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Dermatomycosis in squamate reptiles

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Preface

I would like to thank my supervisor Prof. dr. Frank Pasmans for helping me write this thesis and giving feedback. I would also like to thank my family for all the support during the writing process.

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1. Abstract

Schimmels behorende tot het *Chrysosporium* anamorph van *Nannizziopsis vriesii* (CANV) complex zijn de laatste jaren aan het licht gekomen als veroorzaker van dermatomycoses bij verschillende soorten reptielen. De schimmels kunnen onderverdeeld worden in drie verschilllende subgroepen: *Nannizziopsis*, *Paranannizziopsis* en *Ophidiomyces*. *Nannizziopsis* is voornamelijk belangrijk bij hagedissen en veroorzaakt bij baardagamen (*Pogona vitticeps)* het zogenaamde 'yellow fungus' disease'. *Paranannizziopsis* lijkt voornamelijk van belang bij brughagedissen (*Sphenodon punctatus)* en bepaalde aquatische slangen. *Ophidiomyces* veroorzaakt 'snake fungal disease' (SFD) bij zowel in gevangenschap gehouden slangen als bij wilde slangen. Er is nog maar weinig bekend over de pathogenese en epidemiologie. De schimmels gedragen zich vaak als primaire pathogenen, in tegensteling tot andere schimmels die vaak enkel als opportunistische infectie kunnen optreden. Verspreiding gebeurt waarschijnlijk via de omgeving maar ook door onderling contact tussen dieren. Het effect van SFD op wilde populaties is nog niet geheel bekend, maar vooral bedreigde populaties van de massasauga (*Sistrurus catenatus)* lijken onder druk te komen staan door deze infectie. Het bekomen van een juiste diagnose is niet altijd even evident, maar meestal wordt een combinatie van cultuur of PCR in combinatie met histopathologie aangeraden. Een behandeling met anti-fungale middelen kan ingesteld worden, voriconazole lijkt hierbij het veiligste product. Er zijn echter nog onbeantwoorde vragen rond veiligheid en effectiviteit van dit product bij verschillende reptielensoorten. Ook lokale behandeling kan bijkomend gedaan worden. De prognose voor deze infectieziekte is ondanks behandeling niet goed, preventie is daarom belangrijk, vooral bij in gevangenschap gehouden dieren. Quarantaine, toegangscontrole en desinfectie van materiaal kunnen hierbij een belangrijke rol spelen.

The last couple of years fungi belonging to the Chrysosporium anamorph of *Nannizziopsis vriesii* (CANV) complex have come to light as cause of dermatomycosis in several reptile species. Fungi belonging tot his complex can be devided in to three lineages or clades: *Nannizziopsis, Paranannizziopsis* and *Ophidiomyces*. In lizards *Nannizziopsis* is the most common and causes 'yellow fungus diseae' in bearded dragons (*Pogona vitticeps)*. *Paranannizziopsis* is mainly of interest in tuatara (*Sphenodon punctatus*) and in several aquatic snakes. *Ophidiomyces* is the cause of 'snake fungal disease' (SFD) in captive and wild snakes. There is very little known about the pathogenesis and epidemiology. In contrast to other fungi who mainly act as opportunistic pathogens, CANV-fungi appear to act as primary pathogens. The fungus spreads through the environment as well as through contact between individuals. The effect of SFD on wild snake populations is not yet fully understood. Sensitive populations of he eastern massasauga rattlesnake (*Sistrurus catenatus*) seem most affected. Obtaining a correct diagnosis is not always easy, usually a combination of culture or PCR with histopathology is recommended. Treatment with antifungals is possible, voriconazole is the safest option. There are however still some uncertainties surrounding safety and efficacy of voriconazole in different reptile species. Topical antifungals can aid in treatment. Despite several treatment options, prognosis remains poor. Therefore prevention is important, especially in reptiles kept in captivity. Quarantine, entry-control and desinfection of materials can play an important role as preventative measures.

2. Introduction

Demarcation of the subject

Dermatological diseases are common in reptiles and multiple underlying causes have been identified. Fungal infections are one of the possible underlying causes of cutaneous lesions in reptiles and have likely been underdiagnosed because lesions are often indistinguishable from those caused by bacteria (Paré et al., 2006b). As a rule fungal infections in reptiles have been regarded as opportunistic and secondary to suboptimal environmental conditions such as malnutrition, high humidity, overcrowding and stress (Hoppmann and Barron, 2007; Paré et al., 2006b). Breaches in layers of the integument may also act as a point of entry for infection and consequently predispose the skin for fungal infection (Allender et al., 2015a; Lorch et al., 2015; Paré et al., 2006a). For example *Aspergillus, Fusarium, Geotrichium, Microsporum* and many other species have been isolated from skin lesions in reptiles.

However, there is one species complex of fungi that seem to set themselves apart from these opportunistic fungi by posing more of a threat to reptiles than the species listed above. Fungi belonging to the species complex of *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV) have emerged as significant and major reptile fungal pathogens (Paré, 2014). Fungi belonging to these *Nannizziopsis* were originally considered to be ubiquitous organisms found in the soil causing infections in invertebrates (Mitchell and Walden, 2013). However, over the past several years multiple reports have shown the CANV as cause of outbreaks of superficial and deep mycoses in captive reptiles as well as, more recently, in free ranging snakes in the north-eastern United States (Allender et al., 2015b). They have been recovered from sick reptiles belonging to a broad variety of taxa including crocodilians, lizards, snakes and tuataras (Paré and Sigler, 2016).
Infection can result in a range of symptoms varying from local skin lesions to systemic disease. Crust formation, hyperkeratosis, vesicles, and necrosis are commonly seen lesions of the skin (Sigler et al., 2013). Infections are typically severe and often fatal. They also seem to be contagious among animals housed together or in close proximity to each other (Paré, 2014). Not only does this species complex set itself apart by being a rather aggressive pathogen, the CANV also shines a different light on the assumption that fungal infections in reptiles are always opportunistic and secondary to suboptimal environmental conditions or other causes of reduced immune status. The CANV seems to be able to behave as a primary pathogen in several reptile species. Koch's postulates have been fulfilled for at least CANV for veiled chameleons (*Chamaeleo calyptratus) (Paré et al., 2006a)*. One study also fulfilled Koch's postulates for *Ophidiomyces ophiodiicola* in corn snakes (*Pantherophis guttatus*), although inoculation was always preceded by damaging the skin (Lorch et al., 2015). Furthermore, when the mycobiota of the skin of different reptiles was evaluated, it was shown that the CANV were rare compared with other common skin inhabitants of reptiles (Paré et al., 2003). The rarity of CANV as a common skin inhabitant evokes the thought that it does not involve an opportunistic fungal infection, but it may present as an obligate pathogen that infects reptiles after exposure (Paré, 2014).

