconazole at 10 mg/kg PO SID (Bowman et al. 2010). As the metabolic rate of tuatara is lower at their preferred body temperature than that of most other reptiles when in their POTZ, a lower dosage of itraconazole was indicated than has been used in other reptile species. The Minimum Inhibitory Concentration (MIC) of itraconazole required to inhibit growth of the tuatara *P. australasiensis* isolates tested was also low, at <0.03-0.12 mg/L (see Chapter 3), further suggesting that a relatively low dosage of itraconazole would be appropriate. As itraconazole dose rates of 3 mg/kg had been clinically effective previously with no observed adverse effects, this dosage was selected.

Drug formulation and administration

The itraconazole formulation used in this study was an oral 10 mg/mL liquid (Sporanox 10 mg/mL Oral Solution, Janssen-Cilag, NZ). The liquid formulation allowed ease of administration and accurate dosing. Medication was administered orally as described in Chapter 2.

Blood sampling intervals

There is no published data on the absorption or elimination of oral antifungals in reptiles. This necessitated the estimation of appropriate sampling intervals based on assumptions made from pharmacokinetic data in other animals, and factoring in the lower metabolic rate of tuatara compared to these animals. Sampling intervals were shorter just after medication administration, in an effort to capture the point of maximal concentration, and longer towards the end of sampling, as less data points were likely to be required to capture elimination. The number of sampling points was also limited by concerns for the potential negative welfare impacts associated with repeat handling of the animals, and the amount of blood that could be taken from each animal during the short time period of the study. With these factors in mind, it was decided to sample at 6 time points during the single dose study. These were at 2, 4, 8, 12, 24 and 48 hours following medication administration. Each animal was administered medication 10 minutes apart, and blood sampling was performed as close to the planned time after medication administration as possible. Sampling times were recorded to the nearest minute, and these data were used in the pharmacokinetic modelling calculations.

4.2.2 Multiple dose studies

Dose determination

For the purpose of direct comparison, the same dosage was used at both temperatures for the multiple dose studies. All animals in the study weighed over 500 grams (range 501-1016g), so this was the weight that was chosen for which the dosage required to achieve the target concentration of 2.4 mg/L would be determined. The predicted itraconazole dosage to achieve the target concentration in a 500g animal at 20°C is 2.57 mg/kg once daily (see Section 4.2.2.4).

A dose rate of 2.5 mg/kg PO SID was selected, for ease of calculation and administration. Health screening was conducted every two weeks to monitor tuatara for any signs of itraconazole toxicity. This consisted of weighing, physical examination, haematology and biochemistry measurements every two weeks until the end of the study.

Drug formulation and administration

Itraconazole was administered as per the single dose studies, using itraconazole 10 mg/mL liquid (Sporanox 10 mg/mL Oral Solution, Janssen-Cilag, NZ). This was administered once daily as described in Section 4.2.1.2. Itraconazole was to be administered at 2.5 mg/kg PO SID for 42 days, however abnormalities detected during health screening resulted in cessation of drug administration on day 21 in the 12°C study, and on day 13 in the 20°C study (see Section 4.3.2). The medication was given at approximately the same time each day for each animal (with up to 5 minutes variation between days), between the hours of 7am and 8am. Tuatara were fed once weekly at 12°C, and twice weekly at 20°C. Tuatara were fed in the afternoon, in an effort to

minimise any effect of food on absorption of medication. The gastric pH and gastric emptying time of tuatara is unknown.

Blood sampling intervals

Blood samples were collected just prior to a dose being given, to measure trough concentrations. The first day of medication was designated as day zero, and it was planned to take samples for trough itraconazole concentrations on days 2, 4, 6, 13, 20, 27, 24 and 41. This sampling regime altered when antifungal concentration and health screening results were received during the study, as described below.

In the 12^{o}C study samples for determination of antifungal concentrations were taken on days 2, 4, 6, 13 and 20. Health screening results from day 13 revealed mild elevations in bile acids in two animals (possibly within normal variation, see Chapter 6). Blood could not be obtained for health screening from one animal, so a sample was obtained one week later on day 20, showing marked elevation in bile acids. At this stage all animals were re-sampled for health screening, and abnormal results were obtained in four animals (Section 4.3.2.4). Results from the first two weeks showed that itraconazole concentrations were increasing with no evidence of approaching steady-state concentrations and, as a result, the decision was made to cease itraconazole administration. The last dose of itraconazole was given on day 21, and samples were taken to measure drug elimination on days 28, 34, 41 and 55.

Blood samples were taken for health screening at 6-14 day intervals for each animal, until all values had returned to normal. Normal haematology and biochemistry ranges for tuatara were developed as part of this research (Chapter 6). Reference ranges were

obtained from a small population, so a result was only considered to be definitively abnormal if it was more than three standard deviations from the mean.

In the 20°C study, elevated bile acids were detected on health screening at day 13. The last dose of itraconazole was given on day 13, with the last sample for trough concentration taken on day 14. Samples during drug elimination were taken on days 17, 20, 23 and 27. Blood samples were taken for health screening on days 13 and 20 for each animal, and on days 27, 41 and 89 for animals with abnormal results requiring further monitoring.

There was a fifteen-week washout period between multiple dose studies at the two ambient temperatures. Blood samples collected one week before the commencement of the 20°C study confirmed there was no detectable itraconazole or hydroxy-itraconazole present in the bloodstream of any participating tuatara.

4.3 Results

4.3.1 Results for single dose studies

4.3.1.1 Single dose studies conducted at 12°C ambient temperature

Plasma antifungal concentrations

Itraconazole was administered to four tuatara at a dose rate of 3 mg/kg PO, at time zero. Plasma samples were analysed using LCMS/MS and were run in duplicate to ensure accuracy, with the averages displayed below. (Table 4.1 and Figures 4.1 and 4.2).

Table 4.1 Itraconazole (Itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) in four tuatara at time from itraconazole administration (hours) at 12°C.

Animal ID	Sex	Drug	2h	4h	8h	12h	24h	48h
101	F	Itra	0.317	0.769	0.632	0.707	0.799	1.025
		OH-itra	0	0	0.034	0.041	0.152	0.113
104	M	Itra	0.206	0.405	0.675	0.877	1.445	1.785
		OH-itra	0	0	0.030	0.050	0.115	0.216
105	F	Itra	0.173	0.318	0.565	0.570	0.803	0.799
		OH-itra	0	0	0	0	0.080	0.172
107	F	Itra	0.0860	0.160	0.212	0.187	0.862	0.910
		OH-itra	0	0	0	0	0.039	0.089

Figure 4.1 Itraconazole (—●—) and hydroxy-itraconazole (-■-) plasma concentration (mg/L) at time from itraconazole administration (hours) for each animal at 12°C.

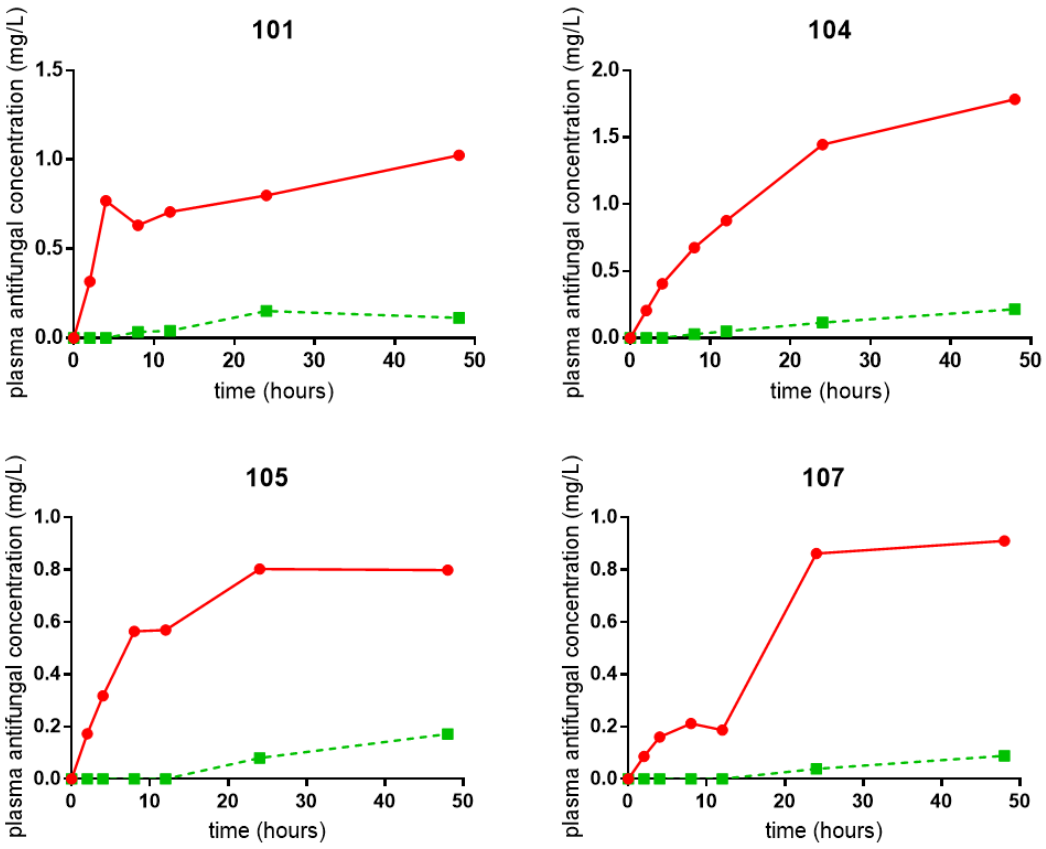
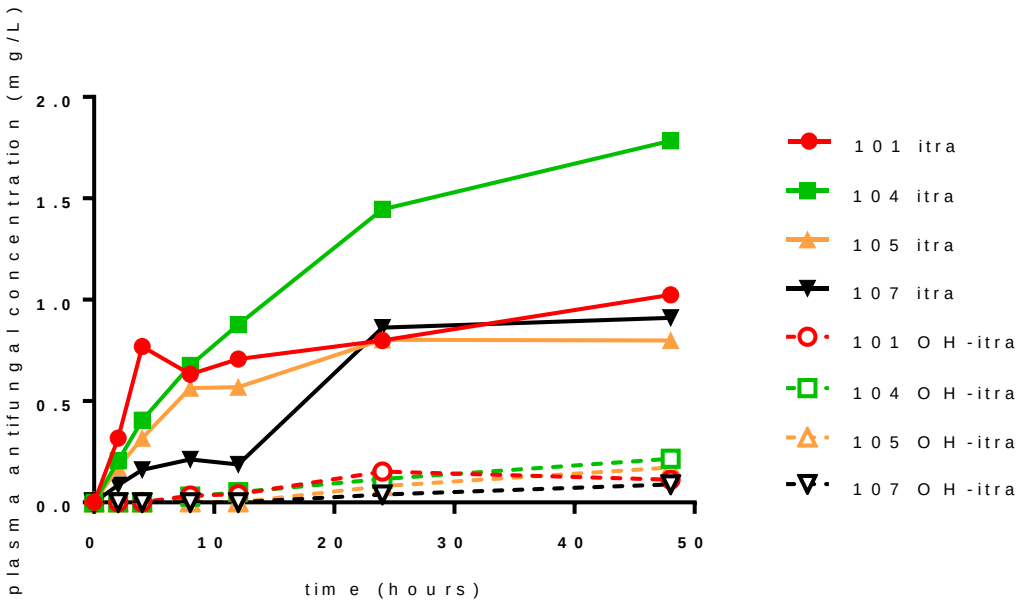


Figure 4.2 Itraconazole (itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from itraconazole administration (hours) from all animals at 12°C.



Pharmacokinetic data and analysis

No animals were observed to have a concentration-time profile in the terminal elimination phase for itraconazole or hydroxy-itraconazole during the 48 hour period of the study, though this may be at least partially due to the sparse sampling towards the end of the study period. Only one of the four subjects (105) had an itraconazole concentration that was lower at 48 hours than at the previous sampling timepoint at 24 hours. Thus it was not possible to calculate the elimination constant (k_{el}), area under the concentration versus time curve from 0h to infinity ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$), clearance (CL), or volume of distribution (V) at 12°C. The only parameter able to be calculated was, for both drugs, AUC_{0-48} (Table 4.2)

Approximate maximal plasma concentration (Cmax) and time to maximal plasma concentration (Tmax) were not able to be determined, as the majority of subjects were not in the terminal elimination phase at the last time point.

Table 4.2 AUC for itraconazole and hydroxy-itraconazole at 12°C.

Animal ID	Itraconazole AUC_{0-48} (mg/L*h)	OH-itraconazole AUC_{0-48} (mg/L*h)
101	21.93	4.56
104	58.78	5.18
105	32.18	3.50
107	29.44	3.52
Mean ± SD	35.58 ± 13.9	4.19 ± 0.7

Pharmacokinetic modelling results

Using mixed effects modelling (see Section 2.11), it was possible to estimate the pharmacokinetic parameters and itraconazole dosages required to reach the plasma target concentration of 2.4 mg/L at 12°C for tuatara of different weights (Table 4.3). Due to the lack of good elimination data, the modelling findings may not be as accurate as desired.

Plasma antifungal concentrations

Itraconazole was administered to the same four tuatara as described in Section 4.2.1. Results are summarised below (Table 4.4, Figures 4.3 and 4.4).

Table 4.3 Pharmacokinetic modelling results for attaining steady-state plasma concentrations of 2.4 mg/L of itraconazole at 12°C.

Weight (kg)	Volume (L)	Clearance (L/h/kg)	Dose rate (mg/kg/day)
0.1	0.29	0.0099	0.571
0.2	0.58	0.0083	0.480
0.3	0.87	0.0075	0.434
0.4	1.15	0.0070	0.404
0.5	1.44	0.0066	0.382
0.6	1.73	0.0063	0.365
0.7	2.02	0.0061	0.351
0.8	2.31	0.0059	0.340
0.9	2.60	0.0057	0.330
1	2.89	0.0056	0.321

4.3.1.2 Single dose studies conducted at 20°C ambient temperature

Table 4.4 Itraconazole (Itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from itraconazole administration (hours). NS = no sample.

			Time after dose (hours)					
Animal ID	Sex	Drug	2	4	8	12	24	48
101	F	Itra	0.375	1.465	0.997	0.737	0.853	0.693
		OH-itra	0.018	0.067	0.242	0.245	0.315	0.395
104	M	Itra	NS	0.552	1.405	1.290	1.405	0.605
		OH-itra	NS	0.048	0.342	0.326	0.593	0.504
105	F	Itra	0.208	0.632	0.629	0.546	0.535	0.278
		OH-itra	0	0	0.095	0.127	0.279	0.219
107	F	Itra	0.164	0.436	0.839	NS	0.386	0.347
		OH-itra	0	0	0.079	NS	0.444	0.242

Figure 4.3 Itraconazole (-●-) and hydroxy-itraconazole (-■-) plasma concentration (mg/L) at time from itraconazole administration (hours) for each animal at 20°C.

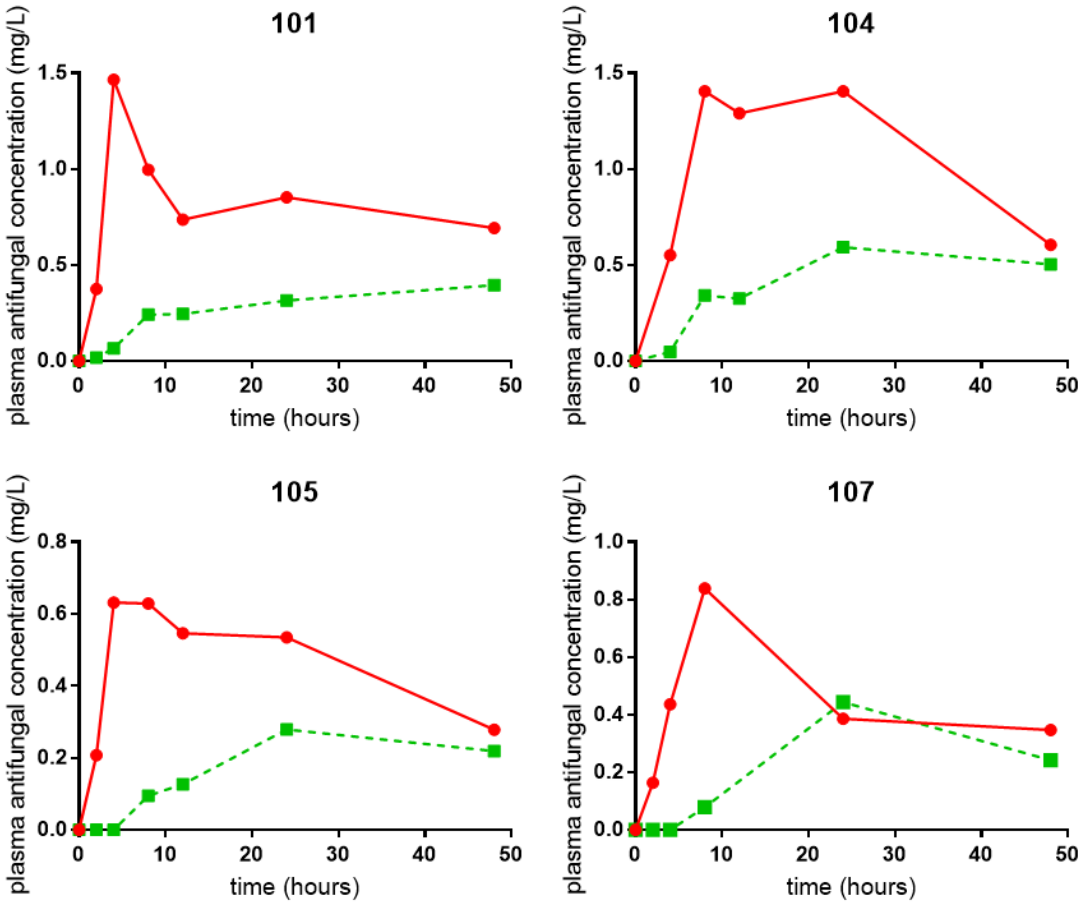
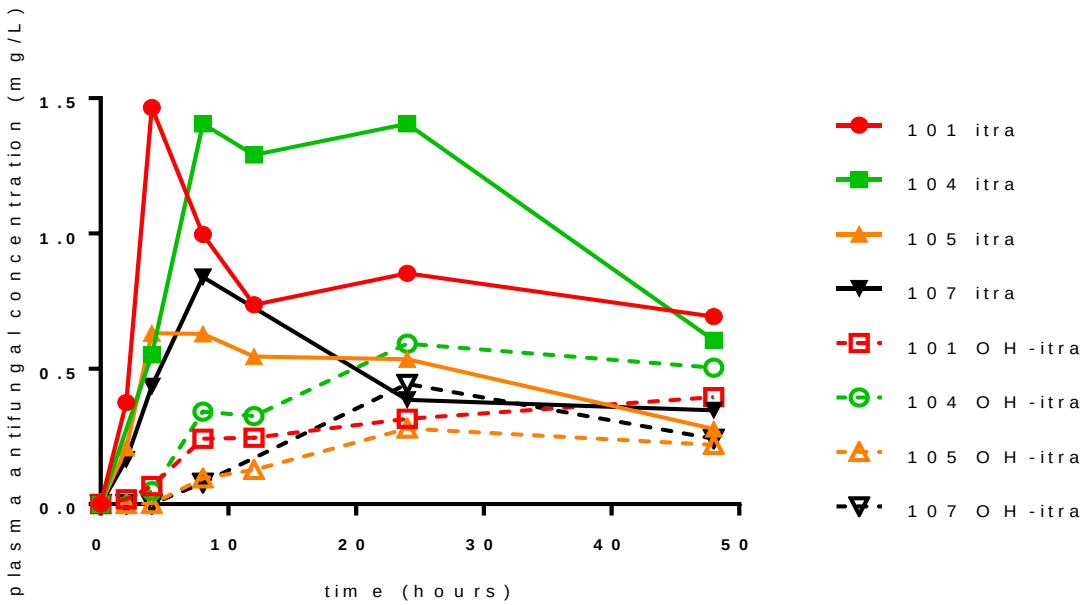


Figure 4.4 Itraconazole (itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from itraconazole administration (hours) from all animals at 20°C.



Pharmacokinetic data and analysis

All four subjects had passed the peak of itraconazole concentrations by the last time point. The extrapolated percentage of $AUC_{0-\infty}$ was higher than 20%, precluding the accurate calculation of the elimination constant, elimination half-life, clearance and volume of distribution. Three of the subjects had passed the peak of hydroxy-itraconazole concentrations. Because only two time points were measured after the peak concentration, it was not possible to accurately calculate pharmacokinetic parameters and indices other than AUC_{0-48} (Table 4.5).

Table 4.5 Selected pharmacokinetic parameters for itraconazole and hydroxy-itraconazole at 20°C.

Animal ID	Itraconazole AUC_{0-48} (mg/L*h)	Itraconazole $AUC_{0-\infty}$ (mg/L*h)	Itraconazole AUC % extrapolated	OH-itraconazole AUC_{0-48} (mg/L*h)
101	38.70	84.40	54.1	13.58
104	50.71	77.01	35.1	21.19
105	22.18	32.35	31.4	9.06
107	21.93	38.69	43.3	6.59
Mean ± SD	33.38 ± 12.1	58.11 ± 22.9	40.98 ± 8.7	12.60 ± 5.6

Approximate time to maximal plasma concentration (Tmax), and approximate maximal plasma concentration (Cmax) of itraconazole were variable. Tmax appears to occur 4-8 hours following oral administration of itraconazole, and Cmax appears to be between 0.632 and 1.450 mg/L. It is not appropriate to estimate Tmax and Cmax for hydroxy-itraconazole, as there was sparse sampling when these events were occurring.

Pharmacokinetic modelling results

Using mixed effects modelling (see Section 2.11), it was possible to estimate the drug dosages required to reach the itraconazole target concentration of 2.4 mg/L at 20°C for tuatara of different weights. It was also possible to estimate pharmacokinetic parameters of itraconazole for different animal weights at these temperatures (Table 4.6). Due to the lack of good elimination data at both temperatures, but particularly at 12°C, the modelling findings may not be as accurate as desired.

Table 4.6 Pharmacokinetic modelling results for attaining steady-state plasma concentrations of 2.4 mg/L of itraconazole at 20°C.

Weight kg	Volume (L)	Clearance (L/h/kg)	Dose rate (mg/kg/day)
0.1	0.29	0.067	3.84
0.2	0.58	0.056	3.23
0.3	0.87	0.051	2.92
0.4	1.15	0.047	2.72
0.5	1.44	0.045	2.57
0.6	1.73	0.043	2.46
0.7	2.02	0.041	2.36
0.8	2.31	0.040	2.29
0.9	2.60	0.039	2.22
1	2.89	0.038	2.16

4.3.1.3 Statistical comparisons

Itraconazole

The only value suitable for statistical comparison between temperatures for itraconazole was $AUC_{0\text{-}48}$, and this was not significantly different (p= 0.75).

Hydroxy-itraconazole

The only value suitable for statistical comparison between temperatures for hydroxy-itraconazole was AUC_{0-48}, and this was not significantly different (p = 0.058).

4.3.1.4 Combined pharmacokinetic modelling results

Combined pharmacokinetic parameters derived from the single dose studies are provided in Table 4.7. Although not practically applicable to tuatara, data for a 70kg animal is included in the results as this is the standard employed in human medicine to allow comparisons between medications, as generally speaking, pharmacokinetic parameters differ on an allometrically scalable basis (Huang and Riviere 2014).

Table 4.7 Estimates of modelling-derived itraconazole PK parameters from single dose studies.

Parameter	Estimate
Clearance at 20°C (L/h/70kg)	1.26
Km (mg/L)	0.01
Volume of distribution (L/70kg)	150
Absorption half-life (hours)	7.95
Fold decrease in CL relative to 20°C	15

4.3.2 Results for multiple dose studies

4.3.2.1 Multiple dose studies conducted at 12°C ambient temperature

Plasma antifungal concentrations

Itraconazole was administered to six tuatara at a dose rate of 2.5 mg/kg PO once daily until the last dose on day 21, and blood samples were obtained as described in Section

4.2. Samples were analysed using LCMS/MS, and results are displayed below (Table 4.8 and Figures 4.5 and 4.6). Animal 104 experienced adverse effects from the medication and was moved to a warmer ambient temperature to hasten drug elimination on day 33, so drug concentrations were not measured after this date.