Recent molecular analysis and following taxonomic revisions reassigned the CANV to the family *Onygenaceae*, order *Onygenales*. The complex is now divided in three different phylogenetic lineages: *Nannizziopsis*, *Paranannizziopsis* and *Ophidiomyces*. Currently the two most prevalent species are *Nannizziopsis guarroi* and *Ophidiomyces ophiodiicola* (Paré and Sigler, 2016; Schmidt, 2015).
N.guarroi has been identified as the primary etiologic agent of a deep granulomatous dermatomycosis in inland bearded dragons (*Pogona vitticeps*) commonly referred to as "yellow fungus disease" (YFD) (Paré et al., 2006b; Schmidt, 2015). Lesions initially appear as single or multiple areas of necrotic hyperkeratotic epidermis of the head, limbs or body. Infection often progresses to involve underlying muscles and bones and infection is often fatal (Paré et al., 2006b). *N.guarroi* has also been reported frequently in green iguana's (*Iguana iguana)* (Abarca et al., 2010; Paré and Sigler, 2016; Schmidt, 2015; Schneider et al., 2018).
O.ophiodiicola has been identified as the causative agent of snake fungal disease (SFD), an emerging fungal pathogen of north American endemic, as well as captive snakes (Allender et al., 2015a). It is seen as one of the true fungal pathogens associated with free ranging wild epidemics and is placed alongside chytridiomycosis caused by *Batrachochytrium* in global frog population declines (Skerratt et al., 2007)

and *Pseudogymnoascus destructans* causing white nose syndrome in bats (Blehert et al., 2009). *O.ophiodiicola* infects only snakes and causes wide spread morbidity and mortality across the eastern United States (Allender et al., 2013; Sigler et al., 2013). It has been hypothesized that environmental conditions such as increased rainfall and frequent mild winters might be the explanation for increased prevalence and/or severity (Allender et al., 2013). However two other studies investigating the causative role of *O.ophiodiicola* in SFD showed that abrasions of the epidermis were important in developing infection (Allender et al., 2015a; Lorch et al., 2015). The true impact of SFD on populations is discussable and presents another gap in literature. The global distribution of the fungus is unknown, however a recent survey of snake populations in the United Kingdom and the Czech Republic have also found *O.ophiodiicola* in snakes displaying signs of snake fungal disease in European wild snakes (Franklinos et al., 2017).

The last genus *Paranannііzziopsis* currently consists of four species, three of which were recovered exclusively from captive tentacle snakes (*Erpeton tentaculatum*) in North America (Paré and Sigler, 2016; Sigler et al., 2013). The fourth *Paranannііzziopsis* species, *Paranannііzziopsis australasiensis* has been reported to infect lizards, snakes and tuataras, but has only been isolated in Australia and New Zeeland (Humphrey et al., 2016; Masters et al., 2016; Paré and Sigler, 2016).

Another true fungal pathogen appears to be *Chamaeleomyces granulomatis*. This fungus has been reported in a stock of captive-bred chameleons (*Chamaeleo calyptratus*) and causes a disseminated systemic mycosis (Schmidt, 2015; Sigler et al., 2010).

Situating the problem

Even though it is clear that dermatomycoses in reptiles caused by CANV are an emerging, significant problem, there is still of lot of information that is lacking. One main issue is that the source of infection in the reported cases of infection caused by CANV has not yet been identified. As stated earlier, studies do indicate that CANV is not a common constituent of the mycobiota of the reptilian skin and Koch's postulates have been fulfilled in veiled chameleons (Paré et al., 2006a). It's prevalence in the environment, perhaps a probable a source of infection, is not known as well as it's virulence factors. The CANV do seem to be able to act as a primary pathogen, however studies also suggest that environmental stressors are likely predisposing factors (Allender et al., 2015b; Lorch et al., 2015; Paré et al., 2006b). Additionally the range of susceptible reptile species is still ill defined.

For SFD there are some additional questions that remain unanswered. For example it is still undetermined whether the fungus is an introduced pathogen or whether it is native to North-America an currently emerging due to recent climate change or other yet to be determined factors (Franklinos et al., 2017). Other fundamental questions such as the source of infection, mode of transmission, environmental influence and effective treatment options still need to be investigated. Estimating the prevalence in populations also appears to be a challenge as there are large numbers of false negative results when using qPCR for identification (Hileman et al., 2018). More sensitive techniques might be necessary to correctly asses the epidemiology.
Along with the uncertainties described above, little is understood about the progression of the disease and how it influences individual and population fitness. Mortality and morbidity seem to vary between populations and species (Allender et al., 2015b; Hileman et al., 2018). Clinical signs are often recorded at a single time point, providing little information on the affected animals history, disease development and over time, mechanisms of the host for coping with the infections and indirect consequences on snake health and survival (Lorch et al., 2015).

All things considered further research is needed to investigate the source, epidemiology, clinical symptoms and lesions, diagnostics, and prophylactic and therapeutic possibilities for all species of CANV. These factors are necessary to gain a better understanding of the host-pathogen relationship.

Objectives of this study

The goal of this literature study is to gather, bundle and critically asses known information about fungal species causing dermatomycosis in squamate reptiles as well as identify gaps in research that demand more research in the future. Understanding the disease ecology is the first step to determining the approach to management and characterizing the epidemiology. All these are necessary to gain a better understanding of the interaction between the animals and the pathogens and come to a suitable prophylactic or curative treatment or identify possible preventive measures (such as disinfection of materials, quarantine, entry control for captive reptiles or strategies for tackling the threat of snake fungal disease). *Chamaeleomyces granulomatis* will not further be discussed in detail as the focus of this thesis will be on fungi belonging to the CANV-complex.

3. Etiology

Fungi are eukaryotic, non-photosynthetic micro-organisms widely distributed in the environment. They are heterotrophs that produce exoenzymes and obtain nutrients by absorption. Traditional classification is based mainly on morphology and sexual reproduction. Recently, classification based on DNA comparisons between species is used more frequently. The sexual stage of the fungal species is called the teleomorph, it's asexual stage is referred to as the anamorph. The teleomorph and anamorph stage of the same species often have different names. This dual naming system resulted from many fungi being identified before their sexual reproductive stage was discovered. The anamorphic names are often better known due to this stage being the one causing disease. Fungi present in one of two forms, as moulds or as yeasts. Moulds are recognized by their growth as branching filaments called hyphae. Yeasts are unicellular and have an oval or spherical shape. Some fungi can present as either form and are called dimorphic fungi. Reproduction takes place using spores, which may be either sexual or asexual. Some species can form both types of spores. Fungi grow aerobically and many are strict aerobes. Different groups of fungi have different temperatures needed for optimal growth. Airborne fungal spores germinate when the environmental conditions are suitable to grow for that species. Spores start to swell and increase their metabolic activity before producing tubular projection which develop into branched hyphae. Extension of the hyphae and their lateral branches form an interlacing network of hyphae called a mycelium. Large colonies are often formed with extensions of hyphae at the peripheries. Some species form specialized aerial hyphae which support spore-bearing structures, these aid in the dispersal of mature, asexual spores. Two different types of spores can be distinguished, namely conidia and sporangiospores. Sporangiospores are formed in a sac-like structure on the aerial hyphae called the sporangium. Conidia are formed on conidiophores. The formation of multi-cellular structures called macroconidia and single-celled microconidia from lateral hyphae branches are another form of asexual reproduction. Disintegration of hyphae within keratinized structures form arthroconidia. The sexual stages of fungi are usually demonstrated in specialized laboratories. Mechanisms involved in fungal disease include tissue invasion (mycosis), toxin production (mycotoxicosis) and induction of hypersensitivity. Fungi can be saprophytic, mutualistic or parasitic. Mutualistic fungi have associations with other microorganisms and are non-pathogenic. The parasitic fungi are the classic dermatophytes, causing ringworm in mammals. The large majority of fungi are saprophytic and are involved in decomposition of organic materials. These saprophytes are widespread and cause sporadic opportunistic in animals (Quinn et al., 2002).

Fungal diseases in reptiles have been frequently documented in all orders and are often described as being opportunistic pathogens, only invading living tissues under favorable circumstances (Paré et al., 2006b; Schmidt, 2015). Infections with *Fusarium* spp, *Trichosporon* spp, *Geotrichum* spp and *Penicillium* spp. that have sporadically been described as mostly opportunistic infections (Hoppmann and Barron, 2007) will not be discussed further. The focus of this thesis will be on those fungi that are frequently involved in clinical skin infections in reptiles.

3.1. Fungal taxa causing dermatomycosis in reptiles

3.1.1. The *Chrysosporium* Anamorph of *Nannizziopsis vriesii* complex

The *Chrysosporium* anamorph *of Nannizziopsis vriesii* complex or CANV-complex is a complex of fungi often encountered as a pathogen present in dermatological lesions in reptiles. In the past fungi present in reptiles have often been deemed as secondary pathogens, purely taking advantage of weakened defence-systems (Hoppmann and Barron, 2007; Mitchell and Walden, 2013; Paré et al., 2006b; Schmidt, 2015). The CANV-complex seems to defy this statement, and in the last decade or so they have come to light as prominent reptile fungal pathogens in captive as well as in wild reptiles (Cabañes et al., 2014; Mitchell and Walden, 2013). However, it might not truly be an emerging reptile pathogen, as it has probably been misdiagnosed, undiagnosed or unrecognized for years (Paré, 2014). The fungal isolates that are so often found in dermatological lesions have been named as the CANV-complex due to morphological similarities of this fungus to the anamorph state of *N.vriesii*. The sexual stage (teleomorph) has however not yet been found in any of the cases, making it difficult to assess the true

relation to *N.vriesii* (Cabañes et al., 2014; Sigler et al., 2013). However, recent molecular studies have given more insight into the relationship and taxonomic classification (Sigler et al., 2013; Stchigel et al., 2013). The species belonging to this complex are a member of the order of the Onygenales, family *Onygenaceae* (Sigler et al., 2013). Other fungal species like *Blastomyces*, *Coccidioides*, dermatophytes, *Histoplasma* and *Paracoccidioides* belong to this same order (Cabañes et al., 2014). Reports of infection with CANV can be found worldwide with cases documented in North America, Europe, Asia and Australasia (Sigler et al., 2013). Most of the infections from this complex have been in the squamate reptiles (consisting of lizards, snakes and amphisbaenians). To a lesser extent infections have also been reported in crocodilians and tuataras.

Recently, numerous revisions have been made in fungal classification. Molecular and other techniques are used in order to re-examine species leading to taxonomic revisions and even revealing new, undescribed species (Paré et al., 2006b). Using small subunit ribosomal ribonucleic acid (SSU) and internal transcribed spacer (ITS) analyses, the isolates have been grouped into three individual clades or lineages. These lineages are distinct form all *Chrysosporium* species and other taxa. The three lineages include the *Nannizziopsis, Paranannizziopsis* and *Ophidiomyces* (Sigler et al., 2013).