Table 4.8 Trough itraconazole (Itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from first day of itraconazole administration (days) at 12^{o}C. * = sample taken on day 33. The final itraconazole dose was administered to all animals on day 21.

			Days after first dose								
Animal ID	**Sex**	**Drug**	**2**	**4**	**6**	**13**	**20**	**28**	**34**	**41**	**55**
101	F	Itra	1.55	3.95	6.85	16.0	14.2	8.21	5.99	4.72	1.38
		OH-itra	0.181	0.565	1.22	2.95	3.17	3.47	3.89	3.36	2.42
104	M	Itra	1.82	5.02	7.44	15.8	22.9	20.7	15.7*	NS	NS
		OH-itra	0.203	0.587	1.67	3.76	4.76	6.83	8.33	NS	NS
105	F	Itra	2.13	3.29	5.62	NS	13.3	10.6	11.7	7.91	7.4
		OH-itra	0.135	0.884	1.25	NS	2.43	3.74	6.17	4.08	4.03
107	F	Itra	1.89	3.20	3.77	6.96	10.9	7.02	4.86	3.38	1.82
		OH-itra	0.172	0.376	0.72	1.87	1.86	2.49	2.98	2.56	2.3
109	F	Itra	1.43	3.76	5.49	9.16	14.0	9.02	7.93	5.43	3.72
		OH-itra	0.445	1.00	1.11	2.85	4.43	4.99	5.51	6.84	7.57
112	M	Itra	2.46	4.61	6.24	15.1	18.7	15.0	13.3	11.7	7.94
		OH-itra	0.328	0.373	0.708	2.13	3.14	3.74	4.44	5.03	5.33

Figure 4.5 Itraconazole (-●-) and hydroxy-itraconazole (-■-) plasma concentration (mg/L) at time from itraconazole administration (days) for each animal at 12°C.

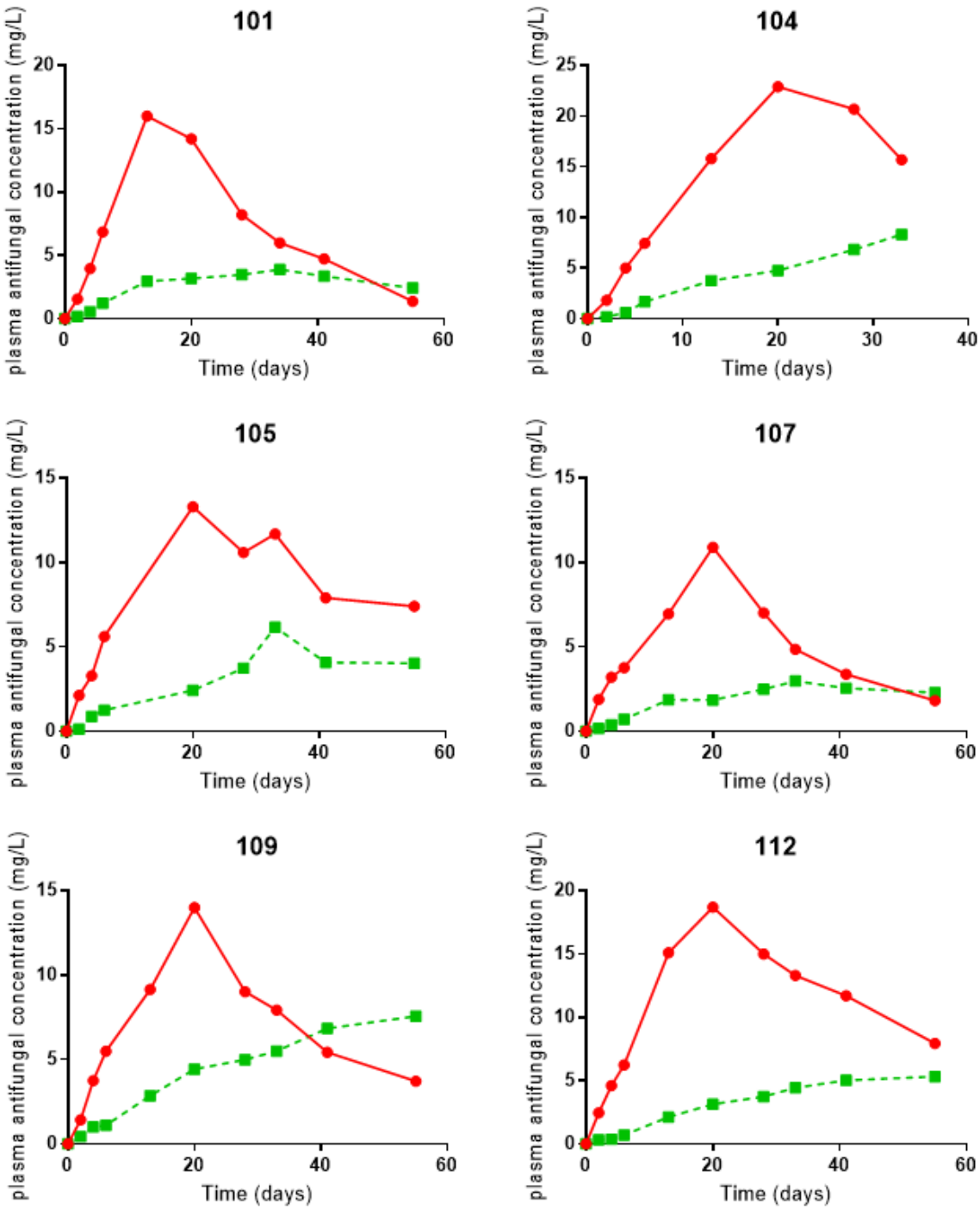
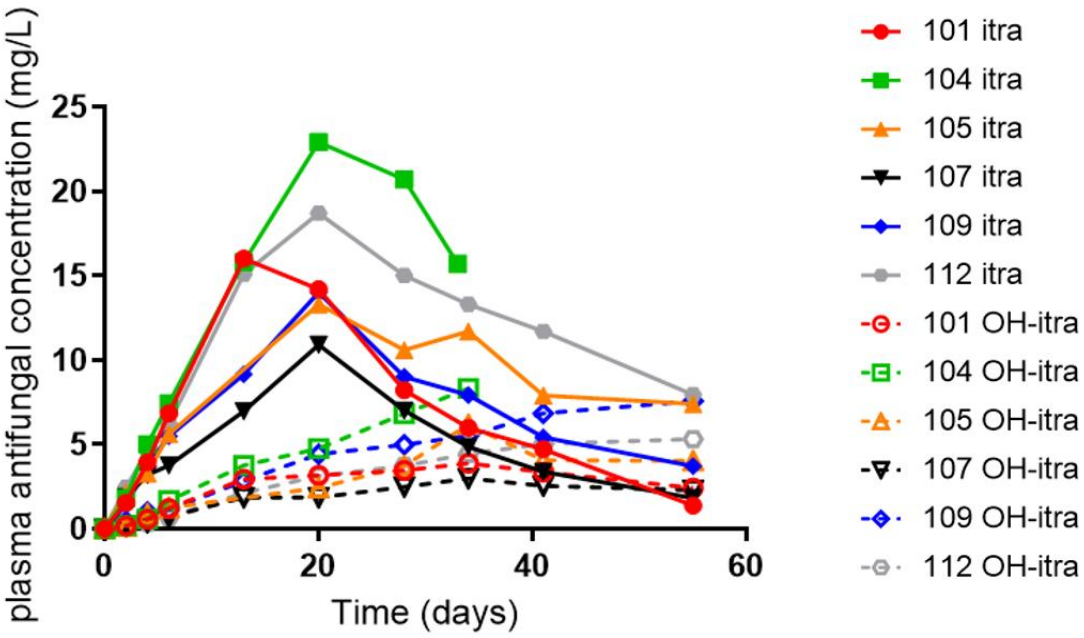


Figure 4.6 Itraconazole (itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from itraconazole administration (days) from all animals at 12°C.



Pharmacokinetic data and analysis

The only index that could be calculated using traditional PK analysis (Chapter 2) was elimination half-life (Table 4.9). The mean ± SD half-life of itraconazole was 22.0 ± 9.4 hours. The mean ± SD half-life of hydroxy-itraconazole was 34.7 ± 11.9 hours.

Table 4.9 $t_{1/2}$ of itraconazole and hydroxy-itraconazole at 12°C. NC = not calculated.

Animal ID	Itraconazole $t_{1/2}$ (hours)	Hydroxy-itraconazole $t_{1/2}$ (hours)
101	10.50	30.51
104	NC	NC
105	35.77	14.18
107	14.04	39.58
109	20.37	49.42
112	29.33	39.58
Mean ± SD	22.00 ± 9.4	34.65 ± 11.9

Pharmacokinetic modelling results

The modelling data below (Table 4.10) describe select pharmacokinetic parameters (clearance and volume of distribution), half-life and the mg/kg per day dosage of itraconazole required to achieve a plasma target concentration of 2.4 mg/L for tuatara weighing between 0.1-1.0kg maintained at 12°C. Dose rates range from 0.21-0.38 mg/kg.

Table 4.10 Model predictions of pharmacokinetic parameters and itraconazole dose rate required to attain steady-state target concentration of 2.4 mg/L at 12°C.

Weight kg	Volume (L)	Clearance (L/h/kg)	Dose rate (mg/kg/day)
0.1	0.27	0.0065	0.38
0.2	0.53	0.0055	0.32
0.3	0.80	0.0050	0.29
0.4	1.06	0.0046	0.27
0.5	1.33	0.0044	0.25
0.6	1.59	0.0042	0.24
0.7	1.86	0.0040	0.23
0.8	2.13	0.0039	0.22
0.9	2.39	0.0038	0.22
1	2.66	0.0037	0.21
70	186.00	0.0013	0.07

Health screening results

Health screening was conducted during the study as described in Section 2.3.2. Abnormalities in haematology, bile acids and uric acid are outlined below.

Haematology

Elevations in white blood cell count were the only haematologic abnormality found. Results are summarised below (Table 4.11). The reference range for the tuatara white cell count is 0.7-3.1 x 10^9/L, with some seasonal and sex variation (Section 6.3.1).

Table 4.11 Summary of white cell counts (* 10^9 cells) for multiple dose studies conducted at 12°C. NS = no sample. * = sample obtained on day 33. ~ = sample obtained on day 20. The final itraconazole dose was administered to all animals on day 21. Results considered definitively abnormal are coloured in red.

		White cell count (x 10^9 cells) at number of days after first dose					
Animal ID	**Sex**	**13**	**22**	**28**	**34**	**41**	**55**
101	**F**	0.6	1.8	2.6	NS	NS	NS
104	**M**	2.1	5.5	8.6	9.1*	7.9	2.1
105	**F**	0.8~	0.8	0.9	NS	NS	NS
107	**F**	1.6	3.4	2.7	NS	NS	NS
109	**F**	0.7	1.5	1.8	NS	NS	NS
112	**M**	3.1	8.6	8.6	8.2	7.0	2.1

Haematology performed on day 13 was considered within normal limits for all animals (Section 6.3.1). Throughout the experiments other haematology values (haematocrit, haemoglobin, mean corpuscular haemoglobin concentration, and fibrinogen) remained within normal limits. The elevations in white cell counts in animals 104 and 112 are displayed in further detail below (Table 4.12).

Table 4.12 White cell count elevations in animals 104 and 112. Differential results considered definitively abnormal are coloured in red.

			Heterophils		Lymphocytes		Monocytes		Eosinophils		Basophils	
Animal ID	Day	Total WCC x 10^9	x 10^9	%	x 10^9	%	x 10^9	%	x 10^9	%	x 10^9	%
104	22	5.5	2.4	43	0.7	13	2.3	41	0.1	2	0.1	1
	28	8.6	3.6	42	1.4	16	3.4	39	0.3	3	0	0
	33	9.1	3.2	35	1.0	11	4.6	50	0.3	3	0.1	1
	41	7.9	2.9	37	2.3	29	2.3	29	0.1	1	0.3	4
112	22	8.6	5.2	60	1.9	22	1.5	17	0.1	1	0	0
	28	8.6	6.3	73	0.5	6	1.7	20	0.1	1	0	0
	34	8.2	5.4	66	1	12	1.6	20	0.1	1	0.1	1
	41	7.0	3.9	55	1.4	20	1.4	20	0.4	5	0	0
Reference range		0.7-3.1	0.1-1.1	15.2-53.4	0.2-1.0	11.2-53.8	0.1-0.7	5.4-35.4	0-0.5	1.2-21.8	0.0-0.3	0.0-9.8

The elevations in white cell counts were predominantly increases in heterophils and monocytes, indicative of granulocytic inflammation. Subject 112 had reactive monocytes on day 34, characterised by cytoplasmic vacuolation. In the other samples, white blood cell morphology remained normal despite elevations in cell count.

Biochemistry

Of the biochemistry panel, bile acids and uric acid concentrations were elevated in several animals. The lower and upper limits of quantitation for bile acids using the Abaxis Vetscan were 35 and 200 µmol/L respectively. Due to the limits of quantitation it was not possible to definitively determine a bile acids reference range for tuatara (see Section 6.3.2). The reference range for uric acid is 51.8-163.6, with some differences based on sex (Section 6.3.2). Biochemistry results are summarised below in Table 4.13.

Table 4.13 Summary of bile acids and uric acid concentrations (µmol/L) for multiple dose studies conducted at 12°C. - = no sample. * = sample obtained on day 20. The final itraconazole dose was administered to all animals on day 21. Results considered definitively abnormal are coloured in red.

			Days after first dose					
Animal ID	**Sex**	Biochemical analyte	13	22	28	34	41	55
101	**F**	Bile acids	<35	52	<35	48	-	-
		Uric acid	152	486	138	168	-	-
104	**M**	Bile acids	64	<35	<35	-	-	-
		Uric acid	292	436	540	-	-	-
105	**F**	Bile acids	100*	63	-	48	60	62
		Uric acid	245*	195	-	103	79	58
107	**F**	Bile acids	<35	56	<35	<35	-	-
		Uric acid	34	139	54	128	-	-
109	**F**	Bile acids	<35	<35	<35	-	-	-
		Uric acid	24	31	29	-	-	-
112	**M**	Bile acids	55	>200	151	119	54	<35
		Uric acid	59	114	132	147	106	54

Bile acids

Biochemistry showed mild elevations in bile acids in two animals (104 and 112) on day 13. Normal bile acids concentrations had not been determined for tuatara at this time, however during health screening all tuatara had bile acids ranging from less than 35 (the lower limit of quantitation) to 41 µmol/L (see Chapter 6). Animal 104 had bile acids of 64 µmol/L, and animal 112 a concentration of 55 µmol/L, however at the time these increases were considered mild and possibly post-prandial elevations related to food intake. It was not possible to obtain blood from one animal (105) at this time, so a sample was taken on day 20 from this animal for health screening. This revealed bile acids of 100 µmol/L, and this was considered likely to be a pathological increase. Following this, all six tuatara underwent repeat health screening on day 22 of the study, which detected elevations in bile acids in four animals (101, 104, 105, and 112). While three of these elevations were considered mild and possibly within normal limits, one animal (112) had a concentration exceeding 200 µmol/L (the upper limit of quantitation).

Uric acid

Uric acid was elevated in three animals (101, 104 and 105) at different periods throughout the study. Normal uric acid concentrations had not been determined for tuatara at this time, but the reference range has now been established as 51.8-163.6 µmol/L (see Chapter 6).

4.3.2.2 Multiple dose studies conducted at 20°C ambient temperature

Plasma antifungal concentrations

Itraconazole was administered to six tuatara at a dose rate of 2.5 mg/kg PO, once daily until the last dose on day 13. Blood samples were obtained as described in Section 4.2.2. Samples were analysed using LCMS/MS, and results are displayed below. (Table 4.14 and Figures 4.7 and 4.8).

Table 4.14 Itraconazole (Itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from first day of itraconazole administration (days) at 20°C. The final itraconazole dose was administered to all animals on day 13.

			Days after first dose administered						
Animal ID	**Sex**	**Drug**	**6**	**13**	**14**	**17**	**20**	**23**	**27**
101	**F**	Itra	4.696	5.824	5.328	4.559	2.281	1.879	1.250
		OH-itra	2.837	4.031	2.651	1.950	2.448	1.624	2.488
104	**M**	Itra	6.921	10.621	11.734	8.224	5.393	4.728	2.403
		OH-itra	3.405	6.129	4.595	4.387	5.409	4.66	4.158
105	**F**	Itra	0.622	7.315	6.121	4.315	5.470	2.977	2.720
		OH-itra	0.266	3.530	3.356	3.254	3.620	2.742	3.691
107	**F**	Itra	2.842	4.893	3.928	3.010	1.827	1.747	1.310
		OH-itra	1.184	3.651	2.802	2.950	2.055	2.352	2.055
109	**F**	Itra	3.279	4.921	4.053	3.452	2.436	2.206	0.946
		OH-itra	2.467	3.645	3.630	4.091	4.509	3.059	4.208
112	**M**	Itra	3.934	6.606	6.164	5.696	3.403	3.397	3.158
		OH-itra	2.143	2.748	4.004	3.945	4.318	4.538	4.713

Figure 4.7 Itraconazole (-●-) and hydroxy-itraconazole (-■-) plasma concentration (mg/L) at time from itraconazole administration (days) for each animal at 20°C.

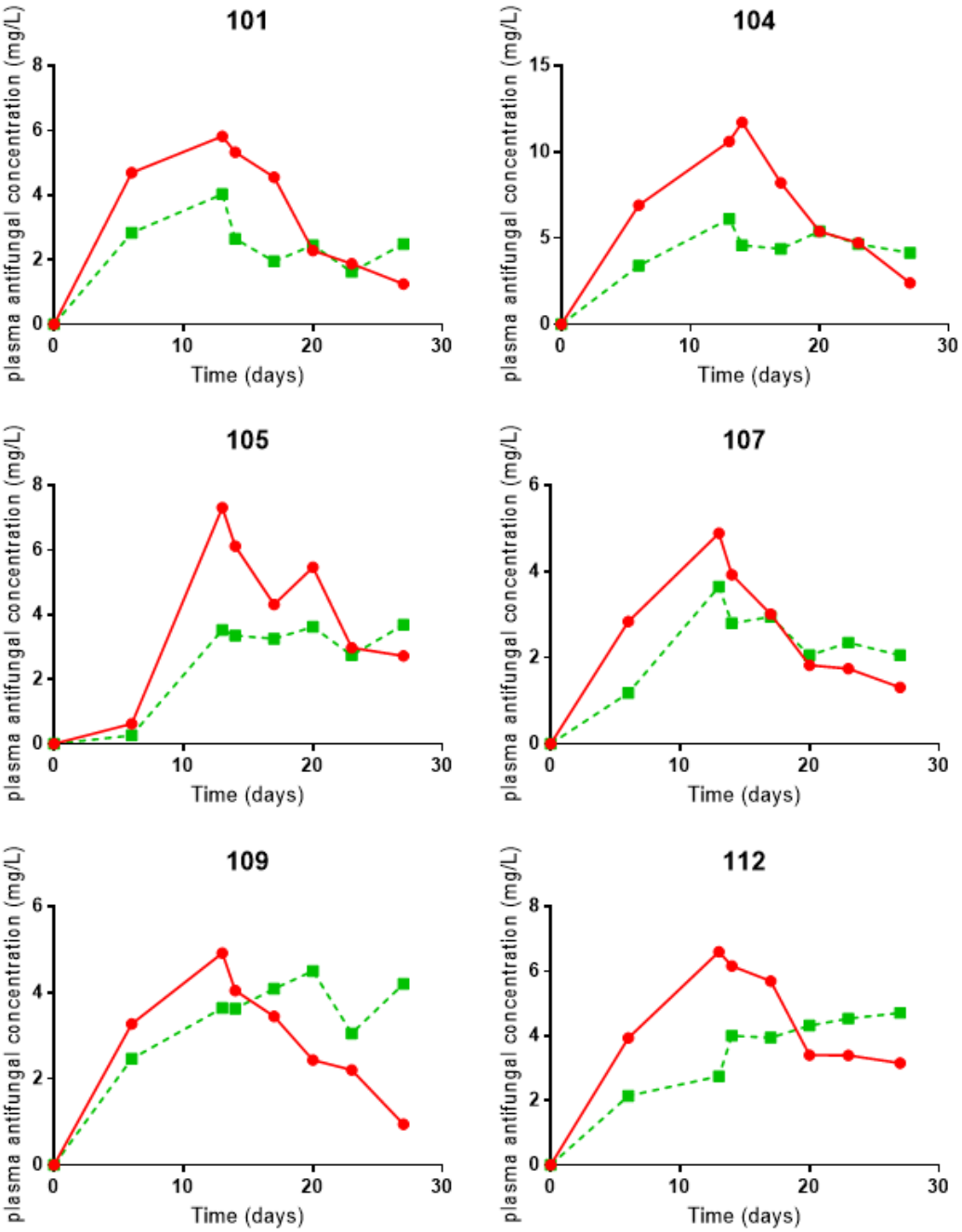
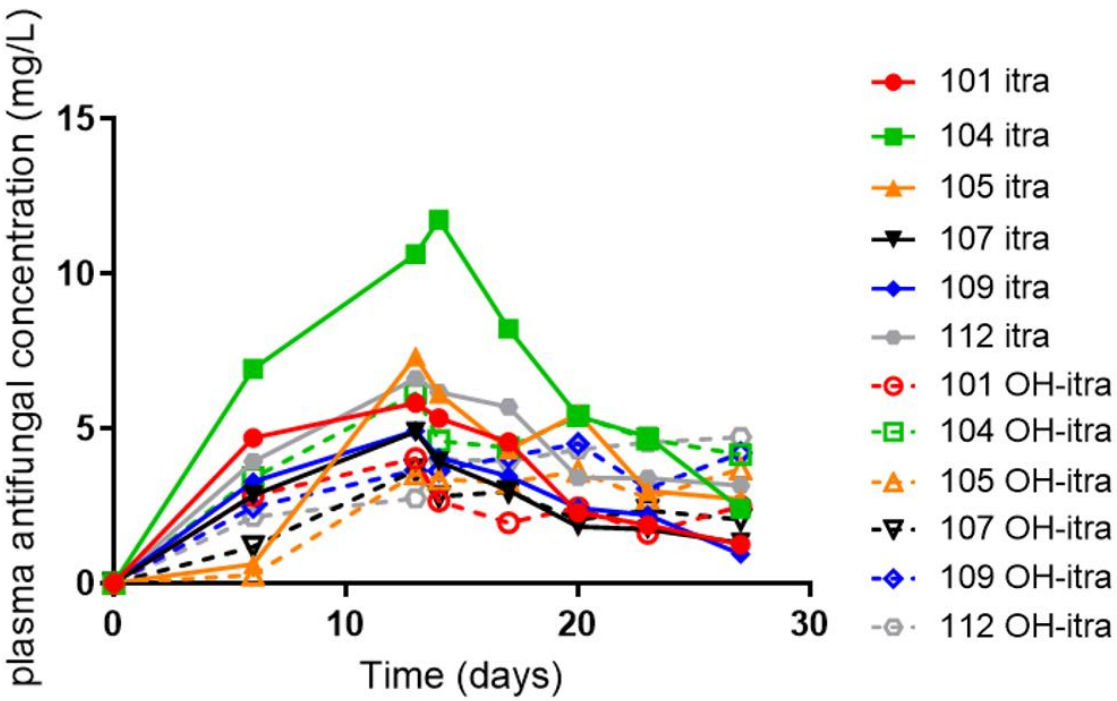


Figure 4.8 Itraconazole (itra) and hydroxy-itraconazole (OH-itra) plasma concentrations (mg/L) at time from itraconazole administration (days) from all animals at 20°C.



Pharmacokinetic data and analysis

The only index that could be calculated using traditional pharmacokinetic analysis was half-life. The mean and standard deviation of itraconazole half-life was 7.65 ± 2.2 hours. The mean half-life of hydroxy-itraconazole could not be calculated, as it was only undergoing elimination in one animal during the sampling period. The half-life in this animal was 19.7 hours.

Table 4.15 $t_{1/2}$ of itraconazole and hydroxy-itraconazole at 20°C. NC = not calculated.