Currently there are twelve species described in the *Nannizziopsis* clade: *Nannizziopsis dermatitidis, Nannizziopsis crocodili, Nannizziopsis barbata, Nannizziopsis guarroi, Nannizziopsis draconii, Nannizziopsis chlamydospora, Nannizziopsis arthrosporioides, Nannizziopsis pluriseptata, Nannizziopsis hominis, Nannizziopis obscura, Nannizziopsis infrequens* and *Nanniziopsis vriesii* (Sigler et al., 2013; Stchigel et al., 2013). These species have been described in lizards and humans. *N.guarroi* (previously known as *Chrysosporium guarroi*) and *N.dermatitidis* appear to be the major pathogens for lizards. Bearded dragons (*Pogona vitticeps*) and green Iguanas (*Iguana iguana*) mainly seem susceptible to *N.guarroi*. Chameleons and gecko's seem to be most susceptible to *N.dermatitidis. N.hominis, N.obscura* and *N.infrequens* have only been isolated from humans, not from reptiles. This suggests that zoonotic transmission is not of great concern and handling of reptiles infected with these pathogens is not dangerous for healthy individuals (Cabañes et al., 2014; Sigler et al., 2013; Stchigel et al., 2013). Morphological and physiological descriptions as well as molecular data for all these species are available.

The lineage of the *Paranannizziopsis* is distinguished from the other two clades by the uncommon occurrence or absence of arthroconidia (Sigler et al., 2013). At this moment known species include *Paranannizziopsis crustacea, Paranannizziopsis californiensis, Paranannizziopsis australasiensis and Paranannizziopsis tardicrescens. P.crustacea* and *P.californiensis* have both been isolated from outbreaks in tentacled snakes kept in zoological constitutions in North America. *P.australasiensis* has a more ill-defined host range affecting tuataras, file snakes and a lizard, all cases occurred in Australasia (Sigler et al., 2013). Most recently *P. tardicrescens* has been identified as the newest member of this clade from several snakes species in North America (Rainwater et al., 2019).

The final clade consists only of *Ophidiomyces ophiodiicola* which has thus far only been isolated from snakes, both wild and captive (Sigler et al., 2013).

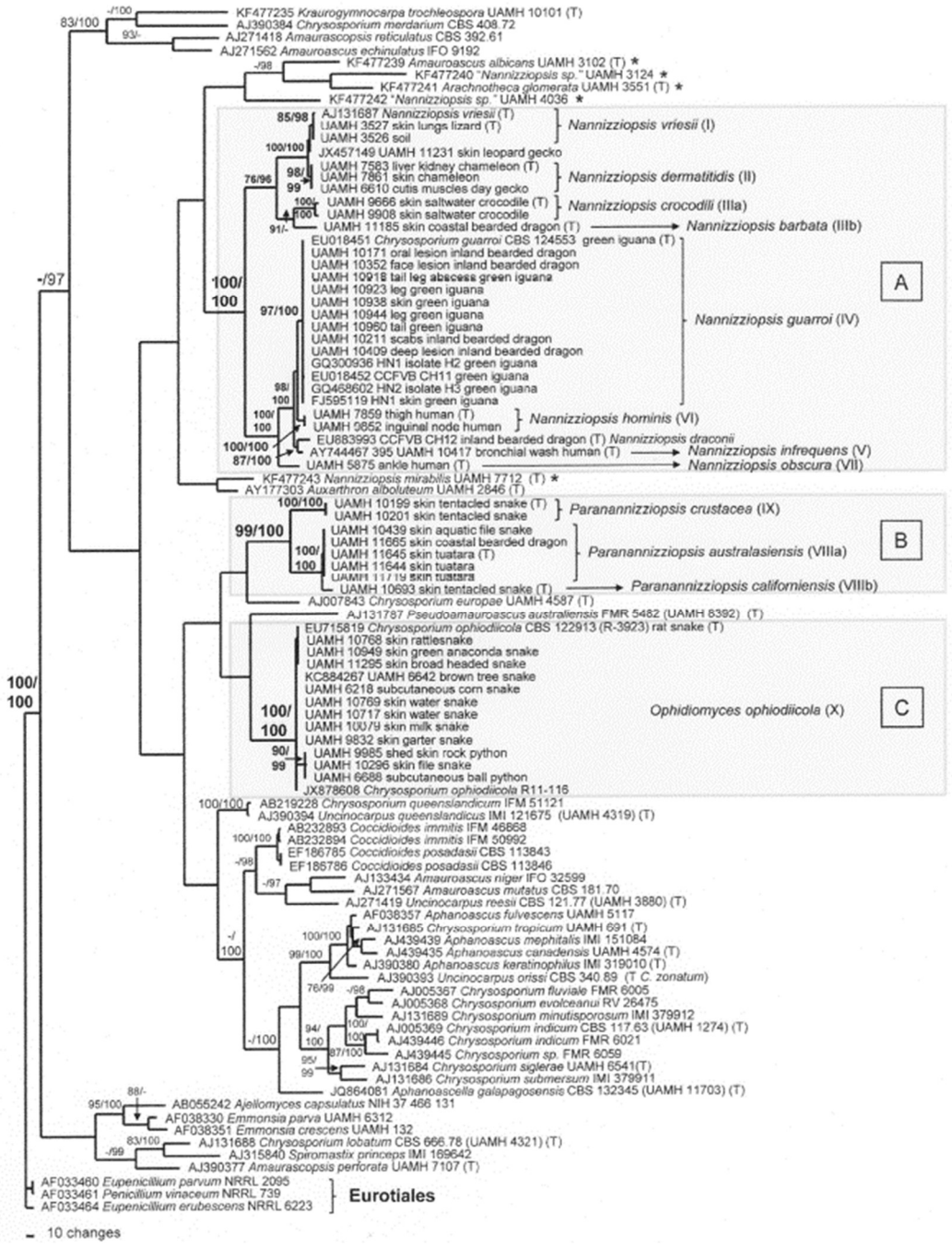


Fig 1. Recent molecular characterization lead to a new phylogenic characterization with division in three major clades: *Nannizziopsis* (A), *Paranannizziopsis* (B) and *Ophidiomyces* (C) (Sigler et al., 2013)

3.1.2. Dermatophytosis

Dermatophytosis is commonly seen in mammals, but rarely in reptiles. Fungi belonging to the genera *Trichophyton* and *Microsporum* are the only fungi causing a true dermatophytosis. From these dermatophytes only *Trichophyton mentagrophytes* has been reported (Paré et al., 2006b). The only confirmed case is of a co-infection with mites in Iran in Iguanas. The lizards showed a mixed infection of mites (*Geckobiella donnae*) and a disseminated cutaneous infection with *T.mentagrophytes* var. *mentagrophytes (Sharifzadeh et al., 2016).* Furthermore, the fungus has been implicated as the cause of dermal lesions in a Ball phyton (*Python regius*). However this fungus has yet to be re-examined to confirm its identification (Paré et al., 2006b). *T.mentagrophytes* var. *interdigitale* was also isolated from green Iguanas causing severe dermatophytosis (Khosravi et al., 2012). Other reports often involve geophilic keratinophilic fungi that may be isolated as contaminants and the pathogenic role of the fungus was not confirmed (Paré et al., 2006b).

3.1.3. *Chamaeleomyces granulomatis* and *Chamaeleomyces viridis*

Another true fungal pathogen appears to be *Chamaeleomyces granulomatis*. This fungus has been reported in a stock of captive-bred chameleons (*Chamaeleo calyptratus*) and causes a disseminated systemic mycosis. Lesions are often seen on the tongue and pharynx. *Chamaeleomyces viridis* gives a similar clinical appearance and has caused disseminated systemic mycoses in carpet chameleons (*Furcifer lateralis*), panther chameleons (*Furcifer paradalis*) and bearded dragons (Schmidt, 2015; Sigler et al., 2010). Disease caused by this fungus will not be discussed further, the focus of this thesis will be only on fungi of the CANV-group.

4. Clinical presentation

4.1. Nannizziopsis

4.1.1. Symptoms and lesions

Clinical signs of reptiles infected with CANV can differ between species, but are mostly associated with the integument. In some cases the clinical signs are order or genera specific (Mitchell and Walden, 2013).

In bearded dragons (*Pogona vitticeps*) a disease syndrome commonly referred to as 'yellow fungus disease' (YFD) has emerged with *Nannizziopsis guarroi* suggested as the primary etiological agent (Paré and Sigler, 2016). The disease gets its name from the lesions that first occur as multifocal yellow discoloration of the skin (Mitchell and Walden, 2013). Any part of the body can be affected, but the yellow crusts are mainly found on the lateral sides of the head, around the oral cavity, on the tail and pericloacal (fig. 2)(Hellebuyck and Martel, 2012; Johnson, 2004; Mitchell and Walden, 2013). The animals are often alert and have a good appetite (Abarca et al., 2009; Bowman et al., 2007). As the disease progresses the lesions transform into vesicles, bullae and eventually become necrotic with a dark thickened crust (Hellebuyck and Martel, 2012). The formed crusts may fall of exposing the underlying dermis. Over time infection can extend through the dermis into deeper layers and may disseminate, often resulting in a fatal outcome (Bowman et al., 2007; Cabañes et al., 2014; Johnson, 2004; Mitchell and Walden, 2013; Paré and Sigler, 2016). It is important to note that in bearded dragons yellow patches are not exclusive to this disease, bacterial and viral infections can give a similar appearance (Johnson, 2004). Systemic infections with fungal granuloma's involving the liver have been described in bearded dragons, but appear to be rare (Schmidt-Ukaj et al., 2014; Schmidt, 2015). Other fungi associated with YFD in bearded dragons are *N.draconii and N.chlamydospora* (Johnson et al., 2011; Stchigel et al., 2013).

Fig. 2 bearded dragon with lesions around the oral cavity (Bowman et al., 2007)

N.guarroi has also been described as a pathogen in several green Iguanas in Spain and South Korea. The symptoms in Iguanas are similar to those in bearded dragons with cases of superficial dermatomycoses and other more severe cases with the infection spreading into deeper layers such as muscle and bones. The tail and limbs are commonly affected (fig. 3) (Abarca et al., 2008; Cabañes et al., 2014; Han et al., 2010; Hellebuyck and Martel, 2012).

Fig. 3 Affected green iguana (Hellebuyck and Martel, 2012)

In chameleons *Nannizziopsis dermatitidis* is a potent fungal pathogen, causing extensive and severe skin lesions. Focal black or grey discoloration can be observed as well as (Paré et al., 1997).
All published cases thus far with fungi of the genus *Nannizziopsis* have been in captive lizards, with exception of *N.guarroi* isolated from an unnamed snake in the USA. (Stchigel et al., 2013). There are no reports yet of *Nannizziopsis* in wild, free-roaming lizards.

4.1.2. Host range

When looking at the CANV-complex there is a wide variation of reptiles who are susceptible for infection. Within the genus of *Nannizziopsis* there is a broad range of different species of fungi as well as a broad host-range. Some species seem to be host or genera specific, as demonstrated in table 1 lizards appear to be most affected by *Nannizziopsis.*

Fungus	(reported) susceptible species		Wild/captive, Country	
	Common name	***Scientific name***		
Nannizziopsis				
N.guarroi	Green iguana	*Iguana iguana*	Captive, Germany, Spain, South Korea	(Abarca et al., 2008; Abarca et al., 2010; Han et al., 2010; Johnson, 2004; Schneider et al., 2018; Sigler et al., 2013)
	Inland bearded dragon	*Pogona vitticeps*	Captive, Germany, USA	(Abarca et al., 2009; Hedley et al., 2010; Johnson et al., 2011; Le Donne et al., 2016; Paré and Sigler, 2016; Schmidt-Ukaj et al., 2014; Schneider et al., 2018; Sigler et al., 2013)
	Common agama	*Agama agama*	Not specified, USA	(Stchigel et al., 20130
	European green lizard	*Lacerta viridis*	Captive, Germany	(Schneider et al., 2018)