Animal ID	Itraconazole $t_{1/2}$ (hours)	OH-itraconazole $t_{1/2}$ (hours)
101	5.88	NC
104	5.92	19.66
105	7.25	NC
107	8.16	NC
109	6.49	NC
112	12.20	NC
Mean ± SD	7.65 ± 2.2	NC

Pharmacokinetic modelling results

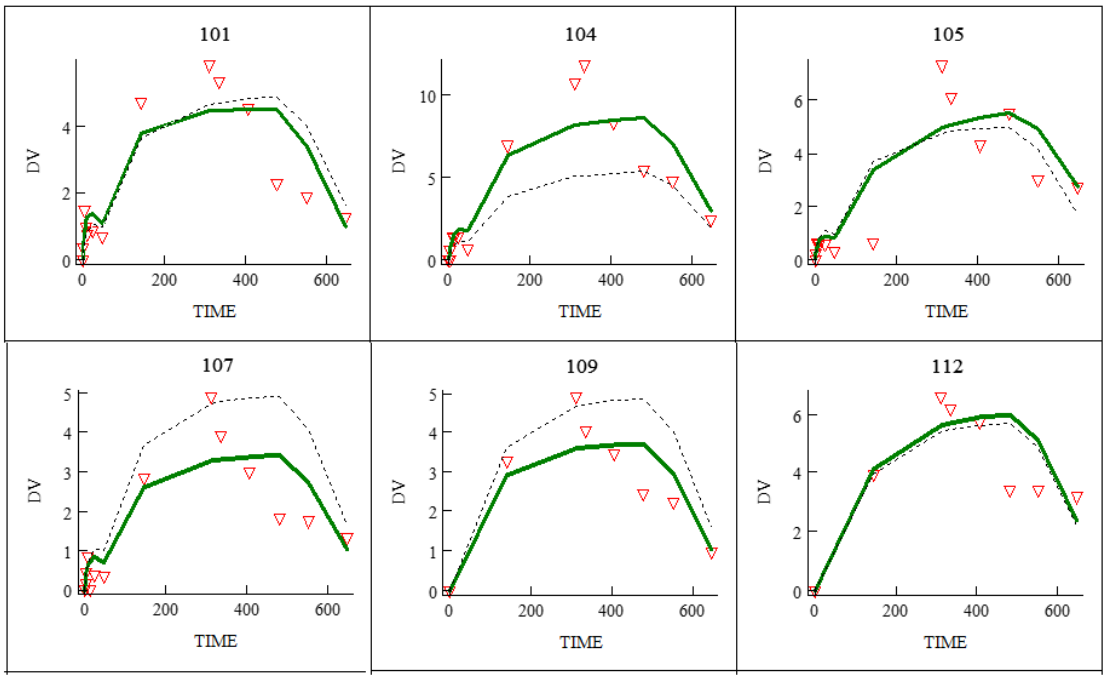
The modelling data below (Table 4.16) describe the mg/kg per day dosage of itraconazole required to achieve a plasma target concentration of 2.4 mg/L for tuatara in a weight range from 0.1-1.0kg maintained at 12°C. Dose rates range from 0.60-1.08 mg/kg. A loading dose is provided as an optional first dose to facilitate achieving steady state concentrations more rapidly. Pharmacokinetic parameters vary with weight, but for a 1kg animal to reach an itraconazole target concentration of 2.4 mg/L, volume of distribution is estimated at 2.66L and clearance at 0.010 L/h.

Modelling based on both the single and multiple dose data illustrate the predicted and observed concentrations of itraconazole at the administered dosage of 2.5 mg/kg for each animal (Figure 4.9).

Table 4.16 Model predictions of pharmacokinetic parameters and itraconazole dose rate required to attain steady-state target concentration of 2.4 mg/L at 20°C.

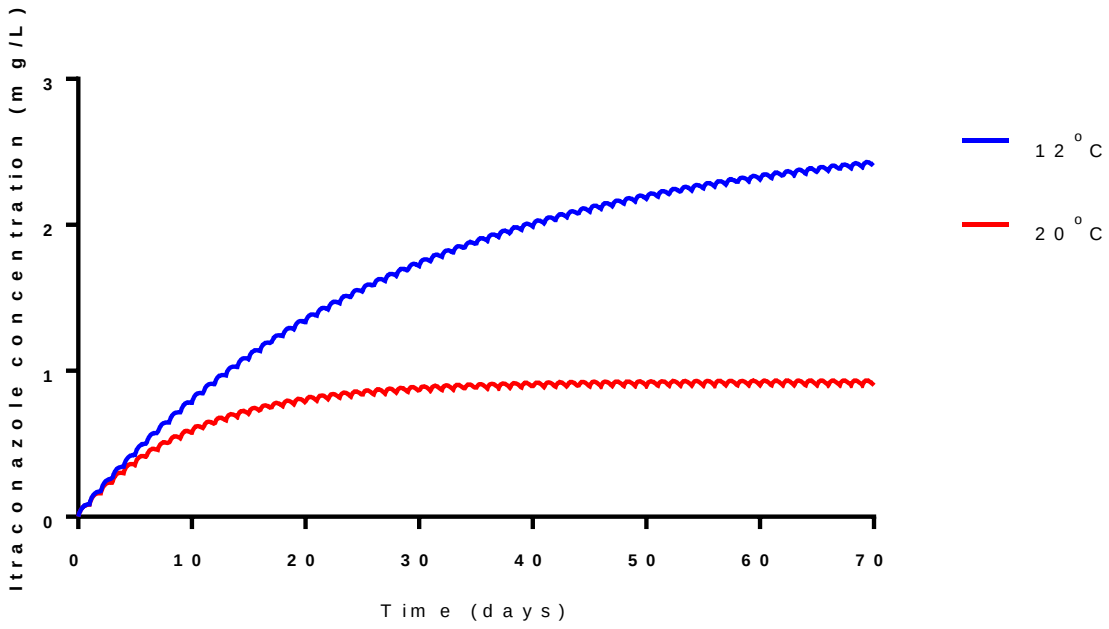
Weight (kg)	Volume (L)	Clearance (L/h/kg)	Loading dose (mg)	Dose rate (mg/kg/day)
0.1	0.27	0.019	0.64	1.08
0.2	0.53	0.016	1.28	0.90
0.3	0.80	0.014	1.91	0.82
0.4	1.06	0.013	2.55	0.76
0.5	1.33	0.012	3.19	0.72
0.6	1.59	0.012	3.83	0.69
0.7	1.86	0.011	4.46	0.66
0.8	2.13	0.011	5.10	0.64
0.9	2.39	0.011	5.74	0.62
1	2.66	0.010	6.38	0.60
70	186.00	0.004	446.40	0.21

Figure 4.9 Predicted and observed itraconazole concentrations at 20°C following dosing at 2.5 mg/kg SID. Green line: Individual prediction. Dashed line: population prediction. Red symbols: observed concentrations.



Pharmacokinetic modelling simulations were run to illustrate the difference between predicted itraconazole concentrations at both temperatures. An example is provided below (Table 4.10), where a 0.75kg tuatara is administered 0.25 mg/kg of itraconazole once daily. 0.25 mg/kg is the dose rate predicted to result in steady-state concentrations of 2.4 mg/L at 12°C. As drug elimination is slower at colder temperatures, higher itraconazole concentrations are reached at 12°C than at 20°C.

Figure 4.10 Time vs concentration profile of a 0.75 kg tuatara administered 0.25 mg/kg itraconazole SID at 12°C and 20°C.



Health screening results

Health screening was conducted during the study as described in Section 2.3.2. Abnormalities in haematology, bile acids and uric acid are outlined below. Itraconazole administration ceased on day 13.

Haematology

White blood cell numbers remained within normal limits during this part of the study. Animal 105 had occasional reactive monocytes on day 27, accompanied by mild anisocytosis and occasional binucleated red blood cells. All other haematology and biochemistry values for this animal were normal. No other animals had any haematologic abnormalities during this part of the study.

Biochemistry

Of the biochemistry panel, the only abnormal values during treatment were bile acids and uric acid. Due to the limits of quantitation it was not possible to definitively determine a bile acids reference range for tuatara (see Section 6.3.2). The reference range for uric acid is 51.8-163.6, with some differences based on sex (Section 6.3.2). Biochemistry results are summarised in Table 4.17.

Animal 112 lost weight during the study, and continued to lose weight despite cessation of itraconazole administration. On day 23 the decision was made to move him back to his normal enclosure in the hope that being in a more natural environment would aid his return to health. Two days after moving to the new enclosure he had regained much of his lost weight, suggesting the weight loss may have been caused by inappetence or dehydration. As the animal was improving it was decided to not disturb him for an additional seven weeks, after which time his health screening results had returned to normal.

Table 4.17 Summary of bile acids and uric acid concentrations (µmol/L) for multiple dose studies conducted at 20°C. - = no sample. * = animal moved to ambient temperature. The final itraconazole dose was administered to all animals on day 13. Results considered definitively abnormal are coloured in red.

			Days after first dose				
Animal ID	**Sex**	**Biochemical analyte**	**13**	**20**	**27**	**41**	**89**
101	**F**	Bile acids	42	<35	-	-	-
		Uric acid	341	177	-	-	-
104	**M**	Bile acids	50	80	<35	-	-
		Uric acid	227	427	60	-	-
105	**F**	Bile acids	<35	<35	-	-	-
		Uric acid	92	107	-	-	-
107	**F**	Bile acids	77	38	<35	-	-
		Uric acid	110	135	108	-	-
109	**F**	Bile acids	<35	<35	-	-	-
		Uric acid	132	80	-	-	-
112	**M**	Bile acids	>200	194	132	87*	<35*
		Uric acid	128	100	115	93	102

4.3.2.3 Statistical comparisons

The elimination half-life of itraconazole was suitable for statistical comparison. Following log transformation, using the paired t-test the p-value was 0.0083, indicating the $t_{1/2}$ at 12°C and 20 °C were significantly different ($p<0.05$).

4.3.2.4 Combined pharmacokinetic modelling results of single and multiple dose studies at both temperatures

Bootstrap estimates of other model parameters and confidence intervals were able to be determined, and these are illustrated in the table below (Table 4.18).

Table 4.18 Select population pharmacokinetic parameters for tuatara receiving itraconazole. RUV = residual unexplained variability. RSE = relative standard error expressed as a percentage.

Statistics	Mean	95% CI	RSE %
Clearance (L/h/70kg)	0.259	0.200-0.315	11
Volume of distribution (L/70kg)	192.3	150.4-236.8	13
Absorption half-life (hours)	5.30	3.461-7.733	21
Fold decrease in clearance relative to 20°C	2.864	2.438-3.489	10
RUV proportional	0.189	0.089-0.284	29
RUV additive (mg/L)	0.158	0.002-0.306	69

Residual unexplained variability (RUV) is a measure of random error, which is always present in modelling and is unpredictable. Quantifying RUV gives a measure of how reliable a model is, and using the correct structural model decreases RUV.

4.4 Discussion

4.4.1 Pharmacokinetics and temperature

Single dose studies were both conducted using itraconazole dosages of 3 mg/kg, while multiple dose studies were both conducted using dose rates of 2.5 mg/kg. This allowed direct comparison of the effect of temperature on pharmacokinetic parameters. Results

show that these dosages were excessive at both temperatures, and resulted in high itraconazole plasma concentrations and significant adverse effects.

Time to maximal plasma concentration

In single dose studies at 12°C, time to maximal plasma concentration was not able to be estimated, as most animals were not in the itraconazole terminal elimination phase at 48 hours. At 20 °C the Tmax was estimated to be between 4-8 hours. While it was not appropriate to make statistical comparisons because of the lack of definitive data at 12 °C, it is apparent there is a marked difference in Tmax at the two temperatures using the 3 mg/kg dose rate. The Tmax of itraconazole in reptiles has not been reported previously, but in humans was 5.7 hours (Willems et al. 2001), and in birds was 4 hours in domestic pigeons, 3.7 hours in black-footed penguins, and varied between 3.7-6.9 hours in Amazon parrots (Lumeij et al. 1995, Orosz et al. 1996, Smith et al. 2010). These studies administered itraconazole of various formulations, at different dose rates to this study, with some animals fed and others fasted, making direct comparisons inappropriate.

Absorption half-life

The absorption half-life of itraconazole in tuatara was estimated at 5.30 hours based on modelling analysis. This is slow absorption, and indicates that 50% of the administered oral dose is absorbed in 5.3 hours. Absorption is considered to be a largely passive process, and not markedly influenced by temperature, so the estimate is the same for both 12°C and 20°C.

Elimination half-life

In the multiple dose studies itraconazole had a mean ± SD elimination half-life of 22.0 ± 9.4 hours at 12 ^{o}C, and 7.65 ± 2.2 hours at 20 ^{o}C. This difference was statistically significant (p=0.0083), indicating that half life was significantly lower at 20^{o}C than 12^{o}C. Modelling indicated that elimination kinetics were first-order (non-saturable), as observed in other species.

Clearance

Pharmacokinetic modelling predicted clearance at 12 ^{o}C of 0.010L/h, and at 20 ^{o}C 0.0037L/h for a 1kg animal with a target itraconazole concentration of 2.4 mg/L (Tables 4.9 and 4.14). The clearance differs between temperatures by a factor of 2.86, which is very similar to the observed proportional difference in mean elimination half-lives at both temperatures (Tables 4.8 and 4.13) of 2.88.

Modelling and simulation to estimate itraconazole dosage regimens in tuatara

The observed itraconazole concentrations matched reasonably well with those predicted by modelling, indicating that itraconazole has predictable pharmacokinetics, and there can be a reasonable level of confidence in the modelling predictions.

It is apparent from these data administering the same dosage of medication at different temperatures produces markedly different plasma itraconazole concentrations. This indicates that ambient temperature control is an essential part of a hospitalisation and treatment protocol using itraconazole in tuatara. Simulations show that at 12^{o}C, itraconazole is not predicted to reach steady-state concentrations by the end of simulations at 70 days, as it continues to accumulate. This makes 12^{o}C an inappropriate temperature at which to treat tuatara with itraconazole, as therapeutic concentrations

would not be reached in an appropriate timeframe, and accumulation of the drug means adverse effects become more likely as treatment progresses. Therefore it is recommended animals are treated at 20°C, the higher end of their POTZ.

4.4.2 Adverse effects

Adverse effects were noted in both the 12°C and 20°C multiple dose studies, with elevated bile acids being the most common problem. Bile acids are produced by the liver to aid digestion, and are considered a specific test for hepatic insufficiency (Campbell 2014). In a study in healthy green iguanas (*Iguana iguana*), pre-prandial bile acids were 7.5 ± 7.8 µmol/L, and post-prandial bile acids were 33.3 ± 22.0 µmol/L (McBride et al. 2006). At the time of our study there were no reference ranges for bile acids in tuatara, and healthy tuatara at Auckland Zoo had previously had bile acids ranging from <35 (the lower limit of detection of the analyser) to 41 µmol/L. Gastric emptying time in tuatara is unknown, and animals in the study were able to feed at any time of day, so no distinction between pre- and post-prandial bile acids concentrations could be made during the study. At the time of the study bile acids concentrations lower than 40 µmol/L were considered normal, between 40-60 µmol/L marginal, and above 60 µmol/L elevated, based on clinical experience and extrapolation from other reptile species.

At 12°C, elevations in bile acids (64 µmol/L) were noted in one animal on day 13 (subject 104, male), this was associated with a plasma itraconazole concentration of 15.8 mg/L, and a hydroxy-itraconazole concentration of 3.76 mg/L. On day 20 subject 105 (female), was found to have elevated bile acids (100 µmol/L); this was associated with an itraconazole plasma concentration of 13.3 mg/L, and a hydroxy-itraconazole concentration of 2.43 mg/L. On day 22 another animal (subject 112, male) was found to

have elevated bile acids (>200 µmol/L), the closest itraconazole measurement in this animal was 18.7 mg/L on day 20 (the last drug dose was administered on day 21), and the hydroxy-itraconazole concentration was 3.14 mg/L. Other tuatara had plasma itraconazole concentrations ranging from 10.9-22.9 mg/L and hydroxy-itraconazole concentrations of 1.86-4.76 mg/mL on day 20, and did not show elevations in bile acids. Subject 104's bile acids normalised to <35 µmol/L on day 22, despite itraconazole treatment only ceasing on day 21, raising the possibility that the observed elevation on day 13 was not directly related to antifungal therapy.

In the 20°C multiple dose study two animals (subjects 107 – female, and 112 – male) had elevations in bile acids on day 13, with corresponding itraconazole concentrations of 4.893 mg/L and 6.606 mg/L, and hydroxy-itraconazole concentrations of 3.651 and 2.748 mg/L respectively. Subject 112 had persistently elevated but decreasing bile acids until day 41. One tuatara (subject 104) had marginal bile acids of 50 µmol/L on day 13, and went on to develop high bile acids of 80 µmol/L on day 20. Unaffected tuatara had itraconazole concentrations between 4.921-10.621 mg/L, and hydroxy-itraconazole concentrations of 3.53-6.129 mg/L.

Elevations in bile acids occurred earlier in the 20°C study, despite lower plasma itraconazole concentrations at the time compared to the 12°C study (4.893-6.606 mg/L versus 13.3-18.7 mg/L respectively). Hydroxy-itraconazole concentrations were similar in both studies when adverse effects were noted (2.748-3.651 mg/L and 2.43-3.14 mg/L). It is possible that hydroxy-itraconazole, or a different metabolite of itraconazole, is responsible for the increased bile acids (rather than the parent compound), as hydroxy-itraconazole attained higher concentrations more rapidly in the 20°C study. It

is also possible that, despite apparently normal bile acids concentrations on health screening prior to the 20°C study, that the 12°C study had caused subclinical liver damage, making the liver more sensitive to itraconazole in the 20°C study.

In humans and rats, itraconazole has been noted to cause dose-dependent hepatocellular necrosis, bile duct hyperplasia, cholestasis and biliary cirrhosis, with elevation in liver enzyme activity and bilirubin (Talwalkar et al. 1999, Somchit et al. 2004, Lou et al. 2011). In one human case report, symptoms and biochemistry abnormalities including elevated bilirubin and liver enzymes continued to worsen after the cessation of itraconazole treatment, ultimately resolving four months after stopping itraconazole (Talwalkar et al. 1999). In bearded dragons administered itraconazole 5 mg/kg PO SID, five out of seven bearded dragons died, with elevations in AST reported in four of these animals (van Waeyenberghe et al. 2010); no histologic abnormalities were found in the liver on post-mortem. Bile acids were not reported in this study, and neither was CK (muscle damage can raise both CK and AST).

Uric acid concentrations were also increased in several animals in both the 12°C and 20°C studies. Uric acid is the primary product of protein catabolism in tuatara (Hill and Dawbin 1969), and is produced by the liver and excreted via the kidneys. Uric acid can be elevated in renal disease and in dehydrated animals. Prior to this study there were no published normal concentrations of uric acid in tuatara, however our subsequent analyses showed concentrations of 42-232 µmol/L were detected in clinically healthy, well hydrated animals (Chapter 6). During the multiple dose studies, uric acid concentrations between 40-250 µmol/L were considered normal, 250-350 µmol/L

marginal, and above 350 µmol/L abnormal, based on clinical experience at Auckland Zoo.

In the 12°C study, one animal (subject 104) had marginally increased uric acid concentrations on day 13, of 292 µmol/L, and progressed to more dramatic elevations on days 22 and 28, with concentrations of 436 µmol/L and 540 µmol/L, respectively. This animal also lost weight during this period, which was regained within two days of returning him to his normal enclosure, indicating that dehydration was likely the cause of both weight loss and the uric acid elevation. Tuatara obtain most of their water from dietary sources (Cree 2014), and it is possible that itraconazole therapy resulted in nausea and inappetence, as this is a commonly reported adverse effect in humans (Manire et al. 2003). It is also possible the study environment and regular handling may have contributed to altered behaviour and dietary intake, however this was not observed in voriconazole-treated animals (Chapter 5). Subject 101 also showed elevated uric acid (486 µmol/L) at the same time when marginal bile acids (52 µmol/L) were noted, on day 22. These had both resolved at the next sampling point on day 28.

In the 20°C studies subject 101 had marginal uric acid (341 µmol/L) on day 13 (the last day of medication administration), which had normalised by day 20. Subject 104 had elevated uric acid (427 µmol/L) on day 20, which had normalised on day 27.

Animals with elevated bile acids did not always have concurrently elevated uric acid and vice versa.

White cell counts were elevated in two animals (subjects 104 and 112, both male) in the 12°C study. White blood cells are part of the immune system, and elevations commonly

indicate inflammation. Both tuatara had elevations in heterophils and monocytes, indicative of granulocytic inflammation. These elevations were first noted on day 22, and persisted through day 41, but had resolved by day 55. During the 20^{o}C study white cell numbers remained normal for all animals, however animal 105 had one instance of reactive monocytes on day 27 which were not observed again.

Overall, subjects 104 and 112 suffered the most severe adverse effects throughout both studies, as evidenced variously by elevated white cells, bile acids and uric acid concentrations. These animals had the highest plasma itraconazole concentrations and were the heaviest of the group, meaning that on an allometrically-scaled basis, these animals received more itraconazole (despite the same mg/kg dosage) than their smaller counterparts. It is possible that this higher dosage is responsible for the increased incidence of adverse effects, however sex-specific differences in itraconazole metabolism cannot be ruled out without further studies involving more animals of varying weights.

It appears that in tuatara, itraconazole toxicity manifests primarily as presumed cholestasic disease characterised by elevations in bile acids and, in more severe cases, white blood cells. Uric acid elevations, likely related to dehydration, were also present in several cases.

4.4.3 Itraconazole : hydroxy-itraconazole ratio

Hydroxy-itraconazole and itraconazole have a similar anti-fungal spectrum of activity, and very few fungi differ significantly in their *in vitro* susceptibility to the two antifungals (Odds and Bossche 2000). In human subjects, hydroxy-itraconazole plasma concentrations and AUC exceed that of itraconazole, after both single and multiple

doses (Meinhof 1993, Barone et al. 1998, Suarez-Kurtz et al. 1999). This was also the case in Humboldt penguins (Bunting et al. 2009). In Kemp's ridley sea turtles, hydroxy-itraconazole concentrations were markedly lower than itraconazole concentrations (Manire et al. 2003), while in horses, hydroxy-itraconazole was not detected at all following itraconazole administration, indicating a different pathway was involved in itraconazole metabolism (Davis et al. 2005).

In tuatara, hydroxy-itraconazole concentrations were significantly lower than itraconazole concentrations at all time points in the single dose studies. In multiple dose studies at 12^{o}C, hydroxy-itraconazole concentrations were observed to continue to increase for 13 days in all animals following cessation of treatment and, in two animals, were still increasing at the end of sampling, 33 days after stopping treatment. Hydroxy-itraconazole concentrations exceeded itraconazole concentrations in three animals at the end of the sampling period. Similar data were obtained in the 20^{o}C studies, with hydroxy-itraconazole concentrations exceeding those of the parent drug 4 days after finishing treatment in one animal, 6 days after finishing treatment in 4 animals, and in all 6 animals at the end of sampling, 14 days after cessation of therapy. These prolonged elevations in hydroxy-itraconazole concentration, along with itraconazole's persistence in skin (Cauwenbergh et al. 1998, Heykants et al. 1989), may account for the success of pulse therapy documented in humans (De Doncker et al. 1997) as the hydroxy-itraconazole concentrations observed at the end of sampling were above the target concentration of itraconazole.

4.4.4 Recommended treatment protocol

From these data it can be concluded that it is not appropriate to treat tuatara at 12°C with itraconazole, due to the prolonged time taken to approach steady-state, the accumulation of drug, and the potential for adverse effects that this presents. Tuatara undergoing itraconazole treatment should be maintained at 20°C and the treatment regime based on their weight (Table 4.19), with careful monitoring of haematology and biochemistry every 2 weeks, or more frequently if symptoms such as inappetence or weight loss become apparent. Particular attention should be paid to changes in bile acids, uric acid and white cell count. Elevations in bile acids should result in the cessation of therapy, with careful monitoring and supportive care if necessary until all haematology and biochemistry parameters return to normal. Administration of a loading dose will reduce the time required to reach target plasma itraconazole concentrations.

Table 4.19 Recommended daily itraconazole dosage for tuatara weighing between 0.1-1.0kg maintained at 20°C.

Weight (kg)	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
Loading dose (mg)	0.64	1.28	1.91	2.55	3.19	3.83	4.46	5.10	5.74	6.38
Maintenance dosage (mg/kg)	1.08	0.90	0.82	0.76	0.72	0.69	0.66	0.64	0.62	0.60

4.5 Conclusions

These studies show that tuatara should be treated at the high end of their POTZ, at 20°C. Itraconazole has the potential to cause elevations in bile acids, uric acid and white blood

cell concentrations, and haematology and biochemistry should be monitored at regular intervals in tuatara undergoing treatment. Ideally, further pharmacokinetic studies and clinical trials at the recommended dose rates should be conducted to ensure target concentrations are met and desired clinical outcomes achieved. The proposed itraconazole treatment regimes based on MIC and PK data from this study have the potential to improve outcomes and reduce toxicity when treating tuatara for fungal infections.

Chapter 5: Voriconazole pharmacokinetics in tuatara

5.1 Introduction

Voriconazole is a second-generation triazole antifungal, synthetically derived from fluconazole (Theuretzbacher 2006). It is commonly used in human medicine to treat and prevent invasive aspergillosis and Candida infections in immunocompromised patients. In mammals voriconazole undergoes hepatic metabolism, and does not have any pharmacologically active metabolites (Theuretzbacher 2006). Voriconazole has complex clinical pharmacology in humans, with non-linear pharmacokinetics and variable hepatic metabolism by CYP2C19 enzymes. Studies in humans and most animals show that voriconazole has saturable metabolism at therapeutic doses, though linear pharmacokinetics were observed in children under 12 and in Amazon parrots (Theuretzbacher 2006, Sanchez Migallon-Guzman et al. 2010).

There are few reports of voriconazole use in reptiles in the literature, however oral voriconazole has been used successfully to treat infections caused by members of the CANV complex in lizards at a dosage of 10 mg/kg SID (Hellebuyck et al. 2010, van Waeyenberghe et al. 2010). This voriconazole dose regimen appeared well tolerated and effective in the majority of cases, though one bearded dragon treated with this regimen died. In this case it was not clear on histopathologic examination if the mortality was caused by voriconazole-related hepatotoxicity or a consequence of fungal invasion (L. Van Waeyenberghe, pers. comm.).

The aim of this study was to investigate the pharmacokinetics of voriconazole after administration of the oral suspension. Single and multiple dose studies were conducted at high and low ends of the tuatara's POTZ to investigate variability in pharmacokinetics with temperature.

5.2 Methods

Single dose studies were conducted first, and the results of these informed the methods of the multiple dose studies. For general methods relating to animal selection, environmental conditions, drug assay method and pharmacokinetic analysis, see Chapter 2.

Mixed effects pharmacokinetic modelling was performed using NONMEM v7.3.0 with the Fortran Compiler v11. Modelling for voriconazole concentration-time date in tuatara assumed one compartment pharmacokinetic model, mixed-order elimination and first-order absorption (see Chapter 2 and Appendix 6).

Animal ethics approvals were obtained from Murdoch University, Auckland Zoo and the Department of Conservation (see Section 2.2).

Plasma antifungal concentrations were determined using Liquid Chromatography and tandem Mass Spectrometry (LCMS/MS). Drug assay methods and HPLC conditions are described in Section 2.6 and Appendices 3 and 4.

5.2.1 Single dose studies

Dose determination

Tuatara have a low metabolic rate compared to other reptiles (Thompson and Daugherty 1998), and voriconazole has a low MIC (<0.008-0.015 mg/L, Section 3) for studied *P. australasiensis* isolates. For these reasons a voriconazole dosage of 5 mg/kg was selected for single dose studies.

Drug formulation and administration

The voriconazole formulation was a powder for reconstitution with tap water (VFEND powder for oral suspension, Pfizer, NZ). It is designed to be reconstituted to 40 mg/mL. However, after verifying with technical staff at Pfizer that absorption and uniformity of suspension would not be affected, a concentration of 15 mg/mL was chosen. The lower concentration ensured more accurate dosing, and volumes administered were similar to those for the itraconazole-treated animals (Section 4.2.1). Medication was administered orally as described in Chapter 2.

Blood sampling intervals

Blood sampling intervals were the same as those in the itraconazole single dose study (Chapter 4), at 2, 4, 8, 12, 24 and 48 hours following oral voriconazole dosing.

5.2.2 Multiple dose studies

Dose determination

All animals in the study weighed over 400 grams (range 458-899 g), so this weight was chosen as the weight for which the target concentration of 0.16 mg/L would be determined. The predicted dose rate to achieve the target concentration of 0.16 mg/L in a 400 g animal at 20 ^{o}C is 0.17 mg/kg/day (Table 5.6), based on the PK modelling from the single dose studies. This is far lower than the 10 mg/kg used in other reptile studies (Hellebuyck et al. 2010, van Waeyenberghe et al. 2010), and lower than the 5 mg/kg used in the single dose studies. There were no adverse effects attributed to the use of voriconazole at 10 mg/kg in the aforementioned studies, so this suggests that voriconazole has a high margin of safety.

As there is the potential for voriconazole to be used in tuatara to treat fungal organisms with MICs higher than that of the current *P. australasiensis* isolates, it was elected to use a higher dosage than the predicted 0.17 mg/kg/day. A dose rate of 1 mg/kg once daily was selected for the 12^{o}C study for the reasons outlined above, and based on the ease of medication administration at this dose. Because a different dose was being used to that in the single dose studies, and given voriconazole's non-linear pharmacokinetics, it was expected that pharmacokinetic parameters and indices would be significantly different in the multiple dose studies than in the single dose studies.

Pharmacokinetic modelling simulations conducted with the aid of the 12^{o}C multiple dose studies indicated that using the same dosage of 1 mg/kg at 20^{o}C was likely to result in very low, possibly undetectable concentrations of voriconazole in most animals. The modelling predicted that a dose rate of 1.8 mg/kg would be required for animals to reach plasma voriconazole concentrations above the target of 0.16 mg/L at 20^{o}C. Consequently a dose rate of 2 mg/kg was chosen for ease of drug dose calculation for this part of the study.

Drug formulation and administration

The VFEND powder for oral suspension (Pfizer, NZ) was reconstituted to a concentration of 4 mg/mL for ease of administration, and so drug volumes were similar to those used in the itraconazole studies (Chapter 4). Medication was stored at room temperature and disposed of after 14 days, as per manufacturer recommendations, and a new suspension was made up for the following two weeks.

In the 12^{o}C study, voriconazole was to be administered at 1 mg/kg PO SID for 42 days. However as there was no evidence of plasma concentrations approaching steady state,

drug administration was ceased on day 21. The medication was given at approximately the same time each day for each animal (with up to 5 minutes variation between days), between the hours of 7am and 8am. At 12^{o}C, animals were fed once weekly in the afternoon, to minimise any effect of food on absorption of medication. The gastric pH and gastric emptying time of tuatara is unknown.

In the 20^{o}C study, VFEND was reconstituted to a concentration of 8 mg/mL so that administration volumes were similar to those used in the 12^{o}C study. Medication was administered using the same method as in the 12^{o}C study, for a total of 42 days. Animals were fed twice weekly in the afternoons, on the same days each week.

Blood sampling intervals

In order to measure trough voriconazole concentrations, blood samples were taken just prior to medication administration. The first day of medication was designated as day zero, and it was planned to take samples for trough voriconazole concentrations on days 2, 4, 6, 13, 20, 27, 24 and 41 in the 12^{o}C study. Results from the first two weeks showed that voriconazole concentrations were increasing with no evidence of approaching steady state. It was unlikely that further beneficial pharmacokinetic information would be generated if the study was continued, and the risk of voriconazole toxicity increased with increasing plasma drug concentrations. The decision was made to cease voriconazole administration and take further blood samples to measure drug elimination. The last dose of voriconazole was given on day 21, and samples were taken to measure drug elimination on days 22, 23, 24 and 25. Blood samples were taken for health screening on days 13 and 22.

In the 20°C study, sampling was undertaken on days 6, 13, 20, 27 and 41 to measure trough voriconazole concentrations. Samples to measure elimination were taken on day 41 at 10 and 16 hours after dose administration, and on day 42, 24 hours after the last administered dose.

5.3 Results

5.3.1 Results for single dose studies

5.3.1.1 Single dose studies conducted at 12°C ambient temperature

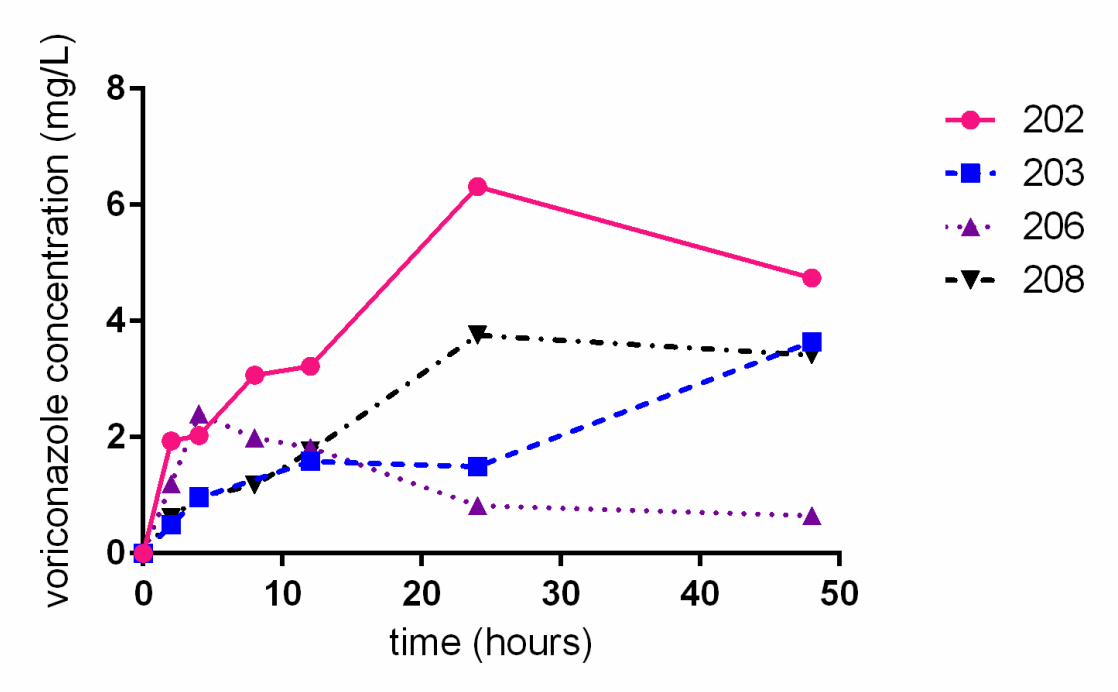
Plasma antifungal concentrations

Voriconazole was administered to four tuatara at a dose rate of 5 mg/kg PO, at time zero. Plasma samples were analysed using LCMS/MS and were run in duplicate to ensure accuracy, with the averages displayed below (Table 5.1 and Figure 5.1)

Table 5.1 Voriconazole plasma concentration (mg/L) at time from voriconazole administration (hours) at 12°C in tuatara. NS = no sample.

Animal ID	Sex	2h	4h	8h	12h	24h	48h
202	F	1.935	2.030	3.070	3.225	6.315	4.745
203	M	0.497	0.956	NS	1.580	1.500	3.645
206	F	1.195	2.405	1.990	1.820	0.822	0.650
208	F	0.612	0.979	1.175	1.765	3.755	3.415

Figure 5.1 Voriconazole plasma concentration (mg/L) at time from voriconazole administration (hours) at 12°C in tuatara.



Pharmacokinetic data and analysis

Two of the four subjects in the 12°C study appeared to be in the terminal elimination phase by the last time point at 48 hours. For subjects 202 and 208, there were only two data points (at 24 and 48 hours) in the elimination curve, and this does not provide enough data to accurately calculate the elimination constant. In subject 203, no elimination was observed 48 hours post- medication administration. As the elimination constant could not be accurately calculated in subjects 202, 203 and 208, it was not possible to obtain data to calculate elimination half-life, $AUC_{0-\infty,}$ apparent clearance, or apparent volume of distribution. The only index able to be reliably calculated for all four subjects was AUC_{0-48} (Table 5.2). Elimination in subject 206 was sufficient to allow calculation of $AUC_{0-\infty,}$ however the extrapolated percentage was >20 %, precluding the calculation of elimination half-life, apparent clearance, and apparent volume of distribution.

Table 5.2 Selected pharmacokinetic indices for voriconazole at 12°C. NC = not calculated.

Animal ID	AUC_{0-48} (mg/L*h)	$AUC_{0-\infty}$ (mg/L*h)	AUC % extrapolated
202	218.6	NC	NC
203	92.3	NC	NC
206	56.2	81.2	30.1
208	131.6	NC	NC
Mean ± SD	124.68 ± 60.43	NC	NC

Approximate maximal plasma concentration (Cmax) and time to maximal plasma concentration (Tmax) were highly variable, and were not able to be determined for subject 203. As maximal concentrations were observed late in the sampling period, it is not appropriate to estimate Cmax and Tmax, as the sampling intervals at this stage were long. The true values of voriconazole Cmax and Tmax for subjects 202 and 208 may have occurred a significant number of hours either side of the sampling points, so these data has not been analysed.

Pharmacokinetic modelling results

Using mixed effect modelling (see Section 2.11), it was possible to estimate the drug dosages required to reach the target concentration of 0.16 mg/L (see Chapter 3) at 12°C for animals of different weights. It was also possible to estimate pharmacokinetic parameters and indices of voriconazole for different animal weights at 12°C (Table 5.3).

Table 5.3 Pharmacokinetic modelling results for attaining steady-state plasma concentrations of 0.16 mg/L of voriconazole at 12°C.

Weight kg	Volume (L)	Clearance (L/h/kg)	Dose rate (mg/kg/ day)
0.1	0.06	0.0188	0.07
0.2	0.11	0.0158	0.06
0.3	0.17	0.0143	0.05
0.4	0.22	0.0133	0.05
0.5	0.28	0.0126	0.05
0.6	0.33	0.0120	0.05
0.7	0.39	0.0116	0.04
0.8	0.44	0.0112	0.04
0.9	0.50	0.0109	0.04
1	0.55	0.0106	0.04

5.3.1.2 Single dose studies conducted at 20°C ambient temperature

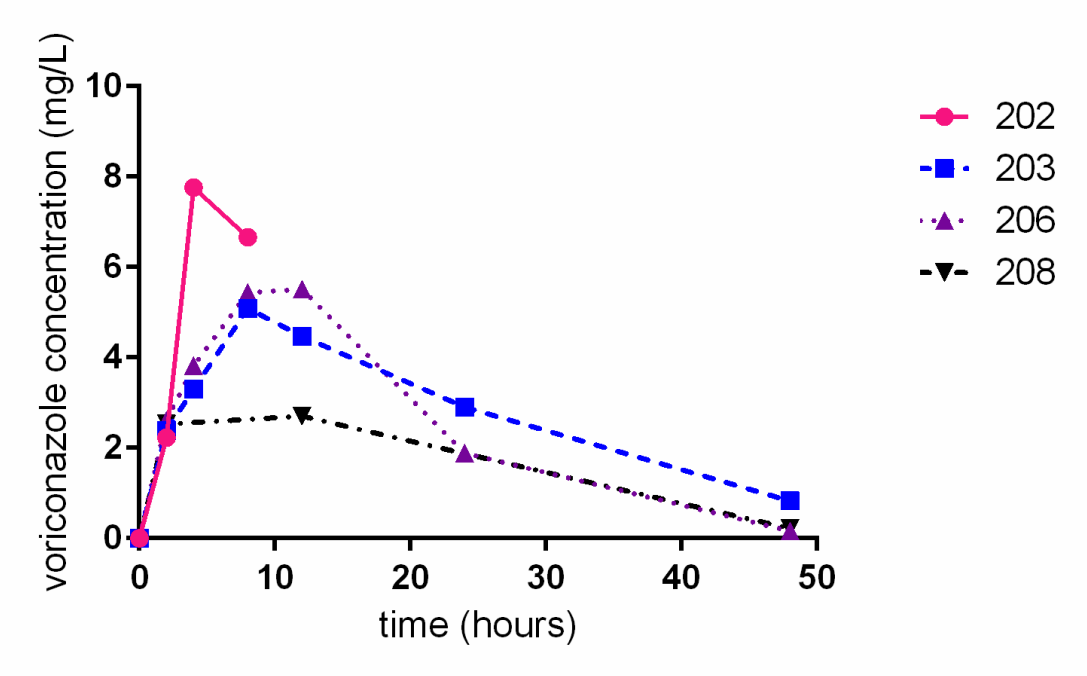
Plasma antifungal concentrations

Samples were run in duplicate to ensure accuracy, with the averages displayed below (Table 5.4 and Figure 5.2).

Table 5.4 Voriconazole plasma concentration (mg/L) at time from voriconazole administration (hours). NS = no sample. * = duplicate samples significantly different, data point not used in calculations.

Animal ID	Sex	2h	4h	8h	12h	24h	48h
202	F	2.230	7.765	6.665	NS	NS	NS
203	M	2.385	3.305	5.085	4.475	2.900	0.828
206	F	2.615	3.820	5.435	5.510	1.890	0.162
208	F	2.525	NS	*3.535	2.700	*3.245	0.222

Figure 5.2 Voriconazole plasma concentration-time profile in the tuatara after 5 mg/kg oral voriconazole dose.



Pharmacokinetic data and analysis

All four subjects were undergoing elimination by the last time point. Sampling for subject 202 was interrupted due to a physical injury sustained during sample collection, and no further data was available for this subject during this part of the study. As only two data points were in this subject's elimination curve, this precluded calculation of all pharmacokinetic parameters and indices for this subject.

The extrapolated percentage of AUC was <20 for subjects 203, 206 and 208, permitting the calculation of elimination half-life, apparent clearance and apparent volume of distribution (Table 5.5). Approximate time to maximal plasma concentration (Tmax), and approximate maximal plasma concentration (Cmax) were moderately variable. Tmax appears to occur 4-12 hours following oral administration of voriconazole. Cmax appears to be between 2.7 and 7.765 mg/L, however this lower value may be misleading, as

plasma concentrations for the associated tuatara (208) were not able to be determined at 4 and 8 hours, as sufficient blood could not be obtained for analysis.

The elimination half-life of voriconazole at 20^{o}C ranged from 7.03-14.55 hours, with a mean ± SD of 10.52 ± 3.1 hours. The apparent clearance ranged from 0.0334-0.0592 L/h/kg, with a mean ± SD of 0.045 ± 0.01 L/h/kg. The apparent volume of distribution ranged from 0.209-0.628 L/h, with a mean ± SD of 0.66 ± 0.18 L/h.

Table 5.5 Selected pharmacokinetic parameters and indices for voriconazole at 20°C. NC = not calculated.

Animal ID	AUC_{0-48} (mg/L*h)	$AUC_{0-\infty}$ (mg/L*h)	AUC % extrapolated	Elimination half-life (hours)	Apparent clearance (L/h/kg)	Apparent volume of distribution (L/kg)
202	NC	NC	NC	NC	NC	NC
203	133.0	150.3	11.6	14.55	0.0334	0.699
206	118.5	120.1	1.4	7.03	0.0415	0.421
208	81.2	84.4	3.8	9.99	0.0592	0.853
Mean ± SD	110.90 ± 21.8	118.27 ± 26.9	5.60 ± 4.4	10.52 ± 3.1	0.045 ± 0.01	0.66 ± 0.18

Pharmacokinetic modelling results

Using mixed effect modelling (see Section 2.11), it was possible to estimate the drug dosages required to reach the target concentration of 0.16 mg/L (Chapter 3) at 20°C for animals of different weights. It was also possible to estimate pharmacokinetic parameters and indices of voriconazole for different animal weights at 20°C (Table 5.6).

Table 5.6 Pharmacokinetic modelling results for attaining steady-state plasma concentrations of 0.16 mg/L of voriconazole at 20°C.

Weight (kg)	Volume (L)	Clearance (L/h/kg)	Dose rate (mg/kg/ day)
0.1	0.06	0.063	0.24
0.2	0.11	0.053	0.20
0.3	0.17	0.048	0.18
0.4	0.22	0.045	0.17
0.5	0.28	0.042	0.16
0.6	0.33	0.040	0.15
0.7	0.39	0.039	0.15
0.8	0.44	0.037	0.14
0.9	0.50	0.036	0.14
1	0.55	0.035	0.14

It can be seen that the dosages required to attain the target steady-state concentration are higher at 20°C than at 12°C, and are higher for animals of lower weights (Tables 5.3 and 5.6). It must be noted that, due to the lack of good elimination data at 12°C, the modelling findings for this temperature may not be as accurate as the 20°C conclusions.

5.3.1.3 Statistical comparisons

The only parameter suitable for statistical comparison between temperatures was AUC_{0-48}. Using the paired t-test the p-value was 0.662, indicating the data at 12°C and 20°C were not significantly different.

5.3.1.4 Combined pharmacokinetic modelling results

Other pharmacokinetic parameters and indices derived from the single dose studies are provided in Table 5.7. Although not practically applicable to tuatara, data for a 70kg animal is included in the below results as this is the standard employed in human medicine to allow comparisons between medications, as generally speaking, pharmacokinetic parameters and indices differ on an allometrically scalable basis (Huang and Riviere 2014).

Table 5.7 Estimates of modelling-derived voriconazole PK parameters and indices from single dose studies.

Statistics	Estimate
Vmax (mg/h/70kg)	2.21
Km (mg/L)	0.01
Volume of distribution (L/70kg)	64
Absorption half-life (hours)	4.22
Fold decrease in CL relative to 20°C	2.93

5.3.2 Results for multiple dose studies

5.3.2.1 Multiple dose studies conducted at 12°C ambient temperature

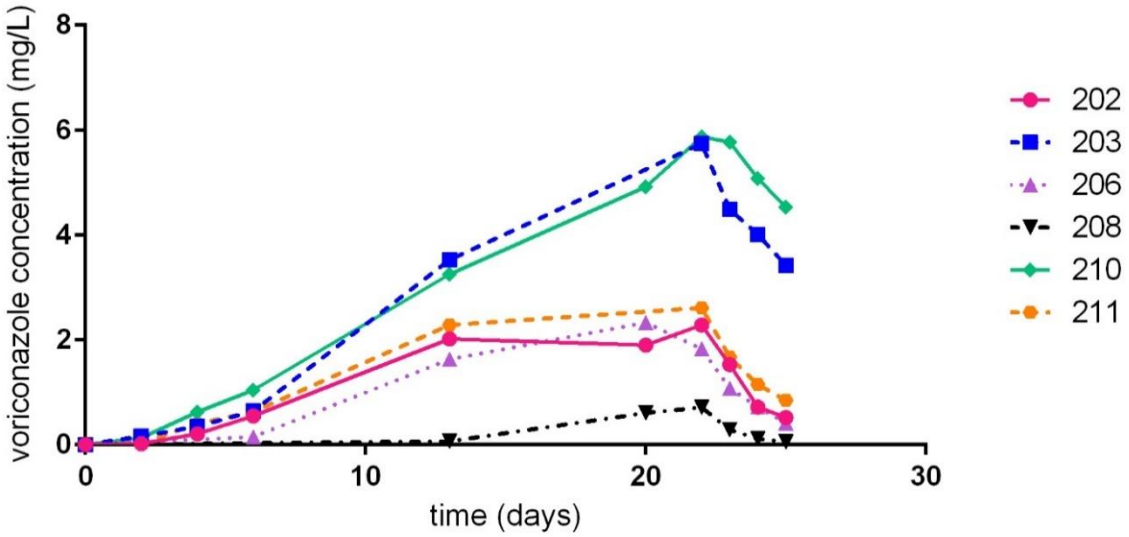
Plasma antifungal concentrations

Voriconazole was administered to six tuatara at a dose rate of 1 mg/kg PO once daily until the last dose on day 21. Blood samples were obtained as described in Section 5.3.1.3. Samples were analysed using LCMS/MS, and results are displayed below (Table 5.8 and Figure 5.3).