N.chlamydospora	Inland bearded dragon	*Pogona vitticeps*	Captive, USA, South-Korea	(Paré and Sigler, 2016; Rhim and Han, 2019; Stchigel et al., 2013)
N.draconii	Inland bearded dragon	*Pogona vitticeps*	Captive, Spain	(Stchigel et al., 2013)
N.barbata	Coastal bearded dragon	*Pogona barbata*	Captive, Australia	(Johnson et al., 2011)
	Eastern water dragon	*Intellagama lesueurii*	Captive, USA	(Stchigel et al., 2013)
N.dermatitidis	Day Gecko	*Phelsuma sp.*	Captive, Madagascar	(Sigler et al., 2013)
	Veiled Chameleon	*Chamaeleo calyptratus*	Captive, Denmark, USA	(Paré et al., 2006a; Sigler et al., 2010; Sigler et al., 2013)
	Leopard gecko	*Euplepharis macularius*	Captive, USA	(Paré et al., 1997; Toplon et al., 2013)
	Parson's chameleon	*Calumma parsoni*	Captive, Canada	(Paré et al., 1997)
	Jewel chameleon	*Furcifer lateralis*	Captive, Canada	(Paré et al., 1997)
	Jackson's chameleon	*Trioceros jacksoni*	Captive, Canada	*(Paré et al., 1997)*
N.pluriseptata	South eastern five-line skink	*Eumeces inexpectatus*	*no details of a pathogenic process or procedures for fungal isolation described, USA	(Stchigel et al., 2013)
N.arthrosporiodes	Water dragon	*Physignathus sp.*	*no details of a pathogenic process or procedures for fungal isolation described, USA	(Stchigel et al., 2013)
N.crocodili	Saltwater crocodile	*Crocodylus prosus*	Captive, Australia	(Thomas et al., 2002)
	Fresh water crocodiles	*Crocodylus johnstoni*	Captive, USA	(Hill et al., 2019)
N.vriesii	Girdled lizard	*Cordylus giganteus*	Captive, Belgium (suspected to be misdiagnosed, likely to have been *N.guarroi*)	(Hellebuyck et al., 2010)
	Ameiva lizard	*Ameiva chaitzami*	Captive, USA	(Martel et al., 2006; Sigler et al., 2013; Stchigel et al., 2013)

Table 1. *Nannizziopsis* species and their respective reported hosts.

4.2. Paranannizziopsis

4.2.1. Symptoms and lesions

P. crustacea, P.californiensis and P.longispora have only been isolated from Tentacled snakes. Infection starts as multiple, small, pale yellow-white skin lesions present on the head and dorsum. The outcome is often fatal due to cutaneous damage causing an osmotic imbalance (Bertelsen et al., 2005). A condition known as “white spot fungus” in file snakes has been described in the pet trade (Paré and Sigler, 2016). Perhaps the same fungus is responsible for this disease, but further research is required. In contrast*, P.australasiensis* has been isolated from a broader host range. Lizards, snakes and even tuatara have been reported to be susceptible to infection. As the name suggests, *P.australasiensis* has only been isolated in Australia and New Zeeland. This fungus has been isolated mostly from tuatara. The disease appears less severe in tuatara than in the file snakes and coastal bearded dragons. The dermatomycosis shows as the characteristic yellow to brown encrustations on the skin. Research remains to be done about the presence of *P.autralasiensis* in wild tuatara on the islands of new-Zeeland (Masters et al., 2016). When monitoring the health of tuataras at Auckland Zoo over several years more than 50% of the tuataras intermittently showed signs of a mild to severe dermatitis with brown discoloration of scales of the ventrum and flanks and at times progressing to a necrotising ulcerative dermatitis. Fungal infection was expected, but a definite aetiological agent was never determined. *P.australasiensis* could have been the underlying pathogen implying that the fungus may have been present in captive tuataras for years, with the possibility of sub-clinically infected animals. Whether it is an imported exotic pathogen or whether it is endemic in New Zealand is not known (Masters et al., 2016).

P.tardicrescens is the most recent isolate causing dermatitis in several snake species from a zoological institution in North-America (Rainwater et al., 2019).

4.2.2. Host range

Species belonging to *Paranannizziopsis* seem to have a much smaller range of hosts compared to *Nannizziopsis.* They have been isolated from reptiles in North America and Australasia (table 2).

Paranannizziopsis	reported susceptible species		Wild/captive, country	
	Common name	**Scientific name**		
P. australasiensis	Tuatara	*Sphenodon punctatus*	Captive, New Zealand	(Humphrey et al., 2016; Masters et al., 2016)
	Coastal bearded dragon	*Pogona barbata*	Captive, New Zealand	(Masters et al., 2016)
	File snake	*Acrochordus sp.*	Captive, Australia	(Sigler et al., 2013)
P.tardicrescens	Wagler’s Viper	*Tropidolaemus wagleri*	Captive, USA	(Rainwater et al., 2019)
	Tentacled snake	*Erpeton tentaculatum*	Captive, USA	(Rainwater et al., 2019)
	Rhinoceros snake	*Rhynchophis boulengeri*	Captive, USA	*(Rainwater et al., 2019)*
P.crustacea	Tentacled snake	*Erpeton tentaculatum*	Captive, Canada	*(Bertelsen et al., 2005; Sigler et al., 2013)*
P.californiensis	Tentacled snake	*Erpeton tentaculatum*	Captive, USA	*(Bertelsen et al., 2005; Sigler et al., 2013)*

P.longispora	Tentacled snake	*Erpeton tentaculatum*	Captive, USA	(Bertelsen et al., 2005; Sigler et al., 2013)

Table.2 Paranannizziopsis species and their respective reported hosts.