Table 5.8 Voriconazole plasma concentration (mg/L) at time from voriconazole administration (days) at 12°C. NS = no sample. * = too low to quantitate.

		Days after first dose								
Animal ID	**Sex**	**2**	**4**	**6**	**13**	**20**	**22**	**23**	**24**	**25**
202	F	0.022	0.208	0.551	2.02	1.9	2.28	1.53	0.722	0.52
203	M	0.16	0.349	0.642	0.353	NS	5.74	4.49	4.01	3.42
206	F	*	NS	0.147	1.63	2.33	1.83	1.08	0.725	0.416
208	F	*	*	*	0.0695	0.607	0.713	0.287	0.113	0.0554
210	M	0.135	0.626	1.04	3.25	4.92	5.87	5.77	5.08	4.53
211	F	0.0312	0.401	0.631	2.28	NS	2.61	1.67	1.15	0.85

Figure 5.3 Vorconazole plasma concentration (mg/L) at time from voriconazole administration (days) at 12°C.



Pharmacokinetic data and analysis

The only index that could be calculated using traditional pharmacokinetic analysis was elimination half-life. The mean ± SD half-life of voriconazole was 2.55 ± 1.78 hours.

Table 5.9 $t^{1/2}$ of voriconazole at 12°C.

Animal ID	**Voriconazole $t_{1/2}$ (hours)**
202	1.34
203	4.16
206	1.43
208	0.81
210	5.73
211	1.85
Mean ± SD	2.55 ± 1.78

Pharmacokinetic modelling results

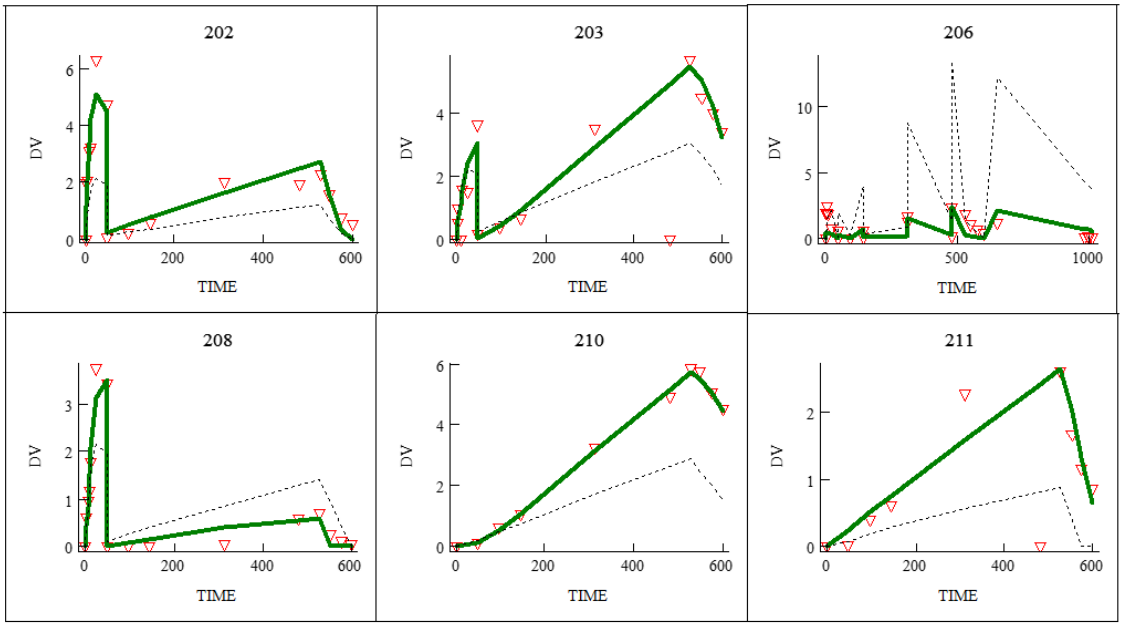
The modelling data below (Table 5.10) describes select pharmacokinetic parameters and indices, and the mg/kg per day dosage of voriconazole required to achieve a plasma target concentration of 0.16 mg/L for tuatara weighing between 0.1-1.0 kg maintained at 12^{o}C. Dose rates range from 0.70-1.25 mg/kg. As in the itraconazole study (Chapter 4), data for a 70kg animal is included to allow standard comparisons between tuatara, humans and other species.

Table 5.10 Model predictions of pharmacokinetic parameters and voriconazole dose rates required to attain steady-state target concentration of 0.16 mg/L at 12^{o}C.

Weight (kg)	Volume (L)	CL/kg (L/h/kg)	Dose rate (mg/kg/ day)
0.1	0.002	0.3262	1.25
0.2	0.005	0.2743	1.05
0.3	0.007	0.2479	0.95
0.4	0.009	0.2307	0.89
0.5	0.011	0.2182	0.84
0.6	0.014	0.2084	0.80
0.7	0.016	0.2006	0.77
0.8	0.018	0.1940	0.74
0.9	0.020	0.1884	0.72
1	0.023	0.1835	0.70
70	1.580	0.0634	0.24

Modelling based on both the single and multiple dose data illustrate the predicted and observed concentrations of voriconazole at the administered dose rate of 1 mg/kg for each animal (Figure 5.4).

Figure 5.4 Predicted and observed voriconazole concentrations at 12°C following dosing at 1 mg/kg SID. Green line: individual prediction. Dashed line: population prediction. Red symbols: observed concentrations.



Health screening results

Health screening was conducted on days 13 and 22 during the study, as described in Section 2.3.2. No significant changes in haematology or biochemistry were detected in any animal.

5.3.2.2 Multiple dose studies conducted at 20°C ambient temperature

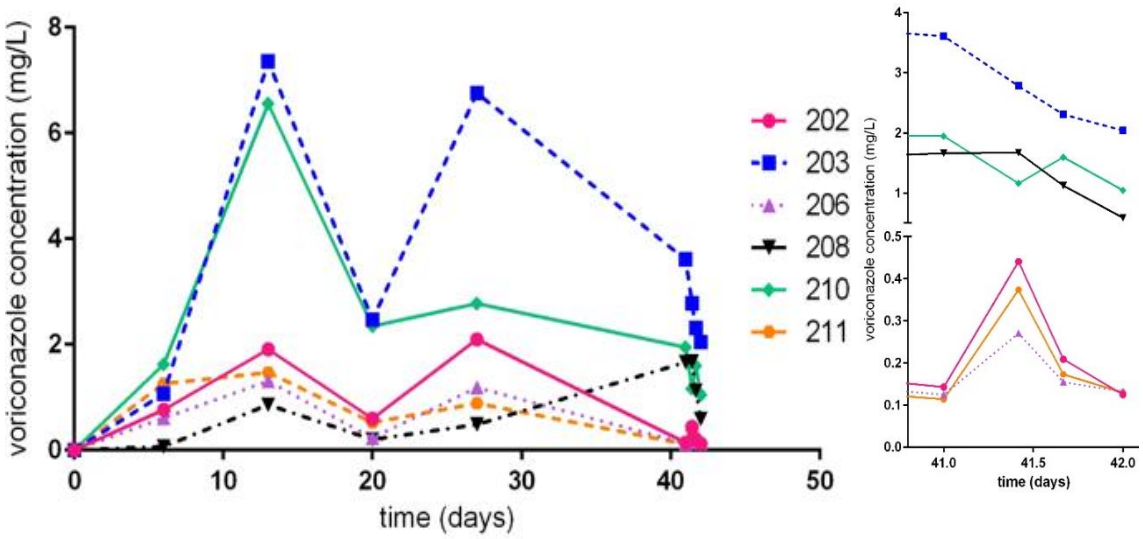
Plasma antifungal concentrations

Voriconazole was administered to six tuatara at a dose rate of 2 mg/kg PO once daily until the last dose on day 21. Blood samples were obtained as described in Section 5.3.1.3. Samples were analysed using LCMS/MS, and results are displayed below (Table 5.11 and Figure 5.5). An unexpected decrease in voriconazole concentrations was observed on day 20.

Table 5.11 Voriconazole plasma concentration (mg/L) at time from voriconazole administration (days).

		Days after first dose							
Animal ID	**Sex**	**6**	**13**	**20**	**27**	**41**	**41.42**	**41.67**	**42**
202	F	0.765	1.907	0.595	2.098	0.143	0.441	0.209	0.125
203	M	1.071	7.356	2.467	6.758	3.611	2.783	2.309	2.04
206	F	0.607	1.31	0.23	1.186	0.125	0.271	0.155	0.132
208	F	0.068	0.857	0.204	0.486	1.662	1.674	1.124	0.591
210	M	1.623	6.557	2.349	2.774	1.948	1.162	1.598	1.047
211	F	1.257	1.472	0.524	0.884	0.114	0.374	0.173	0.13

Figure 5.5 Voriconazole plasma concentration (mg/L) at time from voriconazole administration (days). Inset on right shows close-up of elimination.



Pharmacokinetic data and analysis

The only index that could be calculated using traditional pharmacokinetic analysis was elimination half-life. The mean half-life of voriconazole was 0.60 ± 0.36 hours (Table 5.12).

Table 5.12 $t_{1/2}$ of voriconazole at 20°C.

Animal ID	Voriconazole $t_{1/2}$ (hours)
202	0.33
203	1.31
206	0.58
208	0.39
210	NC
211	0.39
Mean ± SD	0.60 ± 0.36

Pharmacokinetic modelling results

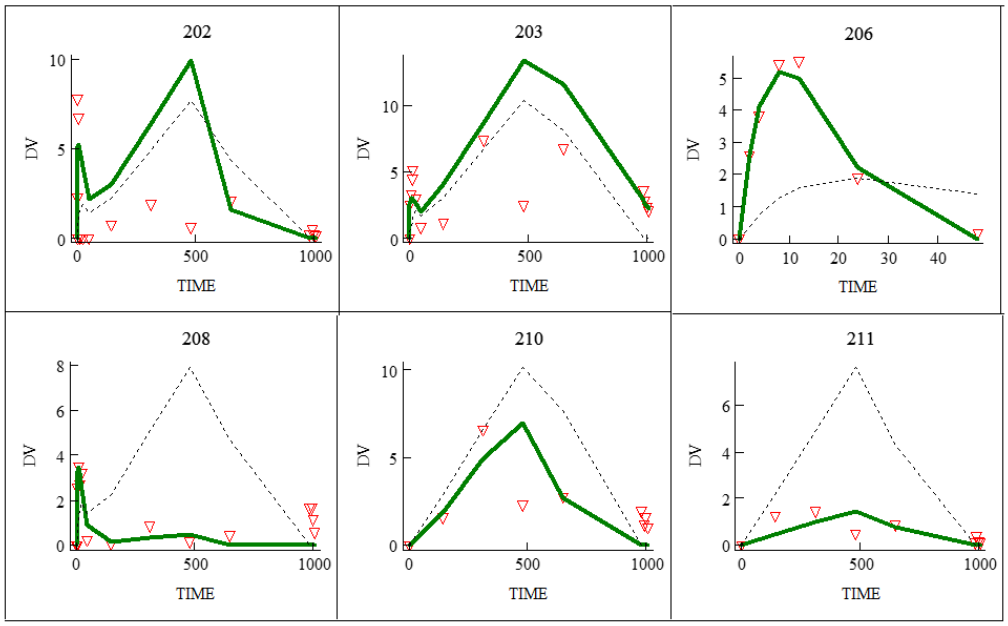
The modelling data below (Table 5.13) describe select pharmacokinetic parameters and the mg/kg per day dosagee of voriconazole required to achieve a plasma target concentration of 0.16 mg/L for tuatara weighing between 0.1-1.0 kg maintained at 20°C. Dose rates range from 1.49-2.64 mg/kg.

Table 5.13 Model predictions of pharmacokinetic parameters and itraconazole dose rates required to attain steady-state concentration of 0.16 mg/L at 20°C.

Weight kg	Volume (L)	Clearance (L/h/kg)	Dose rate (mg/kg/day)
0.1	0.002	0.688	2.64
0.2	0.005	0.579	2.22
0.3	0.007	0.523	2.01
0.4	0.009	0.487	1.87
0.5	0.011	0.460	1.77
0.6	0.014	0.440	1.69
0.7	0.016	0.423	1.63
0.8	0.018	0.409	1.57
0.9	0.020	0.397	1.53
1	0.023	0.387	1.49
70	1.580	0.134	0.51

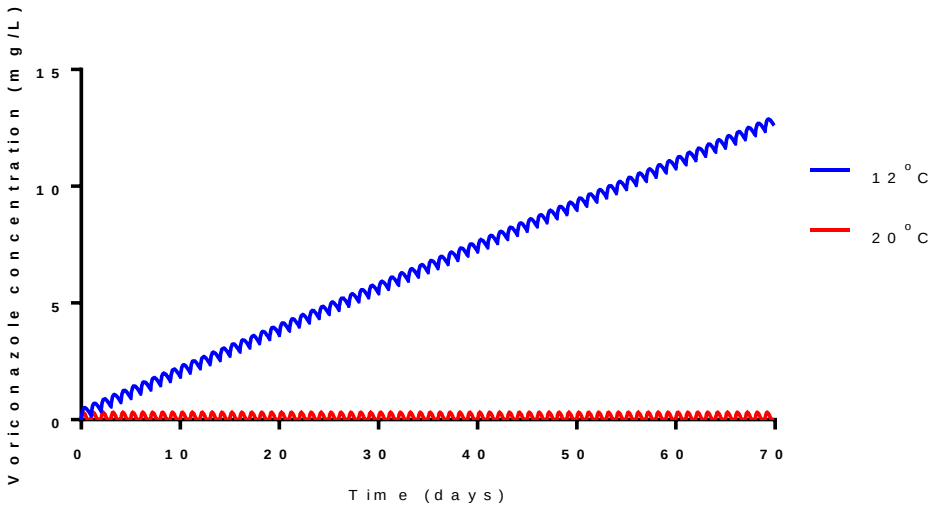
Modelling based on both the single and multiple dose data illustrate the predicted and observed concentrations of voriconazole at the administered dose rate of 2 mg/kg PO SID for each animal (Figure 5.6).

Figure 5.6 Predicted and observed voriconazole concentrations at 20°C following dosing at 2 mg/kg SID. Green line: individual predition. Dashed line: population prediction. Red symbols: observed concentrations.



Pharmacokinetic modelling simulations were run to illustrate the difference between predicted voriconazole concentrations at both temperatures. An example is provided below, where a 0.75kg tuatara is administered voriconazole at 1 mg/kg PO SID (Figure 5.7).

Figure 5.7 Time vs concentration profile of a 0.75 kg tuatara administered 1 mg/kg voriconazole SID at 12°C and 20°C.



Health screening results

Health screening was conducted on days 13, 27 and 41 during the study, as described in Section 2.3.2. No significant changes in haematology or biochemistry were detected in any animal. No significant weight changes were noted in any animal.

5.3.2.3 Statistical comparisons

Elimination half-life was suitable for statistical comparison between temperatures. Following log transformation, using the paired t-test the p-value was 0.0017, indicating the $t_{1/2}$ at 12°C and 20 °C were significantly different ($p<0.05$).

5.3.2.4 Combined pharmacokinetic results of single and multiple dose studies at both temperatures

Bootstrap estimates of other model parameters and indices and confidence intervals were able to be determined, and these are illustrated in the table below (Table 5.14).

Table 5.14 Select population pharmacokinetic parameters and indices for tuatara receiving voriconazole. RUV = residual unexplained variability. RSE = relative standard error expressed as a percentage.

Statistics	Mean	95% CI	RSE %
Vmax (mg/h/70kg)	1.619	1.490-2.091	9
Km (mg/L)	0.030	0.001-0.426	361
Volume of distribution (L/70kg)	77.911	61.675-95.850	12
Absorption half-life (hours)	3.20	1.290-6.618	56
Fold decrease in clearance relative to 20°C	2.133	2.050-2.240	3
Bioavailability decrease factor day 17 20°C	0.737	0.659-0.900	7
RUV proportional	0.268	0.197-0.318	12
RUV additive (mg/L)	0.502	0.275-0.609	20

Vmax is the maximum metabolic rate of voriconazole in tuatara, and depends on the mass of the hepatic metabolic enzymes, which become saturated. Km is the voriconazole plasma concentration at 50% of Vmax. The unexpected decrease in voriconazole concentrations observed on day 20 was accounted for in the modelling by a decrease in bioavailability from day 17 (see Sections 5.3.4.1 and 5.3.5.2). Residual unexplained variability (RUV) is a measure of random error, which is always present in modelling and is unpredictable. Quantifying RUV gives a measure of how reliable a model is, and using the correct structural model decreases RUV.

5.4 Discussion

5.4.1 Pharmacokinetics and temperature

Overall, there were distinct trends in voriconazole plasma concentration at the two different temperatures. Subject 206 had similar voriconazole plasma concentration curves at 12^{o}C and 20^{o}C in the single dose studies. It is unknown why this is the case, as the subject's temperature was measured at the start of each study, and reflected the ambient temperature. As subject 206's curve at 20^{o}C is similar to that of other subjects at 20^{o}C, it is likely that the 12^{o}C data is the anomalous data set. The animal's metabolic rate may have been elevated for an unknown reason, such as increased activity level. With such a small sample size, it cannot be certain that subject 206's 12^{o}C data is a true outlier, so it was included in pharmacokinetic calculations and modelling.

Time to maximal plasma concentration

Single dose studies were both conducted using voriconazole dosages of 5 mg/kg, allowing direct comparison of the effect of temperature on pharmacokinetic parameters

and indices. At 12°C time to maximal plasma voriconazole concentration was highly variable, ranging from 4-24 hours in three animals, with the fourth animal's apparent peak at 48 hours. There was limited sampling after 12 hours, so the peak in the fourth animal's voriconazole concentration may have occurred any time from 24 hours after dosing. At 20°C Tmax was between 4-8 hours for all animals. It was not appropriate to make statistical comparisons for Tmax given the limited sample size and the uncertainty of apparent Tmax at 12°C in one subject, however it is apparent there is a marked difference in Tmax at the two temperatures at a 5 mg/kg dosage. The Tmax of voriconazole in humans is less than two hours (Theuretzbacher et al. 2006), and in various avian species ranged between 1-4 hours (Flammer et al. 2008, Beernaert et al. 2009, Sanchez-Migallon Guzman et al. 2010). In mammals voriconazole Tmax varies significantly, from one hour in female rats to up to 12.9 hours in horses, with mice, dogs, guinea pigs, rabbits and male rats in between (Roffey et al. 2003, Colitz et al. 2007). These studies administered voriconazole at dose rates ranging from 6-30 mg/kg, and the study in Amazon parrots showed a significantly different Tmax when different dosages were used (two hours and four hours at 6 mg/kg and 12 mg/kg respectively), making direct interspecies comparisons using different dosages inappropriate.

Absorption half-life

The absorption half-life was estimated at 3.20 hours based on the modelling. This is a long half-life, and indicates that 50% of the administered oral dose is absorbed in 3.2 hours. The RSE for absorption half-life was 56%, so this value may not be a reliable estimate. Absorption is considered to be a largely passive process, and not markedly influenced by temperature, so the estimate is the same for both 12°C and 20°C.

Elimination half-life

In the single dose studies, the elimination half-life of voriconazole was not able to be calculated at 12^{o}C. At 20^{o}C, elimination half-life had a mean ± SD of 10.52 ± 3.1 hours. In the multiple dose studies voriconazole had a mean ± SD elimination half-life of 2.55 ± 1.78 hours at 12^{o}C with a dose rate of 1 mg/kg, and a mean ± SD half-life of 0.60 ± 0.36 hours at 20^{o}C with a dose rate of 2 mg/kg. As voriconazole displays mixed-order kinetics (saturable elimination) it is not appropriate to directly statistically compare elimination half lives at different dosages, however modelling and simulations confirm that half-life is shorter at 20^{o}C than 12^{o}C, when the same dose is administered. This is likely because although the Vmax remains the same at both temperatures (it reflects a metabolic enzyme mass), the Km (which reflects the affinity of voriconazole for the enzyme) is reduced at lower temperatures, resulting in slower clearance of the drug.

The marked difference in elimination half-life in single and multiple dose studies is a function of the different dosages used in both studies, and illustrates voriconazole's non-linear pharmacokinetics. The observed short half-life in multiple dose studies results in significant fluctuations in drug concentration throughout the day, though this is considered acceptable as long as trough concentrations remain above the target and there are no adverse effects from the transiently high maximal plasma concentrations. Although half-lives were able to be determined using compartmental PK methods, it should be noted that it is not really possible to accurately describe the elimination half-life of a drug like voriconazole where saturable metabolism occurs.

In humans, voriconazole half-life is approximately 6 hours but is dose-dependent (Theuretzbacher et al. 2006), while in birds it ranges from 1.1-10 hours depending on dose and species (Burhenne et al. 2008, Flammer et al. 2008, Beernaert et al. 2009).

The two male tuatara in the study had markedly higher voriconazole plasma concentrations than the females, but the males weighed significantly more than the females, so it is likely this difference was related to weight rather than sex. Allometrically scaled dosage recommendations provided by the modelling would avoid this disparity occurring in the future. True sex differences in voriconazole metabolism cannot be ruled out without further studies.

Clearance

Modelling predicted clearance at 12^{o}C of 0.184L/h, and at 20^{o}C of 0.387L/h for a 1kg animal with a target voriconazole concentration of 0.16 mg/L. There was a 2.13-fold decrease in clearance at 12^{o}C relative to 20^{o}C as estimated by modelling, further emphasising the difference in pharmacokinetics between these two temperatures. Given the level of complexity of the models and the assumptions already being made, it was not possible to determine whether auto-induction of voriconazole metabolism occurred in tuatara, as has been reported in several other species, but not humans (Roffey et al. 2003, Beernaert et al. 2009).

Maximal voriconazole metabolism (Vmax) and Michaelis-Menten constant (Km)

Modelling using the combined single and multiple dose data estimated the maximum metabolic rate of voriconazole as 1.619 mg/h/70kg. In humans Vmax has been estimated at 37.67 mg/h (Hope 2012) and at 43.9 mg/h (Dolton et al. 2014). The maximal metabolic rate of voriconazole is significantly lower in tuatara than in humans, likely as

a function of the tuatara's low physiologic metabolic rate. Similarly, the Km (voriconazole concentration at 50% of Vmax) in tuatara was estimated at 0.030 mg/L; in humans it has been estimated as 2.07 mg/L (Hope 2012) and 3.33 mg/L (Dolton et al. 2014). It must be noted that the RSE for the Km was 361%, meaning the estimates provided by modelling are likely to be unreliable.

Volume of distribution

Modelling estimated the volume of distribution as 64 L/70 kg, and as 0.55L in a 1kg animal. Voriconazole's volume of distribution in humans is 2.0-4.6 L/kg, and in birds ranges from 1.05-3.5 L/kg, varying with species and dose (Burhenne et al. 2008, Beernaert et al. 2009, Flammer et al. 2008, Theuretzbacher et al. 2006). Volume of distribution was lower in tuatara than in other animals, indicating that voriconazole may not distribute as extensively into tissues in tuatara as in other animals.

Modelling and simulation to estimate voriconazole dose regimens in tuatara

The observed voriconazole concentrations matched variably with those predicted by modelling. In some animals concentrations matched very well, in others there was limited association. This is likely a function of the high inter-individual variability observed in voriconazole concentrations and pharmacokinetics, a phenomenon well-recognised in humans (Beernaert et al. 2009). In humans, therapeutic drug monitoring is commonly used as a tool to guide dosage adjustments, however this is rarely practical in veterinary medicine. High variability in humans is attributed to differences in the amount of the hepatic enzyme CYP2C19, which is involved in voriconazole metabolism. It is possible that the tuatara in this study also had widely different amounts of CYP2C19,

however no studies have been conducted on tuatara to determine the composition or amounts of their hepatic enzymes.

5.4.2 Discrepancy in observed and expected voriconazole concentrations on day 20 of multiple dose study

On day 20 of the 20°C multiple dose study there was an unexpected decrease in voriconazole concentrations in all animals, compared to those on the sampling points on either side on days 13 and 27. All samples for days 13, 20 and 27 were re-tested, and the drop in concentration on day 20 was shown to be a true decrease and not a measuring error. There were several potential causes for this: one or more missed medication doses, a temporarily increased metabolic rate likely caused by an increase in ambient temperature, or an error in medication reconstitution resulting in a different voriconazole concentration to the desired 4 mg/mL. A change in ambient temperature was ruled out based on datalogger records. We consider the most likely cause to be one or more missed medication doses, as during the experimental period from days 13-21 there was increased unplanned sampling of itraconazole animals, and it is possible that during this extra activity medication doses were overlooked in the voriconazole animals. The decrease in plasma voriconazole concentrations was modelled by assuming a decrease in bioavailability from day 17, and this allowed satisfactory modelling predictions to be made with the available data.

5.4.3 Recommended treatment protocol

Despite the inter-individual variability in voriconazole concentrations and pharmacokinetics observed in our study, all animals exceeded the target voriconazole

concentration at 20°C and 2 mg/kg SID by day 13, and no animal experienced observable adverse effects despite two animals reaching concentrations above 6.5 mg/L. The modelling predictions for the dose rate required to attain a steady-state concentration (Css) of 0.16 mg/L range from 1.49-2.64 mg/kg SID. At these dosages and with voriconazole's short half-life, modelling predicts that some animals would likely spend significant time with trough concentrations below the therapeutic target. It is also worth noting that many fungal pathogens have voriconazole MICs higher than those found in this study for *P. australasiensis*, including other CANV complex fungi (Van Waeyenberghe et al. 2010), and our recommended dosages may be too low to treat these other pathogens. Modelling simulations show that increasing the voriconazole dosage to maintain trough concentrations above 0.16 mg/L may result in very high voriconazole concentrations in some animals as metabolic enzyme saturation and drug accumulation occurs. During our study no adverse effects were observed at dosages higher than those modelled in the aforementioned simulations, so using higher dosages may be acceptable when combined with careful health monitoring. In humans, trough voriconazole concentrations above 5.5 mg/L resulted in encephalopathy in 31% of patients, and above 8 mg/L had an estimated 90% probability of resulting in neurotoxicity (Pascual et al. 2008). Various studies in humans have recommended trough concentrations remain below 4.0-5.5 mg/L to reduce the likelihood or neurotoxicity (Dolton et al. 2014). In racing pigeons, clinically significant hepatotoxicity was detected in animals with trough voriconazole plasma concentrations of 5.85 (± 3.12) mg/L (Beernaert et al. 2009), and in various species of penguins neurological signs of toxicity were noted in multiple animals with trough concentrations above 5.5 mg/L (Hyatt et al. 2015).

The medication dose rates required to maintain Css of 0.16 mg/L at 20°C for tuatara between 0.1-1.0 kg are shown below (Table 5.15). Given the high inter-individual variability observed, it is possible these dosages may result in subtherapeutic voriconazole concentrations in some animals. In clinical cases this may not be a significant problem as medication dosages can be increased as required, but it would be inappropriate to use these modelling-derived dose rates in clinically normal animals as part of a quarantine treatment protocol. This may be overcome by using higher dosages than those recommended by modelling, however as a small increase in dose will result in a disproportionately large increase in plasma voriconazole concentration due to the non-linear kinetics, close monitoring for adverse effects must be undertaken. It is worth remembering there were no adverse effects detected in either of our studies, despite trough plasma voriconazole concentrations reaching up to 7.4 mg/L.

Table 5.15 Modelling recommended daily voriconazole dosage for tuatara weighing between 0.1-1.0 kg maintained at 20°C.

Weight (kg)	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
Dosage (mg/kg)	2.64	2.22	2.01	1.97	1.77	1.69	1.63	1.57	1.53	1.49

5.5 Conclusions

These studies show that, as with itraconazole, there is significant variation in pharmacokinetics with temperature, and that tuatara should be treated at 20°C, at the high end of their POTZ. Voriconazole has non-linear pharmacokinetics in tuatara, as in many other species. While there were no adverse effects associated with voriconazole

treatment in our study, caution and careful monitoring are warranted if dosages higher than those recommended by modelling are used, as small dose increases can result in large increases in plasma voriconazole concentrations. As in humans, high inter-individual variation in plasma voriconazole concentrations were noted, and this may result in treatment failure or adverse effects in some individuals even when recommended dosages are used. The proposed treatment regimes based on MIC and PK data have the potential to improve clinical outcomes and reduce the likelihood of toxicity.

Chapter 6: Development of haematological and biochemical reference intervals

6.1 Introduction

Reference ranges for haematological and biochemical parameters provide a set of 'normal' blood values with which to evaluate the health of individuals of the same species. Evaluation of reptile haemograms and biochemistry can be problematic, as values can change with season, age, sex, environment and nutritional status (Mader 1999, Campbell 2014). It has been suggested that serial blood values from the same animal, or values from a healthy conspecific held under the same conditions, may have the most use in evaluating blood values in reptiles (Mader 1999, Hernandez-Divers 2006). However in the absence of these, as is often the case in wildlife, reference ranges assist greatly in interpreting values from individual animals.

Tuatara are an iconic endemic New Zealand reptile, but there are only limited haematological and biochemical data available for this species (Boardman and Blanchard 2006, Gartrell et al. 2006, Species360 2013). The Species360 (2013) data provides ranges only for white blood cell numbers, calcium and phosphorous, with wide reference intervals and no information on how the values were derived. These data are compiled from member institutions, and information on sample handling, testing methodology, age and signalment of participant animals is not consistently available, limiting the use of these reference ranges. The data from Boardman and Blanchard (2006) has similar limitations, though testing was performed by a single laboratory and more biochemical parameters were included, and the data from Gartrell et al. (2006) is limited to haematologic parameters from estimated white cell counts.

This chapter provides haematology and plasma biochemistry values for a small population of captive tuatara, collected as part of a wider study on the pharmacokinetics

of antifungal agents reported in this thesis. It also evaluates the data and describes any significant variations in haematologic and biochemistry values based on the variables of sex, gravidity status and season.

6.2 Methods

6.2.1 Study population

The study population contained twelve adult tuatara comprising eight females and four males. Animals ranged in age from 21 years to unknown-aged adults. Several females were gravid at various times throughout the study, as determined by the presence of eggs on radiographic examination, or if eggs were laid in the nine months following health screening. Physical examination and x-ray were carried out as described in Section 2.3.2. All animals were in good health and no pharmacologically active agents had been administered in the two months prior to blood sampling.

Tuatara were housed in naturalistic outdoor enclosures, with no supplementary heating or UV lighting. Blood samples were collected in May-June of 2014 and 2015 (reported as 'winter' samples), and December 2014 (reported as 'summer' samples). Ambient temperatures in Auckland in May-June in both years had a mean of 12.9^{o}C (range -2.3-23.7 ^{o}C), and in December 2014 a mean of 18.4 ^{o}C (range 6.4-26.9 ^{o}C) (National Institute of Water and Atmospheric Research 2016). Several samples were also collected in September 2015, and these were excluded from seasonal analysis.

6.2.2 Blood sampling, processing and analysis

Blood sampling and haematology and biochemistry measurements were performed as described in Chapter 2.

White cell counts and morphology

For some samples, insufficient blood was available for a white cell count using the haemocytometer, and an estimated count from a smear was performed. During the pharmacokinetic studies it was noted that counts from smears were consistently higher than those using the chamber method. Consequently, both haemocytometer and estimated white cell counts were performed on a subset of animals. These samples were from animals receiving medication, so the data is not included in the reference ranges, but was used to illustrate and attempt to quantify the difference in white cell count between the haemocytometer and estimated white blood cell count methods. Haemocytometer counts and estimated counts were performed by the same technician, with the smears submitted from the same sample but under a different ID, so the technician was unaware of the haemocytometer method results.

Photomicrographs of white blood cells were taken using an Olympus BX41 microscope and Olympus DP27 camera with DP2-SAL controller (Olympus, Australia). Dimensions of cells were recorded using the measurement tool in the DP2-SAL controller.

Statistical analysis

The dataset was initially inspected visually for outliers, resulting in the removal of one sample from the data for analysis. All analyses were performed in GraphPad Prism version 6.07 (GraphPad Software, USA). The Shapiro-Wilk test was used to check the

datasets for normality, with tests for significance using the two-tailed t test for normally distributed data, or the Mann Whitney test for non-parametric data. Results were grouped according to sex: male (n=12 for haematology, n=13 for biochemistry) and female (n=21 for haematology, n=22 for biochemistry) for analysis. Results from female tuatara were further grouped according to reproductive status: gravid (n=6 for haematology, n=5 for biochemistry) and non-gravid (n=15 for haematology, n=17 for biochemistry). An animal was considered gravid at the time of blood testing if she laid eggs in the following 9 months. Results were also grouped according to the season in which they were obtained: winter (n=20 for haematology, n=21 for biochemistry) and summer (n=12 for haematology and biochemistry). Means, medians, standard deviations and 10^{th}-90^{th} percentiles were calculated for all groups using GraphPad Prism. Statistical significance was assessed at a level of $p<0.05$.

6.3 Results

All animals underwent health screening on multiple occasions. In all tables n = the number of individual tuatara that were tested for each parameter, and the number that follows in parentheses is the number of samples analysed in total for that parameter.

6.3.1 Haematology

General

A total of 31 individual samples from 12 tuatara were available for analysis. Not all parameters were available for all animals, due to limitations in blood sample volume. Table 6.1 displays the results for all samples. Statistically significant differences in

parameters based on sex or temperature are discussed further below. There were no significant differences in any haematologic parameters based on reproductive status.

Table 6.1 Haematology values for captive tuatara. PCV = packed cell volume, Hb = haemoglobin, MCHC = mean corpuscular haemoglobin concentration. * Some data were outside the range of quantitation, see text for details. † Statistically significant difference based on sex, season or reproductive status, see text for details.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} percentile)	n (samples)
PCV †	%	34.4 (34.0)	6.9	25-52 (26.0-47.4)	12 (25)
Hb	g/L	73.9 (71.5)	12.0	58-102 (60.8-92.9)	12 (28)
MCHC	g/L	220.5 (229.0)	40.4	133-285 (165.4-281.0)	12 (25)
Fibrinogen	g/L	*	*	<0.1-1.9 (*)	12 (24)
Total white cell count †	x 10^9/L	1.68 (1.5)	0.82	0.4-3.5 (0.7-3.1)	12 (27)
Heterophil †	x 10^9/L	0.53 (0.5)	0.35	0.1-1.3 (0.10-1.12)	12 (27)
	%	32.5 (32.0)	14.4	8-63 (15.2-53.4)	12 (31)
Lymphocyte	x 10^9/L	0.56 (0.6)	0.35	0.0-1.6 (0.18-1.00)	12 (27)
	%	32.1 (30.0)	14.6	5-61 (11.2-53.8)	12 (31)
Monocyte	x 10^9/L	0.32 (0.3)	0.23	0.0-1.0 (0.08-0.74)	12 (27)
	%	20.0 (19.0)	10.4	1-47 (5.4-35.4)	12 (31)
Eosinophil	x 10^9/L	0.19 (0.1)	0.19	0.0-0.7 (0.0-0.52)	12 (27)
	%	10.4 (9.0)	7.9	1-35 (1.20-21.80)	12 (31)
Basophil	x 10^9/L	0.08 (0.0)	0.11	0.0-0.4 (0.0-0.3)	12 (27)
	%	4.2 (3.0)	4.4	0-23 (0.0-9.80)	12 (31)

Red blood cells

The packed cell volume ranged from 25-52%, with a mean of 34.4% (Table 6.1). Erythrocytes were ellipsoidal cells with a homogenous pink-red cytoplasm. Occasional small, pink-staining cytoplasmic inclusions were noted; these did not resemble known haemoparasites and were presumed to be artefacts related to slide preparation. No intraerythrocytic parasites or mitotic figures were observed. Mild anisocytosis and polychromasia were occasional findings, and rare binucleated red blood cells were observed. Erythrocyte nucleoli were oval and frequently irregular, with dark blue staining chromatin. Erythrocytes were 23-28µm in length and 12-16µm wide. There was a statistically significant difference in PCV between sexes, with males having a higher PCV than females (p=0.0034) (Table 6.2). There was no significant difference in PCV between seasons.

Table 6.2 Differences in PCV based on sex in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
PCV (sexes combined)	%	34.4 (34.0)	6.9	25-52 (26.0-47.4)	12 (25)
PCV (males)	%	39.1 (36.5)	7.7	30-52 (30.2-51.9)	4 (10)
PCV (females)	%	31.3 (31.0)	4.3	25-39 (25.6-37.8)	8 (15)

Total white blood cells

Total white blood cell numbers ranged from 0.4-3.5 x 10^9/L, with a mean of 1.68 x 10^9/L (Table 6.3). White blood cell morphology and differential counts are discussed further below. There was a statistically significant difference in leukocyte numbers based on

season, with samples taken in winter having higher WBC counts than those in summer (p=0.0112). This is detailed in Table 6.3.

Further analysis revealed that the higher leukocyte counts in winter were attributable to female tuatara. There was a significant difference in white blood cell count in females between summer and winter (p=0.0112), but not in males (Table 6.4). There was no statistically significant difference in total white blood cell numbers attributable to reproductive status.

Table 6.3 Differences in total white blood cell count based on season in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
WBC (combined)	x 10^9/L	1.68 (1.50)	0.82	0.4-3.5 (0.7-3.1)	12 (27)
WBC (summer)	x 10^9/L	1.22 (1.10)	0.46	0.4-1.8 (0.46-1.80)	11 (11)
WBC (winter)	x 10^9/L	2.21 (1.75)	1.2	0.7-3.5 (0.98-3.29)	12 (16)

Table 6.4 Differences in total white blood cell count based on season in a population of captive female tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
WBC (female combined)	x 10^9/L	1.59 (1.40)	0.86	0.4-3.2 (0.64-3.12)	8 (17)
WBC (female summer)	x 10^9/L	1.00 (1.00)	0.38	0.4-1.5 (0.40-1.50)	7 (7)
WBC (female winter)	x 10^9/L	2.01 (2.05)	0.86	0.7-3.2 (0.74-3.19)	8 (10)

Heterophils

Heterophils and lymphocytes were the most common white blood cells observed, though there was considerable variation in the numbers of each cell type between

samples. Heterophils comprised 8-63% of WBCs (Table 6.1) and were large, round cells with pale purple to non-staining eccentrically placed, lobulated nuclei and pink, fusiform-shaped granules (Figure 6.1, A). Heterophils were the largest of the leukocytes seen, with diameters of 18-24µm. There was a statistically significant difference in heterophil numbers (p=0.0075) and differential percentage (p=0.0067) between females in winter and the remaining samples (females in summer + all males). This is detailed in Table 6.5.

Table 6.5 Differences in heterophil numbers based on sex and season in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
Heterophils (combined)	x 10^9/L	0.53 (0.5)	0.35	0.1-1.3 (0.10-1.12)	12 (27)
Heterophils (females in winter)	x 10^9/L	0.79 (0.85)	0.40	0.2-1.3 (0.2-1.29)	8 (10)
Heterophils (females in summer + all males)	x 10^9/L	0.37 (0.40)	0.20	0.1-0.6 (0.1-0.6)	12 (17)
Heterophils (combined)	%	32.5 (32)	14.4	8-63 (15.2-53.4)	12 (31)
Heterophils (females in winter)	%	40.2 (40.5)	10.8	16-54 (20.2-53.1)	8 (12)
Heterophils (females in summer + all males)	%	27.6 (25)	14.5	8-63 (10.0-59.0)	12 (19)

Lymphocytes

Lymphocytes comprised 5-61% of WBCs (Table 6.1). They were small, round cells with purple-staining, round nuclei which took up most of the cell. Scant blue-staining cytoplasm was present around the periphery of the nucleus (Figure 6.1, B). Lymphocytes were the smallest of the leukocytes seen, with diameters of approximately 7-9µm. There was no significant difference in lymphocyte numbers based on sex or season.

Monocytes

There was considerable variation in monocyte number between samples. Monocytes comprised 1-47% of WBCs, but were usually less numerous than heterophils and lymphocytes (Table 6.1). Monocytes were generally round, with a horseshoe-shaped nucleus and blue-staining cytoplasm (Figure 6.1, C). Monocytes were approximately 15-20 µm in diameter. There was no statistically significant difference in monocyte numbers based on sex or season.

Eosinophils

Eosinophils comprised 1-35% of WBCs, but were generally found in lower numbers than heterophils, lymphocytes and monocytes (Table 6.1). Eosinophils were round to oval, with a lobulated blue-staining nucleus, pale blue cytoplasm and round, pink cytoplasmic granules (Figure 6.1, D). Eosinophils were approximately 15-22µm in diameter. There was no significant difference in eosinophil numbers based on sex or season.

Basophils

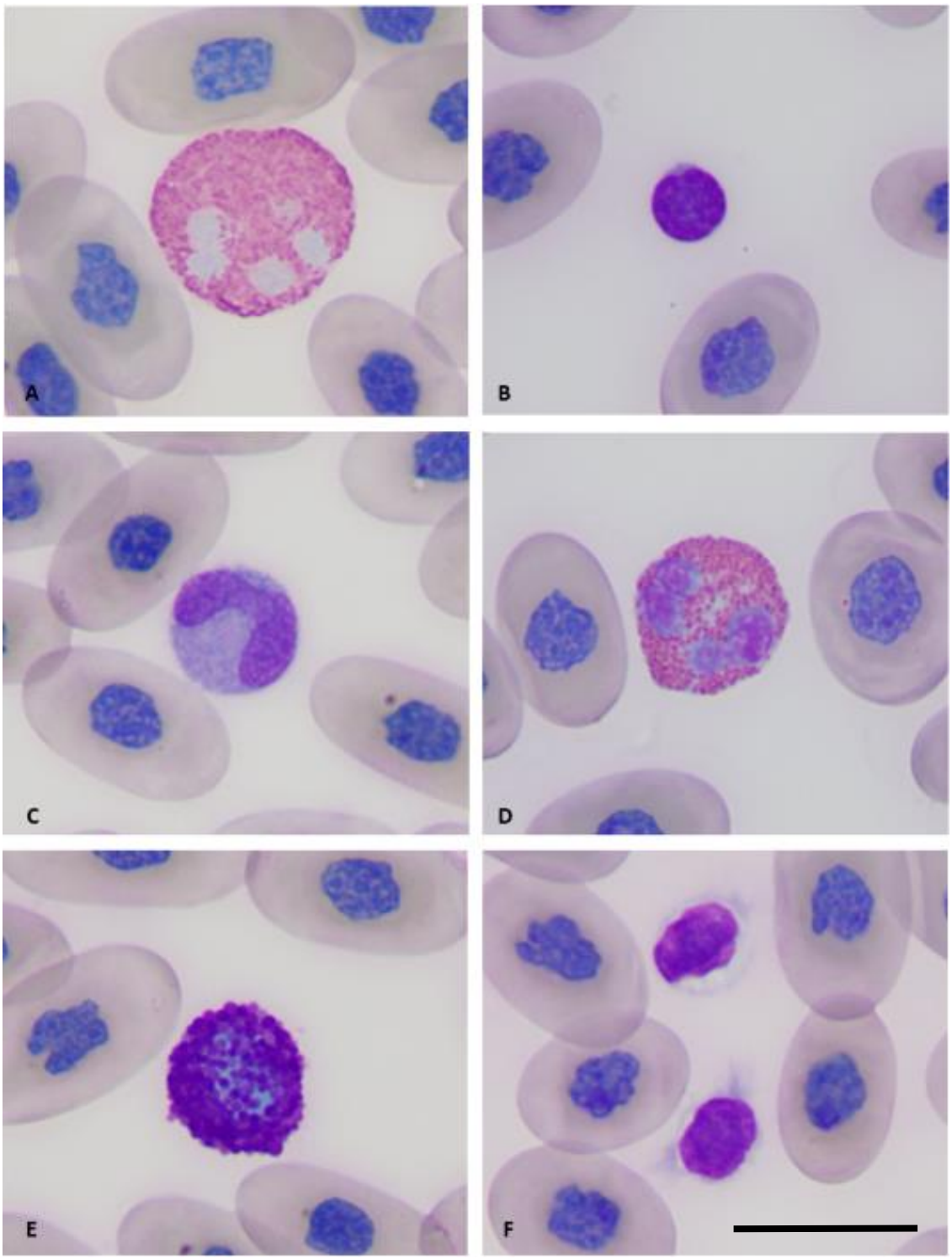
Basophils were the rarest of the white blood cells, comprising 0-23% of WBCs seen (Table 6.1). Basophils were round with prominent dark purple-staining cytoplasmic granules that obscured the nucleus (Figure 6.1, E), and were approximately 13-17µm in diameter. There were no statistically significant differences in basophil numbers based on sex or season.

Thrombocytes and fibrinogen

Thrombocytes were found both in clumps and singly. Thrombocytes resembled lymphocytes, and were round to oval, often with an irregular, sometimes indistinct outline (Figure 6.1, F). Their nuclei were purple-staining and often slightly irregular in

shape. Their cytoplasms were a much paler blue than that of the lymphocytes. Thrombocytes were generally slightly larger than lymphocytes, with a diameter of 9-12µm. There was no significant difference in fibrinogen concentrations based on sex or season.

Figure 6.1 Tuatara white blood cells and thrombocytes, Leishman's stain. A = heterophil, B = lymphocyte, C = monocyte, D = eosinophil, E = basophil, F = two thrombocytes. Bar = 20µm.



White cell count method

A subset of 28 samples had both chamber counts and estimated white cell counts performed on the same sample. These samples were taken from animals undergoing pharmacokinetic studies and are not, therefore, suitable for use in reference ranges, but can be used to illustrate the difference in white blood cell numbers between counting methods. Estimated white cell counts were significantly higher than chamber counts by factors of 2-7 ($p<0.0001$) (Table 6.6).

Table 6.6 Differences in white blood cell count based on counting method.

Count method	Unit	Mean (Median)	SD	Min-Max	n (samples)
Haemocytometer count	x 10^9/L	1.94 (1.80)	1.04	0.4-5.5	12 (28)
Estimated count	x 10^9/L	8.92 (8.80)	4.65	1.0-20.5	12 (28)

6.3.2 Biochemistry

A total of 33 individual samples from 12 tuatara were available for analysis, and results are summarised in Table 6.7. Using the Abaxis Vetscan the lower limit of quantitation for bile acids is 35 µmol/L, and as all but one result were reported as <35, no further data on mean, median or range is available for this parameter. Similarly, several total calcium readings were over the upper limit of quantitation of 4 mmol/L, and several potassium readings were below the lower limit of quantitation of 1.5 mmol/L, precluding calculation of summary statistics. All parameters were tested in all 33 samples, however some readings for bile acids and CK came back as zero. These were below the limit of quantitation as described by Abaxis, and these values are not included in the reference tables or calculations. Statistically significant differences in parameters based on sex, season and reproductive status are discussed further below.

Table 6.7 Biochemistry values for captive tuatara. * Some data were outside the range of quantitation, see text for details. † Statistically significant difference based on sex, season or reproductive status, see text for details.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
AST	IU/L	22.8 (15.7)	15.5	7-80 (9.0-46.4)	12 (33)
Bile acids	µmol/L	*	*	<35-41* (<35)*	12 (30)
CK	IU/L	1548 (1726)	1698	36-6519 (55-4639)	12 (28)
Uric acid †	µmol/L	102.5 (97.0)	44.4	42-232 (51.8-163.6)	12 (33)
Glucose †	mmol/L	5.02 (4.7)	1.10	3.3-7.6 (3.50-6.78)	12 (33)
Total calcium †	mmol/L	2.98 (2.86)*	0.63*	2.1-4.0*	12 (27)
Phosphorous †	mmol/L	1.76 (1.67)	0.59	0.57-3.49 (1.14-2.61)	12 (33)
Total protein	g/L	38.6 (36.0)	8.7	27-64 (28.4-51.2)	12 (33)
Albumin	g/L	20.8 (20.0)	4.2	14-30 (15.4-28.2)	12 (33)
Globulin †	g/L	17.7 (17.0)	5.9	10-37 (11.0-24.0)	12 (33)
Potassium	mmol/L	3.19 (3.4)*	1.10*	<1.5-6.1*	12 (26)
Sodium	mmol/L	133.6 (133.0)	4.2	126-143 (128.0-139.8)	12 (33)

There were no statistically significant differences in AST, bile acids, creatine kinase, total protein, albumin, potassium or sodium concentrations based on sex or season. Glucose concentrations differed between season, while uric acid, total calcium, phosphorous and globulins differed based on sex (Tables 6.8-6.12).