4.3. *Ophidiomyces ophiodiicola*

4.3.1. Symptoms and lesions

Symptoms caused by *O.ophiodiicola* are referred to as "snake fungal disease" (SFD). The clinical signs of this disease can be referred to as a deep granulomatous fungal dermatitis (Paré and Sigler, 2016). Signs are mainly associated with the head, but can occasionally be found on other parts of the body (Mitchell and Walden, 2013). In a study done by Lorch and colleagues captive bred corn snakes (*Pantherophis guttatus*) were inoculated with the fungus on the snout, chin, ventral neck, ventral body and dorsal body. The skin on the chin, ventral neck and dorsal body was abraded using sandpaper to determine whether abrasion of the skin is important in developing infection. Initial clinical signs consisted of swelling, mainly on the snout. On the body swelling was less apparent and more transient than on the head. Scales became oedematous and progressively turned yellow and thickened. Eventually crusts appear with hyperpigmentation. Skin lesions got progressively more severe, until the snakes shed on day 15-20. At the start of ecdysis fluid accumulated between the old and new skin, mainly on and around the inoculation sites. At the head, this pile up of fluid occasionally lead to distortion of the head. Granulomas were sometimes seen, mostly on the head. Granulomas on the head can lead to extensive disfiguring facial lesions, with possible obstruction of the nasolabial pits and misalignment of the mandibular and maxilla (Allender et al., 2011; Lorch et al., 2015). As these lesions progress they may interfere with normal feeding habits causing the snake to become anorexic and emaciated which will eventually have a fatal outcome (Paré, 2014). Infection may spread to the eyes, giving the eye a diffusely opaque look (not related to ecdysis)(Allender et al., 2011; Dolinski et al., 2014; Ohkura et al., 2016). Erythema, vesicles and plaque and crust formation are commonly seen. Granuloma's in deeper layers such as muscles or in organs are possible (Cheatwood et al., 2003). Dysecdysis occurred occasionally and has also been reported in wild Massasauga rattlesnakes (Tetzlaff et al., 2015). The lesions cleared up after ecdysis, yet some scales remained shrunken, deformed or slightly depigmented. As new skin often appears clinically normal after moulting, this suggests that ecdysis clears the infection if it is limited to the superficial epidermis. If the infection has progressed enough before moulting and infected the new epidermis disease may reoccur. At locations where dysecdysis occurs reinfection may develop more easily. Lesions occurred more frequently at the abraded skin sites (100%) versus the non-abraded skin sites (62.5%). Snakes were inoculated again after moulting, each time with a higher dose of conidia. With the higher dose severity of the lesions did not differ, however non-abraded skin was more likely to develop gross lesions. Severity of the disease seems to differ between species. Eastern Massasauga rattlesnakes (*Sistrurus catenatus catenatus*) seem to be particularly sensitive and reports of disease in this species are abundant. On histological examination lesions consisted of necrosis and infiltration of granulocytes in areas of the epidermis. In the necrotic sites fungal hyphae were visible. In the dermis and muscles granulocytic to mononuclear inflammation was frequently seen. Granulomas were sometimes seen, mostly on the head. No disseminated fungal infections were present, these are also rarely seen in wild snakes (Lorch et al., 2015).

4.3.1.1 behavioural changes

Besides the progression of the disease shown clinically and histopathologically in this study, another aspect of the disease came to light. Exposure to *O.ophiodiicola* led to a higher moulting frequency and thus a shorter mean shed interval compared to the non-exposed control snakes. Rapid and successive moulting may be part of the host response in getting rid of the infection. Additionally, infected snakes were also significantly more found exposed in their cages as opposed to the control group who were more likely to be found in their hiding areas. Similar behaviour has been reported in wild ranging snakes, infected massasauga rattlesnakes moved around less compared to other asymptomatic snakes in the area (Tetzlaff et al., 2015).

The behavioural change seen in the experimental study has been further explored in a study concerning free-ranging massasauga rattlesnakes (Tetzlaff et al., 2017). While monitoring several behaviour parameters, a difference in behaviour between infected and non-infected snakes became apparent. Infected snakes moved less and were less exposed than healthy snakes. Uninfected snakes had lower body temperatures at the end of the active season before hibernation, whereas infected snakes still had higher body temperatures at the end of the season (22,5^{o}C and 28,0^{o}C respectively). Infected snakes basked near entrances of overwintering sites when many uninfected snakes were already below ground. Similar behaviour has been reported in wild-ranging timber rattlesnakes (McBride et al., 2015). Increasing body temperature by basking to induce a fever has been reported in snakes coping with infection to reduce pathogen load (Burns et al., 1996). These behavioural changes suggest it might be possible for *O.ophiodiicola* to influence underlying acclimation physiology and/or alter normal behavioural responses to environmental cues that stimulate the snake to overwinter (Tetzlaff et al., 2017). Another explanation for the need for infected snakes to bask relatively more when being closer to the overwintering period, is that immune function is reduced due to cooler temperatures in winter and this is the last opportunity for the snake to preform thermoregulation before this becomes virtually impossible for a couple of months (Tetzlaff et al., 2017).

4.3.2. Host range

In contrast to *Nannizziopsis and Paranannizziopsis* infections with *Ophidiomyces ophiodiicola* are well documented in captive and wild free-roaming snakes as demonstrated in table 3.