Glucose

Glucose was the only parameter to differ significantly between season, with concentrations being higher in samples taken in summer ($p<0.0001$) (Table 6.8).

Table 6.8 Differences in glucose concentration based on season in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
Glucose (combined)	mmol/L	5.02 (4.7)	1.10	3.3-7.6 (3.50-6.78)	12 (33)
Glucose (winter)	mmol/L	4.47 (4.50)	0.73	3.3-5.6 (3.50-5.48)	12 (12)
Glucose (summer)	mmol/L	5.97 (6.10)	1.04	4.3-7.6 (4.42-7.42)	12 (21)

Uric acid

Uric acid concentrations were significantly higher in females than males, with a mean of 80.0 µmol/L in males, and 113.7 µmol/L in females (p=0.037) (Table 6.9).

Table 6.9 Differences in uric acid concentration based on sex in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
Uric acid (combined)	µmol/L	102.5 (97.0)	44.4	42-232 (51.8-163.6)	12 (33)
Uric acid (male)	µmol/L	80.0 (81.0)	18.9	49-104 (51.0-103.6)	4 (11)
Uric acid (female)	µmol/L	113.7 (111.5)	49.3	42-232 (51.1-187.1)	8 (22)

Total calcium

Total calcium concentrations were significantly higher in females than males. There were five females with readings >4.00 mmol/L (the upper limit of quantitation of the Vetscan analyser), so it was not possible to calculate true means and standard deviations for the combined group or for females. However even with these five values excluded, females had a significantly higher total calcium concentration than males (p=0.0021) (Table 6.10). There was no significant difference in calcium concentrations in gravid and non-gravid females.

Table 6.10 Differences in total calcium concentration based on sex in a population of captive tuatara. * Five samples had calcium concentrations >4.00 mmol/L and were excluded from these calculations.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
Calcium (combined)	mmol/L	2.98 (2.86)*	0.63*	2.1-4.0*	12 (27)
Calcium (male)	mmol/L	2.54 (2.46)	0.33	2.10-3.28 (2.13-3.20)	4 (11)
Calcium (female)	mmol/L	3.32 (3.53)*	0.61*	2.19->4.00*	8 (17)

Phosphorous

Phosphorous concentrations were significantly higher in females than males, with a mean of 1.43 mmol/L in males, and 1.92 mmol/L in females (p=0.0228) (Table 6.11). There was no difference between gravid and non-gravid females.

Table 6.11 Differences in phosphorous concentration based on sex in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
Phosphorous (combined)	mmol/L	1.76 (1.67)	0.59	0.57-3.49 (1.14-2.61)	12 (33)
Phosphorous (male)	mmol/L	1.43 (1.41)	0.29	0.97-1.85 (1.00-1.84)	4 (11)
Phosphorous (female)	mmol/L	1.92 (1.79)	0.64	0.57-3.49 (1.29-2.87)	8 (22)

Globulins

Globulin concentrations were significantly higher in males than females, with a mean of 22.1 g/L in males and 15.6 g/L in females (p=0.0021) (Table 6.12).

Table 6.12 Differences in globulins concentration based on sex in a population of captive tuatara.

Parameter	Unit	Mean (Median)	SD	Min-Max (10^{th}-90^{th} Percentile)	n (samples)
Globulins (combined)	g/L	17.7 (17.0)	5.86	10-37 (11.0-24.0)	12 (33)
Globulins (male)	g/L	22.1 (22.0)	5.74	16-37 (16.2-34.6)	4 (11)
Globulins (female)	g/L	15.6 (14.0)	4.67	10-24 (10.3-23.0)	8 (22)

6.4 Discussion

This study is the most comprehensive analysis of tuatara haematology and biochemistry values to date. The development of reference ranges is of considerable value in evaluating the health status of individual animals. In doing so, due consideration must be given to influencing factors such as season, sex, diet and physiologic state when assessing haematology and biochemistry in reptiles (Campbell 2014). The animals in this study were from a single population maintained under identical husbandry conditions, allowing comparison between individuals in the absence of the confounding factors of different diets or environments.

The PCVs of tuatara in this study were in agreement with those reported by Boardman and Blanchard (2006), with tuatara in this study having slightly higher Hb and MCHC. Data from two other sources (Gartrell 2006 and Species360 2013) do not include red cell parameters. Tuatara have among the largest red blood cells of all reptiles (Frye 1991), and the length and width measurements in this study are consistent with those reported elsewhere (Desser 1978, Frye 1991). Male tuatara had significantly higher PCVs than females, which may indicate an increased oxygen carrying capacity in males. Male tuatara are more active than females during the breeding season as they vigorously

defend their territory and actively pursue females (Gillingham et al. 1995), and this may explain the requirement for increased oxygen carrying capacity in males compared to females. Intraerythrocytic haemogregarine parasites (*Hepatozoon tuatarae*) have been observed in other tuatara (Godfrey et al. 2011), but none were seen in this population.

White blood cell numbers were markedly lower than those reported in several other sources (Boardman and Blanchard 2006, Gartrell et al. 2006, Species360 2013). Two of these studies reported only estimated white cell counts from blood films (Boardman and Blanchard 2006, Gartrell 2006), and the Species360 count methods are not reported. As seen in the data here and reported elsewhere (Russo et al. 1986), estimated white cell counts are often significantly higher, and considered less accurate, than those determined by haemocytometer on recently drawn blood. Estimated white blood cell counts from the same sample can vary significantly depending on sample handling and preparation, laboratory technique, and between individual technicians (Campbell 2014). The methodology used in this study aimed to minimise these influences.

Total white blood cell counts were higher in winter than summer. When white cell count was analysed by sex and season, it became apparent the higher concentration in winter was attributable to females rather than males, but there was no significant difference between white blood cell concentrations in winter between gravid and non-gravid females. Other studies in reptiles have reported seasonal variation in white blood cell numbers in reptiles, some higher in winter (Yu et al. 2013) and some higher in summer (Troiano et al. 1997), but the reasons for this are not well established. A report in a single tuatara documented a 30% decrease in circulating white cells when the animal was undergoing hibernation, compared to those obtained in summer (Desser 1979). The

increase in white cells in in the current study was attributable to higher heterophil numbers. These cells were increased in total number and as part of the differential percentage; no other leukocytes showed significant changes in cell number or differential percentage. While the differences in leukocyte numbers between sex and season were statistically significant, care should be taken in interpreting these results as sample sizes were small. Further study is required to ascertain if these results are consistent findings in other captive as well as wild tuatara populations.

Heterophils and lymphocytes were the most common white blood cells and were present on average in almost equal proportions. This is in contrast to most other reptiles, where lymphocytes are considered the predominant leukocyte (Campbell 2014). Other published data show heterophil to lymphocyte ratios of approximately 1.5:1 (Jakob-Hoff 1996) and 2:1 (Species360 2013) in tuatara of unspecified age, and 1:9 in juvenile tuatara (Gartrell et al. 2006). These wide variations illustrate the limitations of reference ranges in reptiles, and reinforce the utility of analysing a sample from a healthy conspecific when evaluating an unwell reptile (Campbell 2014).

The sizes of the various white blood cells measured in this study is in agreement with those published for tuatara (Desser 1978, Frye 1991). Monocytes are usually the largest leukocytes in other reptiles (Campbell 2014), but this is not the case in tuatara. Heterophils were the largest white blood cells, followed by (in descending order) eosinophils, monocytes, basophils and lymphocytes. In comparison with other reptiles, tuatara were found to have the largest eosinophilic granulocytes, basophilic granulocytes and erythrocytes of the reptiles studied (Frye 1991). The aforementioned text did not evaluate relative sizes of monocytes or lymphocytes, and did not

differentiate specifically between heterophils and eosinophils, referring to both as 'eosinophilic granulocytes'. The significance of the larger leukocytes in tuatara is unknown.

The only biochemistry value to show significant seasonal variation was glucose, with higher concentrations recorded in summer in both sexes. As all samples were handled, transported and stored using the same methods prior to testing, it is considered unlikely that an extrinsic factor contributed to the difference in glucose concentrations. Tuatara have a higher metabolic rate and are more active in summer; it is also the time of year when nesting and mating takes place. The increased glucose concentration in summer may be associated with higher activity levels and physiological stress (Scheelings and Jessop 2011) associated with territorial defence and reproduction. It should be noted that although samples were refridgerated and analysed within three hours of acquisition, red cells would still have been utilising glucose during the transport and storage interval. As a result, the glucose ranges reported here may be slightly lower than the actual concentration in circulating tuatara blood.

Several biochemical values were significantly different between sexes. Calcium and phosphorous were higher in females, a phenomenon that is also observed in other reptiles and is related to females' reproductive status (Campbell 2014). In other reptiles elevated calcium occurs during vitellogenesis and shell deposition, and can be used as an indicator that a female is gravid (Simkiss 1967, Campbell 2014). This has not been the case in tuatara, and in one study of 29 wild tuatara, the animal with the highest calcium concentration was a female with atretic ovarian follicles (Cree et al. 1991). Vitellogenesis occurs slowly in wild tuatara over a period of 2-4 years on Stephens Island (Cree 2014),

but tuatara in our captive population have laid eggs in consecutive years, indicating more rapid follicle development and maturation can occur. This may be related to greater food availability and higher metabolic rate in the warmer captive environment at Auckland Zoo when compared to wild populations on the more southerly and cooler Stephens Island. Further study is required to determine the specific timing and cause of calcium and phosphorous elevations in the tuatara's reproductive cycle. Calcium and phosphorous concentrations in this study were similar to those reported by Boardman and Blanchard (2006).

While uric acid concentrations were significantly higher in females than males ($p=0.037$), further analysis showed that the two highest uric acid values were attributable to the one tuatara, and with these values removed there was no significant difference between sexes. Furthermore, there was no significant difference in uric acid concentration based on the reproductive status of female tuatara. Based on these data, there is no current indication that separate reference ranges are required for uric acid concentrations in tuatara based on sex. It is likely that the statistically significant difference in uric acid concentrations was related to the small sample sizes in this study. Uric acid is an end-product of protein catabolism in reptiles, and an indicator of renal function (Campbell 2014). Uric acid concentrations can increase with dehydration and with renal compromise; no tuatara in this study showed evidence of either of these conditions. Uric acid concentrations reported here were similar to those published by Boardman and Blanchard (2006).

Globulins were significantly higher in males than females, however the reason for this is unknown. There was no corresponding increase in leukocyte numbers in males,

suggesting antigenic stimulation is an unlikely reason for the observed higher globulins. Further study is required to determine whether this is a consistent finding, and if so, protein electrophoresis may prove valuable in assessment of globulins in both sexes. There are no other published studies that report globulin concentrations in tuatara.

Bile acids were all recorded as <35 mmol/L, except for one value of 41 mmol/L. Bile acid composition is different between reptile species, and it was initially considered possible that the Vetscan analyser may not be able to detect tuatara bile acids. This was proven not to be the case, as concentrations of over 200 were detected in animals undergoing itraconazole treatment (see Chapter 4). Bile acids are produced by the liver to aid digestion, and are considered a specific test for hepatic insufficiency (Campbell 2014). Pre- and post-prandial 3α-hydroxy bile acid concentrations were measured in male green iguanas (*Iguana iguana*) and were found to be 7.5 ± 7.8 mmol/L and 33.3 ± 22.0 mmol/L respectively (McBride et al. 2006). Additional study is required to further quantitate bile acids concentrations in tuatara, and the relationship of bile acids concentrations to food intake.

It is important to note that biochemistry results in reptiles can differ between analysers. Studies in sea turtles and various squamate species have shown variable correlation between the VetScan and commercial laboratory analysers (Wolf et al. 2008, McCain et al. 2010), though in most cases the differences would not have significantly influenced clinical interpretation of results. The ranges here are best suited for use in captive tuatara kept in conditions similar to those described here, and in samples analysed using the Abaxis Vetscan.

6.5 Conclusions

This study provides preliminary haematologic and clinical biochemical reference ranges for a population of captive tuatara. The data indicate that sex and environmental temperature can influence results, and these factors should be taken into account when assessing blood values from individual animals. As tuatara inhabit environments significantly colder than those experienced by this captive population, it is likely that further significant temperature-related changes in blood values would be detected in other populations. Calcium and bile acids both had measurements outside the limit of quantitation of the analyser used in this study, and it is recommended that further studies be undertaken to determine the true values of these parameters.

Blood values in reptiles can differ significantly based on analytical method used, so this must be consistent when comparing values to reference populations. Of particular note is the use of estimated white cell counts, which can result in far higher values than those obtained using a haemocytometer. This may lead the clinician to misinterpret leukocyte count results if careful attention is not paid to the method used in both study and reference populations. Comparison of blood values obtained using the same method, and ideally the same laboratory and technician, will reduce the chances of this type of error occurring. The captive population in this study was small, so reference ranges and differences based on sex or season must be interpreted with caution. Comparison of blood values with a healthy conspecific that shares the same environment and diet remains the best way to assess the health of an individual reptile (Mader 1999, Hernandez-Divers 2006). Further studies to determine haematologic and biochemical parameters of larger populations of both captive and wild tuatara are warranted to further validate and increase the robustness of data obtained in this study.

Chapter 7: Summary of findings and directions for future research

Tuatara (*Sphenodon punctatus*) are the last extant members of the order Rhyncocephalia, and are unique, cold-adapted reptiles found only in New Zealand. Recently, captive tuatara have been found to be affected by an emerging fungal pathogen, *Paranannizziopsis australasiensis*. *P. australasiensis* causes dermatitis in tuatara. Its presence has prevented the release of captive tuatara to offshore islands, and has negative implications for the long-term health and welfare of the animals. A review of the literature highlighted that little is known about the epidemiology and treatment of infections caused by *P. australasiensis* and related reptile fungal pathogens, with attempts at treatment in other species sometimes resulting in fatal hepatotoxicity (Van Waeyenberghe et al. 2010).

In vitro fungal growth experiments undertaken in this study, indicated that the optimal growth temperature for *P. australasiensis* encompasses the range from 20°C-30°C, with scant growth at 12°C, moderate growth at 15°C, and no growth at 37°C. Isolates of *P. australasiensis* were also subject to MIC testing to determine what doses of voriconazole and itraconazole were required to inhibit growth of the pathogen. The relatively low MICs are similar to reports of susceptibility testing results in related reptile fungal pathogens (Abarca et al. 2008, Hellebuyck et al. 2010, Van Waeyenberghe et al. 2010). These MICs were used to determine target concentrations for plasma antifungal levels for the pharmacokinetic study. There was no significant difference in MICs tested at 12°C and 20°C, indicating that the same target concentrations could be used for the pharmacokinetic studies at different temperatures. The robustness of this process could be improved by conducting MIC testing on more *P. australasiensis* isolates, and by using a haemocytometer rather than an approximation of optical density to measure fungal

spore concentration in the fungal inoculum. However, resource constraints precluded the application of these methods at the time of the study.

Further investigation into the epidemiology of *P. australasiensis* and related infections is warranted, including determining whether *P. australasiensis* is present in wild populations of tuatara, and the ecology of the fungus itself. This work will significantly enhance our understanding of emerging fungal disease ecology, and will inform future conservation management plans for tuatara and other reptiles.

Itraconazole has been used in the treatment of *P. australasiensis* in tuatara, and related reptile fungal pathogens in other reptiles, at dose rates ranging from 3-5 mg/kg PO SID with varying success, and sometimes fatal adverse effects (Van Waeyenberghe et al. 2010, Masters et al. 2016). This study shows that itraconazole-treated tuatara should be maintained at 20^{o}C to facilitate more rapid attainment of target concentrations, and to enhance drug elimination. Administration of a loading dose will assist with reaching target concentrations sooner. Adverse effects may occur at the modelling-derived dosages, and careful monitoring of tuatara weight, and haematologic and biochemistry parameters (particularly bile acids, uric acid and white blood cell count) are recommended during treatment. Observed itraconazole concentrations showed good agreement with pharmacokinetic modelling, and there was limited inter-individual variability in plasma itraconazole concentrations when compared to voriconazole-treated animals.

Prior to this study, voriconazole had not been used in the treatment of *P. australasiensis* in tuatara, but had been used successfully to treat a related fungal infection in two lizard species (Hellebuyck et al. 2010, Van Waeyenberghe et al. 2010). This study shows that,

as with itraconazole treatment, voriconazole-treated tuatara should be maintained at 20°C. However as target concentrations of voriconazole are rapidly achieved, no loading dose is required. There was significant inter-individual variability in voriconazole plasma concentrations between tuatara, a phenomenon that is also observed in humans. This would likely result in some tuatara receiving a given dosage having sub-therapeutic voriconazole plasma concentrations, while other tuatara may have unnecessarily high concentrations. No adverse effects were seen in this study, however it is important that routine health monitoring be undertaken during treatment. Treatment failure may be due to subtherapeutic plasma concentrations, and increasing the administered voriconazole dose may remedy this.

In both itraconazole and voriconazole studies, oral drug absorption was prolonged. Further study of gastric emptying times and the effect of food on drug absorption in tuatara would assist understanding of the factors influencing drug absorption. Intravenous pharmacokinetic data would enable the determination of oral bioavailability, which would assist with more accurate determination of other pharmacokinetic parameters on which to base recommended antifungal dosages.

The findings of this study will be important in the development of a quarantine treatment protocol for tuatara, to ensure they are not subclinical carriers of *P. australasiensis* prior to release to wild sites. Itraconazole has the more predictable pharmacokinetics of the two drugs, so is well suited to this role, but adverse effects may limit its use. Voriconazole appears to be the safer of the two drugs, however variable inter-individual pharmacokinetics mean some animals may reach sub-therapeutic voriconazole concentrations. This may be remedied by increasing the administered

dose, but this increases the likelihood of adverse effects occurring. Further, it would not be appropriate to use voriconazole in this manner, associated with treatment during quarantine periods, until it has been established that voriconazole successfully treats clinical infection of *P. australasiensis* in tuatara. Proprietary voriconazole is currently significantly more expensive than itraconazole, and this must also be taken into account when assessing the utility of both antifungals. Ideally, pharmacokinetic studies would be conducted on clinically affected tuatara, to determine if illness influences the antifungal pharmacokinetics, and to ensure the modelling-derived dose rates n this study are clinically effective in diseased animals.

Establishing haematologic and biochemical reference ranges for a species assists in the evaluation of health and disease in individual animals. The ranges established in this study provide data from a population of known sex structure, environmental and seasonal status. These data provide a basis for future studies in both captive and wild populations, to increase the robustness and utility of these ranges.

This research is the first of its kind to use population pharmacokinetic modelling of antifungal treatments in reptiles, and provides a framework for future research in this area. It demonstrates the utility of population-based modelling in circumstances of sparse data sampling and high variability in drug concentrations, which are often the case in non-domestic species where small sample sizes and volumes can limit the amount of data collected. Fungal diseases have been associated with significant population declines of amphibians, reptiles and mammals worldwide, and it is crucial that safe, evidence-based treatments are developed to combat these emerging pathogens.

Appendices

8.1 Appendix 1 – Culture method for fungal isolates

Media/Reagents

- Enrofloxacin solution 20 µg/ml (working solution)
- Sabourauds Dextrose agar (SDA) plates with and without antibiotics, Mycobiotic agar
- Sabourauds Dextrose agar slopes with cycloheximide - used for sending cultures overseas for confirmation, and to Canterbury Health Laboratories (CHL, Christchurch) for MIC testing.

Method

- Using sterile scalpel, aseptically divide the sample and cut into small pieces ~ 3-4 mm.
- Dip pieces into Baytril solution (allow submersion for ~30secs).
- Remove and further cut into 1-2 mm sizes (aim for 5-6 pieces for each medium used).
- Place pieces onto culture medium (use scalpel end to mark 5-6 areas in the agar first)
- Make sure they are all firmly pressed onto the agar.
- Use sellotape to seal all agar plates.
- Incubate plates/slopes at 25 or 30^{o}C
- Check daily for the first 7-10 days and then for up to 3 weeks.

8.2 Appendix 2 – Preparation of inoculum for antifungal susceptibility testing

Each fungal isolate was subcultured onto an SDA slant, and the following process performed to prepare the inoculum for MIC testing.

- Remove fungus from the slant using a sterile loop
- Deposit in a test tube with 2 mL of saline and mix to produce a uniform suspension
- Adjust amount of saline and fungus present to produce an inoculum with optical density of 1.5 McFarland (approximately 4.5 x 10^8 cells), as assessed visually
- Inoculate 20 µL of solution to a SDA slope as a sterility check
- Inoculate 100 µL to an 11 mL RPMI 1640 solution and mix to form uniform suspension
- Transfer suspension aseptically to a sterile plastic reagent reservoir
- Add 100 µL of suspension to each well in the sensititre plate
- Cover plate with adhesive plastic sheet, to prevent contamination from environmental fungi during incubation

8.3 Appendix 3 – Drug assay procedure

Reagents used in the assays were as follows:

- Ammonium formate solution, 1 mol/L. This was prepared by dissolving 63 g of ammonium formate in deionised water, and making up to 1 L with deionised water.
- Formic acid, 88%
- Eluent A. This was prepared using 4 mL of the 1 mol/L ammonium formate solution and 4 mL of formic acid, and making up to 2 L with deionised water.
- Eluent B. This was prepared using 4 mL of the 1 mol/L ammonium formate solution and 4 mL of formic acid, and making up to 2 L with acetonitrile.

Standards were prepared as follows:

- Voriconazole stock standard, 1.0 mg/mL. 10.0 mg of voriconazole powder was dissolved in 10 mL of methanol, and stored at 4^{o}C until use.
- Itraconazole stock standard, 0.4 mg/mL. 10.0 mg of itraconazole powder was dissolved in 25 mL of methanol, and stored at 4^{o}C until use.
- Hydroxy-itraconazole stock standard, 0.1 mg/mL. 10.0 mg of hydroxy-itraconazole powder was dissolved in 100 mL of methanol, and stored at 4^{o}C until use.
- **Antifungal standards in plasma**. The standards were produced using a mix comprised of 0.1 mL voriconazole stock standard, 0.25 mL itraconazole stock standard, and 1.0 mL hydroxy-itraconazole stock standard, as prepared above. This was made up to 10 mL with drug-free plasma, producing a standard containing 0.1 mg of each antifungal in 10 mL, or 10 mg/L, as ST 10. Other

standards were produced by sequentially diluting ST 10 in drug-free plasma, to produce standards of 5.0, 2.5, 1.25, 0.625 and 0.3125 mg/L. A blank standard, of drug-free serum only, was also prepared.

- **Working internal standard solutions of 1 mg/L**. These were prepared by diluting 50 µL of a stock solution of a commercial preparation of each antifungal (Toronto Research Chemicals Inc, Canada) to 50 mL with acetonitrile. The stock solutions were deuterated standards of voriconazole-d3, itraconazole-d5, and Hydroxy-itraconazole-d5 as 1.0 mg/mL solutions in methanol.

Sample preparation was as follows:

- Place 30 µL of sample, QC, drug free plasma or standard into a 1.5 mL plastic centrifuge tube
- Add 30 µL of working internal standard (1 mg/L in acetonitrile)
- Vortex for 30 seconds and centrifuge at 15,000 g for 5 minutes
- Transfer 20 µL to HPLC autosampler injection vial or 96 well microtitre plate
- Add 280 µL of water and mix gently
- Inject 30 µL into LCMS/MS system

8.4 Appendix 4 – Inter-run and intra-run statistics for assay validation and precision

Below are the inter- and intra-run statistics for voriconazole, itraconazole and hydroxy-itraconazole.

CV = coefficient of variation

Voriconazole

Inter run

Analyte level	Low	Medium	High
Average	0.48	1.07	2.20
Target value	0.5	1.0	2.0
CV%	10.0	5.4	7.6

Intra-run

Analyte level	Low	Medium	High
Average	0.44	0.78	2.05
Target value	0.5	1.0	2.0
CV%	6.72	7.5	4.4

Itraconazole

Inter run

Analyte level	Low	Medium	High
Average	0.58	1.04	2.07
Target value	0.5	1.0	2.0
CV%	7.4	7.0	3.5

Intra-run

Analyte level	Low	Medium	High
Average	0.57	0.99	2.12
Target value	0.5	1.0	2.0
CV%	5.8	6.9	6.6

Hydroxy-itraconazole

Inter run

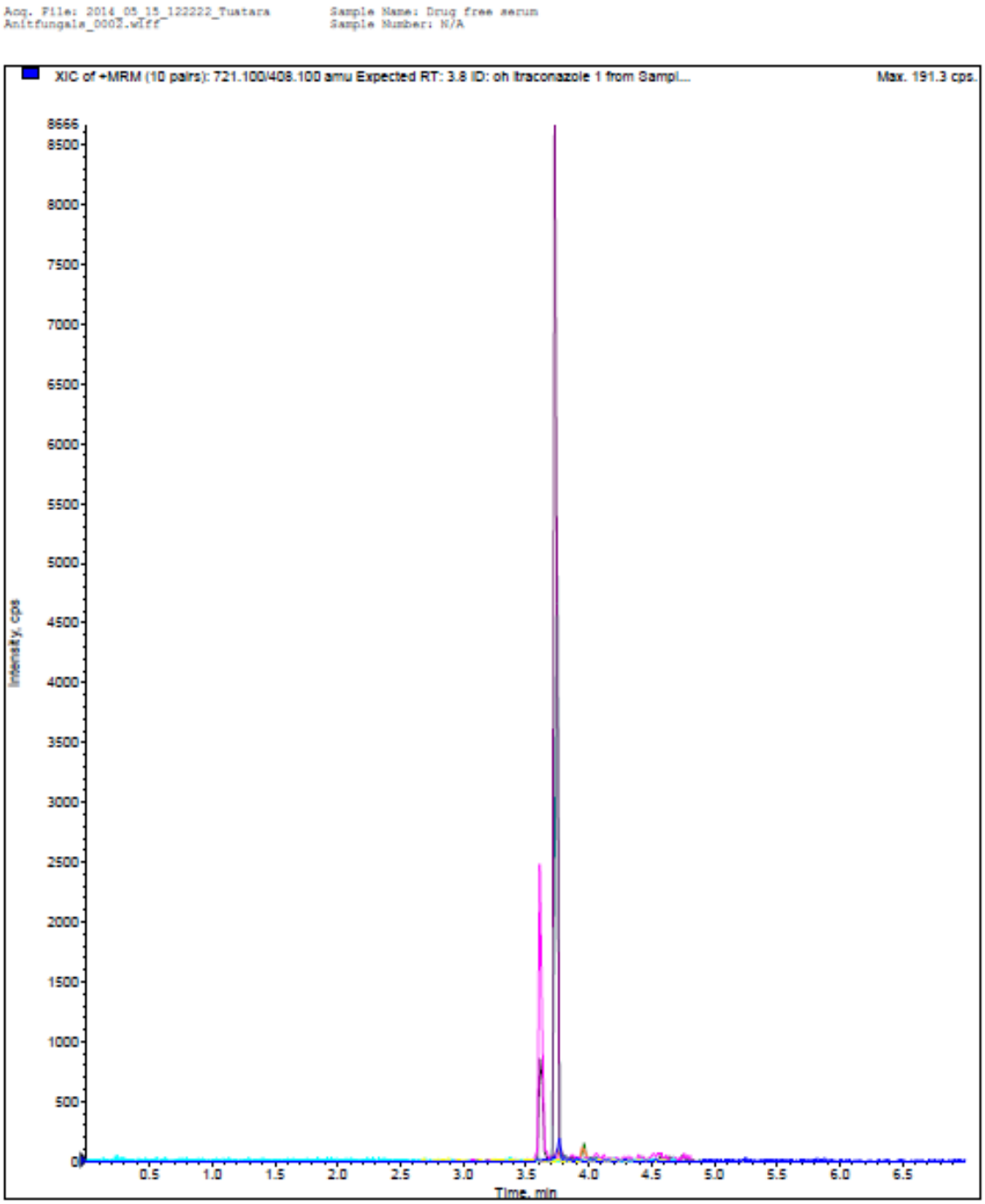
Analyte level	Low	Medium	High
Average	0.52	1.04	2.17
Target value	0.5	1.0	2.0
CV%	12.6	5.8	6.8

Intra-run

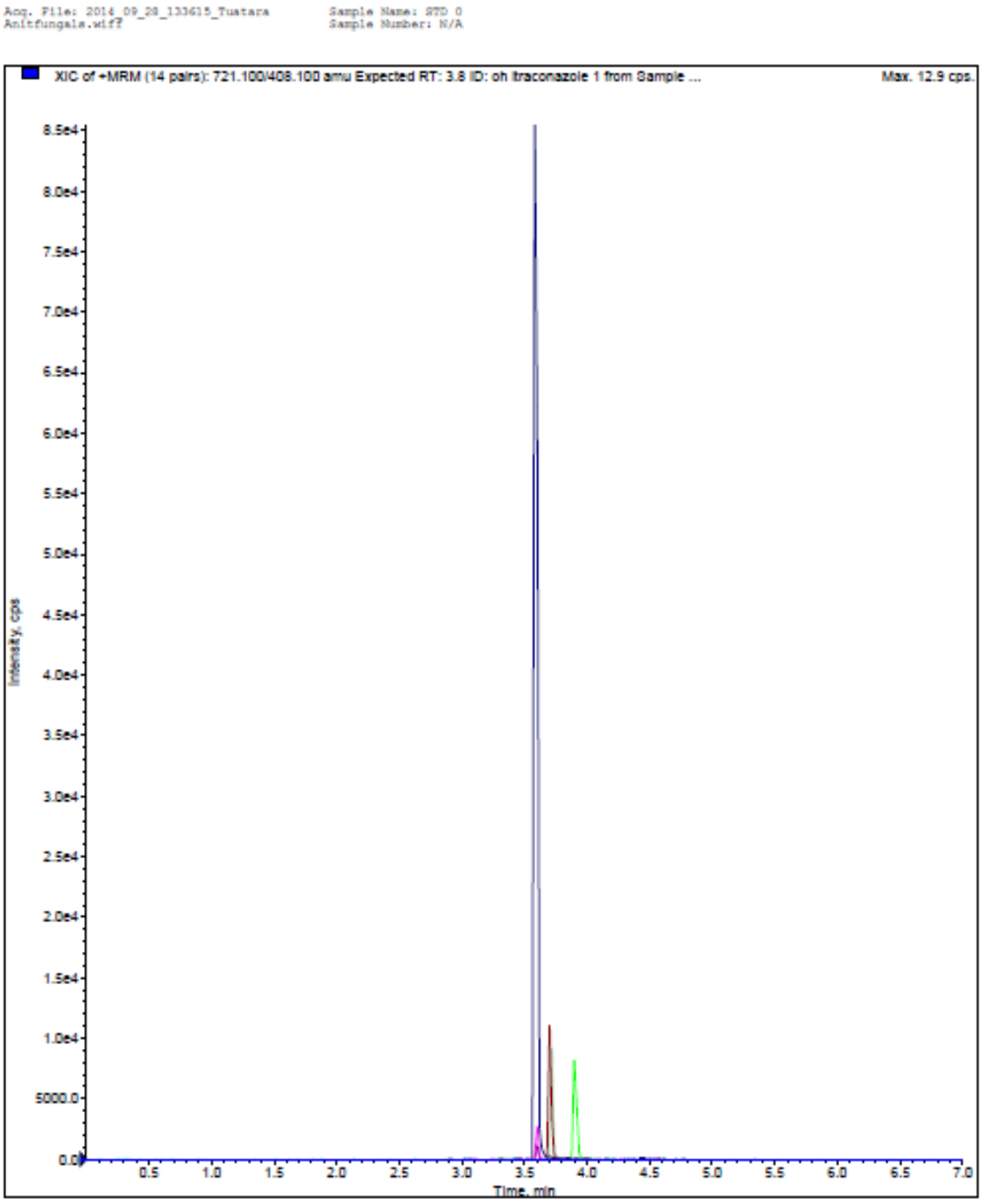
Analyte level	Low	Medium	High
Average	0.52	1.05	2.09
Target value	0.5	1.0	2.0
CV%	6.5	5.9	6.8

8.5 Appendix 5 - Chromatograms of tuatara plasma

8.5.1 Chromatogram of blank tuatara plasma



8.5.2 Chromatogram of blank tuatara plasma plus deuterated internal standards



The highest peak on the blank sample did not correspond to any transition pairs being monitored at the retention time, thus there are no peaks that would be misidentified as antifungal drugs that could interfere with the assays.

8.6 Appendix 6 – Control stream for pharmacokinetic modelling

Mixed effects modelling was performed using NONMEM v7.3.0 with the Intel Fortran Compiler v11. The control stream is provided below:

```
NM-TRAN Control Stream for PK Model for Single Dose Model 03
$PROB Tuatara Sarah Alexander 13 Jan 2014
$INPUT
ID ; numerical ID
Number=DROP Name=DROP SEX=DROP Gravid=DROP DRUG=DROP MGKG=DROP
TYPE=DROP
DAT1=DROP ; dd/mm/yyyy
TIME ; 0 - 48 h
TRT ; 1=itraconozole, 2=voriconazole
TEMP ; Celsius
WTKG ; kg
M1F0 ; male=1, female=0
Pregnant ; 0=No, 1=Yes or Likely
DVID ; 0=dose, 1=itraconazole, 2=voriconazole
AMT ; mg oral
CMT ; 1=itraconozole, 2=voriconazole
DV ; mg/L concentration
MDV ; 0=observation 1=missing observation
$DATA ..\..\Data\Tuatara.csv
;ACCEPT=(TEMP.EQ.20)

$EST METHOD=COND INTER
MAX=9990 NSIG=3 SIGL=9 PRINT=1
MSFO=tuatara.msf
;$COV UNCOND

$THETA
```

```
(0,0.908,) ; POP_CL_IT L/h/70kg
(0,202.,) ; POP_V_IT L/70kg
(0,4.87,)  ; POP_TABS_IT h

(0,0.858,) ; POP_CL_VR L/h/70kg
(0,38.7,) ; POP_V_VR L/70kg
(0,6.92,)  ; POP_TABS_VR h

(0,0.1,) FIX ; RUV_PROP_IT
(0,0.3,) FIX ; RUV_ADD_IT mg/L

(0,0.1,) FIX ; RUV_PROP_VR
(0,0.3,) FIX ; RUV_ADD_VR mg/L

(1,6.73,10)  ; FCL_20_IT
(0,1.,) FIX ; FTAB_20_IT

(1,3.35,10)  ; FCL_20_VR
(0,1.,) FIX ; FTAB_20_VR

$OMEGA BLOCK(3)
0.00001 ; BSV_CL_IT
0. 0.121 ; BSV_V_IT
0. 0. 0.00001 ; BSV_TABS_IT

$OMEGA BLOCK(3)
0.00003 ; BSV_CL_VR
0. 0.00001 ; BSV_V_VR
0. 0. 0.00001 ; BSV_TABS_VR

$OMEGA BLOCK(3)
0.00001 ; BOV_CL_IT_1
0. 0. ; BOV_V_IT_1
```

```
0. 0. 0.364 ; BOV_TABS_IT_1
$OMEGA BLOCK(3) SAME
;; BOV_CL_IT_2
;; BOV_V_IT_2
;; BOV_TABS_IT_2

$OMEGA BLOCK(3)
0. ; BOV_CL_VR_1
0. 0.739 ; BOV_V_VR_1
0. 0. 2.25 ; BOV_TABS_VR_1
$OMEGA BLOCK(3) SAME
;; BOV_CL_VR_2
;; BOV_V_VR_2
;; BOV_TABS_VR_2

$SIGMA 1. FIX ; EPS1

$SUB ADVAN13 TOL=9
$MODEL
COMP (DEPOTIT)
COMP (DEPOTVR)
COMP (CENTRIT)
COMP (CENTRVR)

$PK
IF (TEMP.EQ.12) THEN
  FCLTMPIT=1/FCL_20_IT
  FTABTMPIT=1/FTAB_20_IT
  FCLTMPVR=1/FCL_20_VR
  FTABTMPVR=1/FTAB_20_VR
ELSE
  FCLTMPIT=1
  FTABTMPIT=1
```

```
  FCLTMPVR=1
  FTABTMPVR=1
ENDIF
GRPCLIT=FCLTMPIT*POP_CL_IT*(WTKG/70)**0.75
GRPVIT=POP_V_IT*(WTKG/70)
GRPTABIT=FTABTMPIT*POP_TABS_IT

GRPCLVR=FCLTMPVR*POP_CL_VR*(WTKG/70)**0.75
GRPVVR=POP_V_VR*(WTKG/70)
GRPTABVR=FTABTMPVR*POP_TABS_VR

IF (TEMP.EQ.12) THEN
  BOVCLIT=BOV_CL_IT_1
  BOVVIT=BOV_V_IT_1
  BOVTABIT=BOV_TABS_IT_1
  BOVCLVR=BOV_CL_VR_1
  BOVVVR=BOV_V_VR_1
  BOVTABVR=BOV_TABS_VR_1
ENDIF
IF (TEMP.EQ.20) THEN
  BOVCLIT=BOV_CL_IT_2
  BOVVIT=BOV_V_IT_2
  BOVTABIT=BOV_TABS_IT_2
  BOVCLVR=BOV_CL_VR_2
  BOVVVR=BOV_V_VR_2
  BOVTABVR=BOV_TABS_VR_2
ENDIF

CLIT=GRPCLIT*EXP(BSV_CL_IT+BOVCLIT)
VIT=GRPVIT*EXP(BSV_V_IT+BOVVIT)
TABIT=GRPTABIT*EXP(BSV_TABS_IT+BOVTABIT)

CLVR=GRPCLVR*EXP(BSV_CL_VR+BOVCLVR)
```

```
VVR=GRPVVR*EXP(BSV_V_VR+BOVVVR)
TABVR=GRPTABVR*EXP(BSV_TABS_VR+BOVTABVR)

KAIT=LOG(2)/TABIT
KAVR=LOG(2)/TABVR

$DES

GUTIT=A(1)
GUTVR=A(2)
CENIT=A(3)/VIT
CENVR=A(4)/VVR

RATEIT=KAIT*GUTIT
RATEVR=KAVR*GUTVR

DADT(1)=-RATEIT
DADT(2)=-RATEVR
DADT(3)=RATEIT - CLIT*CENIT
DADT(4)=RATEVR - CLVR*CENVR

$ERROR

CONCIT=A(3)/VIT
CONCVR=A(4)/VVR

IF (DVID.LE.1) THEN
  PROPIT=CONCIT*RUV_PROP_IT
  ADDIT=RUV_ADD_IT
  SDIT=SQRT(PROPIT*PROPIT + ADDIT*ADDIT)
  Y=CONCIT + SDIT*EPS1
ELSE
  PROPVR=CONCVR*RUV_PROP_VR
```

```
  ADDVR=RUV_ADD_VR
  SDVR=SQRT(PROPVR*PROPVR + ADDVR*ADDVR)
  Y=CONCVR + SDVR*EPS1
ENDIF

$TABLE ID TIME TRT TEMP AMT WTKG
CLIT VIT TABIT CLVR VVR TABVR
Y
ONEHEADER NOPRINT FILE=tuatara.fit

NM-TRAN Control Stream for PK Model for Single and Multiple Dose Model 013
$PROB Tuatara Sarah Alexander 20 Dec 2015
$INPUT
ID ; numerical ID
Number=DROP Name=DROP SEX=DROP Gravid=DROP DRUG=DROP MGKG=DROP
TYPE=DROP STUDY=DROP
DAT1=DROP ; dd/mm/yyyy
TIME ; 0 - 48 h
TRT ; 1=itraconozole, 2=voriconazole
S1M2 ; 1=single Dose=1, 2=multiple dose
TEMP ; Celsius
WTKG ; kg
M1F0 ; male=1, female=0
Pregnant ; 0=No, 1=Yes or Likely
DVID ; 0=dose, 1=itraconazole, 2=voriconazole
AMT ; mg oral
CMT ; 1=itraconozole, 2=voriconazole
DV ; mg/L concentration
MDV ; 0=observation 1=missing observation
II ; dosing interval hours
ADDL ; additional doses after first
EVID ; event reset
$DATA ..\..\Data\Tuatara_20.csv
```

```
;ACCEPT=(TEMP.EQ.20)

$EST METHOD=COND INTER
MAX=9990 NSIG=3 SIGL=9 PRINT=1
MSFO=tuatara.msf
;$COV UNCOND

$THETA
(0,0.254,) ; POP_CL_IT L/h/70kg 20C
(0,186.,) ; POP_V_IT L/70kg
(0,5.47,15)  ; POP_TABS_IT h

(0,0,) FIX ; POP_CL_VR L/h/70kg 20C
(0,1.58,25) ; POP_VM_VR mg/h/70kg 20C
(0.001,0.00866,7.5)  ; POP_KM_VR mg/L 20C
(0,79.7,) ; POP_V_VR L/70kg
(0,3.,15)  ; POP_TABS_VR h

(0,0.178,)  ; RUV_PROP_IT
(0,0.198,)  ; RUV_ADD_IT mg/L

(0,0.275,)  ; RUV_PROP_VR
(0,0.541,)  ; RUV_ADD_VR mg/L

(1,2.86,1000)  ; FCL_12_IT
(0,1.,) FIX ; FTAB_12_IT

(1,2.11,1000)  ; FCL_12_VR
(0,1.,) FIX ; FTAB_12_VR

(0.1,0.719,1000)  ; FBIOF_20_VR

$OMEGA BLOCK(2)
```

```
0.0394 ; BSV_CL_IT
0. 0.0112 ; BSV_V_IT
$OMEGA BLOCK(1)
0. FIX ; BSV_TABS_IT
$OMEGA BLOCK(1)
0.0355 ; BSV_F_IT

$OMEGA BLOCK(2)
0.00122 ; BSV_CL_VR
0. 0.0136 ;BSV_V_VR
$OMEGA BLOCK(1)
0.00008 ; BSV_TABS_VR
$OMEGA BLOCK(1)
0.00028 ; BSV_F_VR

$OMEGA BLOCK(2)
0.0139 ; BOV_CL_IT_1
0. 0.0628 ; BOV_V_IT_1
$OMEGA BLOCK(2) SAME
;; BOV_CL_IT_2
;; BOV_V_IT_2
$OMEGA BLOCK(1)
0.636 ; BOV_TABS_IT_1
$OMEGA BLOCK(1) SAME
;; BOV_TABS_IT_2
$OMEGA BLOCK(1)
0. FIX ; BOV_F_IT_1
$OMEGA BLOCK(1) SAME
;; BOV_F_IT_2

$OMEGA BLOCK(1)
0. ; BOV_CL_VR_1
$OMEGA BLOCK(1) SAME
```

```
;; BOV_CL_VR_2
$OMEGA BLOCK(1)
0.0395 ; BOV_V_VR_1
$OMEGA BLOCK(1) SAME
;; BOV_V_VR_2
$OMEGA BLOCK(1)
0.791 ; BOV_TABS_VR_1
$OMEGA BLOCK(1) SAME
;; BOV_TABS_VR_2
$OMEGA BLOCK(1)
0. FIX ; BOV_F_VR_1
$OMEGA BLOCK(1) SAME
;; BOV_F_VR_2

$SIGMA 1. FIX ; EPS1

$SUB ADVAN13 TOL=9
$MODEL
COMP (DEPOTIT)
COMP (DEPOTVR)
COMP (CENTRIT)
COMP (CENTRVR)

$PK
IF (TEMP.EQ.12) THEN
  FCLTMPIT=1/FCL_12_IT
  FTABTMPIT=FTAB_12_IT
  FCLTMPVR=1/FCL_12_VR
  FTABTMPVR=FTAB_12_VR
ELSE
  IF (TEMP.EQ.16) THEN
    FCLTMPIT=2/FCL_12_IT
    FTABTMPIT=FTAB_12_IT/2
```

```
    FCLTMPVR=2/FCL_12_VR
    FTABTMPVR=FTAB_12_VR/2
  ELSE
    FCLTMPIT=1
    FTABTMPIT=1
    FCLTMPVR=1
    FTABTMPVR=1
  ENDIF
ENDIF
GRPCLIT=FCLTMPIT*POP_CL_IT*(WTKG/70)**0.75
GRPVIT=POP_V_IT*(WTKG/70)
GRPTABIT=FTABTMPIT*POP_TABS_IT

GRPCLVR=FCLTMPVR*POP_CL_VR*(WTKG/70)**0.75
GRPVMVR=FCLTMPVR*POP_VM_VR*(WTKG/70)**0.75
GRPKMVR=POP_KM_VR
GRPVVR=POP_V_VR*(WTKG/70)
GRPTABVR=FTABTMPVR*POP_TABS_VR
TBIO=17*24
IF (TIME.GT.TBIO.AND.TEMP.EQ.20) THEN
  FBIOFVR=FBIOF_20_VR
ELSE
  FBIOFVR=1
ENDIF


IF (TEMP.LT.20) THEN
  BOVCLIT=BOV_CL_IT_1
  BOVVIT=BOV_V_IT_1
  BOVTABIT=BOV_TABS_IT_1
  BOVFIT=BOV_F_IT_1
  BOVCLVR=BOV_CL_VR_1
  BOVVVR=BOV_V_VR_1
```

```
  BOVTABVR=BOV_TABS_VR_1
  BOVFVR=BOV_F_VR_1
ENDIF
IF (TEMP.EQ.20) THEN
  BOVCLIT=BOV_CL_IT_2
  BOVVIT=BOV_V_IT_2
  BOVTABIT=BOV_TABS_IT_2
  BOVFIT=BOV_F_VR_2
  BOVCLVR=BOV_CL_VR_2
  BOVVVR=BOV_V_VR_2
  BOVTABVR=BOV_TABS_VR_2
  BOVFVR=BOV_F_VR_2
ENDIF

CLIT=GRPCLIT*EXP(BSV_CL_IT+BOVCLIT)
VIT=GRPVIT*EXP(BSV_V_IT+BOVVIT)
TABIT=GRPTABIT*EXP(BSV_TABS_IT+BOVTABIT)
F1=1*EXP(BSV_F_IT+BOVFIT)

PPVCLVR=EXP(BSV_CL_VR+BOVCLVR)
VVR=GRPVVR*EXP(BSV_V_VR+BOVVVR)
TABVR=GRPTABVR*EXP(BSV_TABS_VR+BOVTABVR)
F2=FBIOFVR*EXP(BSV_F_VR+BOVFVR)

KAIT=LOG(2)/TABIT
KAVR=LOG(2)/TABVR

$DES

GUTIT=A(1)
GUTVR=A(2)
CENIT=A(3)/VIT
CENVR=A(4)/VVR
```

```
GRPMMCLVR=GRPVMVR/(GRPKMVR+CENVR)
CLVR=(GRPMMCLVR+GRPCLVR)*PPVCLVR
RATEIT=KAIT*GUTIT
RATEVR=KAVR*GUTVR

DADT(1)=-RATEIT
DADT(2)=-RATEVR
DADT(3)=RATEIT - CLIT*CENIT
DADT(4)=RATEVR - CLVR*CENVR

$ERROR

CONCIT=A(3)/VIT
CONCVR=A(4)/VVR

IF (DVID.LE.1) THEN
  PROPIT=CONCIT*RUV_PROP_IT
  ADDIT=RUV_ADD_IT
  SDIT=SQRT(PROPIT*PROPIT + ADDIT*ADDIT)
  Y=CONCIT + SDIT*EPS1
ELSE
  PROPVR=CONCVR*RUV_PROP_VR
  ADDVR=RUV_ADD_VR
  SDVR=SQRT(PROPVR*PROPVR + ADDVR*ADDVR)
  Y=CONCVR + SDVR*EPS1
ENDIF
HOURS=TIME
DAYS=TIME/24
IF (S1M2.EQ.2) THEN ; 10 day offset for plotting
  DAYS=DAYS+10
  HOURS=DAYS*24
ENDIF
```

```
IF (TEMP.EQ.16) THEN
  T1220=12 ; Was originally in 12 C group but moved to 16 (ambient)
ELSE
  T1220=TEMP
ENDIF
$TABLE ID TIME HOURS DAYS S1M2 TRT T1220 AMT WTKG
CLIT VIT TABIT CLVR VVR TABVR
Y
ONEHEADER NOPRINT FILE=tuatara.fit
```

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