Ophidiomyces ophiodiicola	**reported susceptible species**		**Wild/captive, Country**	
	Common name	**Scientific name**		
	Timber rattlesnake	*Crotalus horridus*	Wild, USA	(Clark et al., 2011; Lorch et al., 2016; McBride et al., 2012; McBride et al., 2015)
	Eastern Massasauga rattlesnake	*Sistrurus catenatus catenatus*	Wild, USA	(Allender et al., 2011; Allender et al., 2013; Allender et al., 2016; Lindemann et al., 2017; Lorch et al., 2016; Tetzlaff et al., 2015; Tetzlaff et al., 2017)
	Eastern milksnake	*Lampropeltis Triangulum*	Wild, USA	(Lorch et al., 2016; Ravesi et al., 2016)
	Cottonmouth	*Agkistrodon piscivorous*	Captive, USA *after experimental infection	(Allender et al., 2015a; Lorch et al., 2016)
	Corn snake	*Pantherophis guttatus*	Captive *after	(Lorch et al., 2015; Sigler et al., 2013)

			experimental infection, USA	
	Brown tree snake	*Boiga irregularis*	Captive, USA	(Nichols et al., 1999; Sigler et al., 2013)
	Broad banded watersnake	*Nerodia fasciata confluens*	Wild, USA	(Glorioso et al., 2016; Lorch et al., 2016)
	Black rat snake	*Elahe obsolete obsolete*	Captive, USA	(Rajeev et al., 2009)
	Ball python	*Python regius*	Captive, UK	(Sigler et al., 2013)
	Garter snake	*Thamnophis sp.*	Captive, Germany	(Ohkura et al., 2016; Sigler et al., 2013)
	African rock python	*Python sebae*	Captive, USA	(Lorch et al., 2016; Sigler et al., 2013)
	Milk snake	*Lampropelti sp*	Captive, USA	(Sigler et al., 2013)
	File snake	*Acrochordus sp.*	Captive, Australia	(Sigler et al., 2013)
	Salt marsh snake	*Nerodia clarkia*	Wild, USA	(Lorch et al., 2016)
	Eastern diamondback rattlesnake	*Crotalus adamanteus*	Not specified, USA	(Sigler et al., 2013)
	Green anaconda	*Eunectes murinus murinus*	Captive, USA	(Sigler et al., 2013)
	Broad-headed snake	*Hoplocephalus hungaroides*	Captive, Australia	(Sigler et al., 2013)
	North-American racer	*Coluber constrictor*	Wild/captive, USA	(Lorch et al., 2016; Ohkura et al., 2016)
	Red-bellied mud snake	*Farancia abacura*	Wild, USA	(Last et al., 2016; Lorch et al., 2016)
	Eastern black kingsnake	*Lampropeltis nigra*	Wild, USA	(Lorch et al., 2016)
	Common watersnake	*Nerodia sipedon*	Wild, USA	(Guthrie et al., 2016; Lorch et al., 2016)
	Brown watersnake	*Nerodia toxispilota*	Wild, USA	(Guthrie et al., 2016)
	Eastern rat snake	*Pantherophis alleghaniensis*	Wild/captive, USA	(Lorch et al., 2016)
	Red cornsnake	*Pantherophis guttatus*	Captive, USA	(Sigler et al., 2013)
	Eastern foxsnake	*Pantherophis vupinus*	Wild, USA	(Lorch et al., 2016)
	Foxsnake sp.	*Pantherophis sp.*	Wild, USA	(Lorch et al., 2016)

	Bullsnake	*Pituophis catenifer sayi*	Wild, USA	(Lorch et al., 2016)
	Louisiana pinesnake	*Pituophis ruthveni*	Wild, USA	(Lorch et al., 2016)
	Queensnake	*Regina septemvitata*	Wild, USA	(Lorch et al., 2016; Price et al., 2015)
	Western ribbonsnake	*Thamnophis proximus*	Wild, USA	(Lorch et al., 2016)
	Plains gartersnake	*Thamnophis radix*	Wild, USA	(Dolinski et al., 2014)
	Common gartersake	*Thamnophis sirtalis*	Wild, USA	(Lorch et al., 2016)
	Smooth earthsnake	*Virginia valeriae*	Wild, USA	(Lorch et al., 2016)
	Copperhead	*Agkistrodon contortrix*	Wild, USA	(Lorch et al., 2016)
	Dusky pygmy rattlesnake	*Sistrurus miliarius barbouri*	Wild, USA	(Lorch et al., 2016)
	Grass snake	*Natrix natrix*	Wild, Switzerland, UK	(Franklinos et al., 2017; Meier et al., 2018)
	Adder	Vipera berus	Wild, UK	(Franklinos et al., 2017)
	Dice snake	*Natrix tessellata*	Wild, Czechs Republic	*(Franklinos et al., 2017)*

Table 3 Ophidiomyces ophiodiicola and its reported hosts.

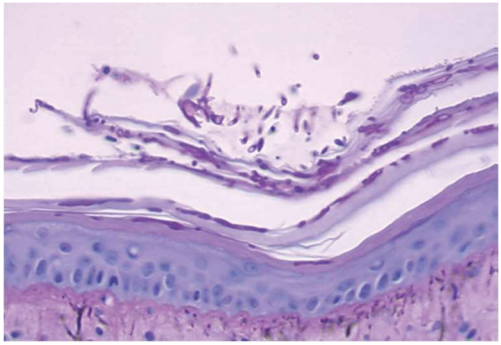
5. Pathogenesis

Dermatological problems are common in reptiles, but are primarily considered the consequence of substandard husbandry or underlying disease (Hellebuyck et al., 2013). Fungi are no exception, numerous fungi have been isolated from skin lesions in reptiles in both epidermis as well as deep layers of the dermis (Harkewicz, 2001). Unsuitable environmental conditions and malnutrition are often contributors to fungal infections (Harkewicz, 2001). However, this general consensus is being challenged by fungi belonging to the CANV complex. Very little is known about the pathogenesis of this disease, but due to the emergence of this pathogen in captive as well as in wild reptiles there is a need for more information on the course and pathogenesis of this disease.

There are a couple of studies that suggest that CANV is not merely an opportunistic infection only taking advantage of necrotic tissue.

5.1, *Nannizziopsis*

Koch's postulates are used to determine whether there is a causative relationship between a microbe and a disease. These postulates have been fulfilled for CANV for at least some reptiles species. The first study testing the potential for CANV to act as a primary pathogen and confirming Koch's postulates was done by Paré and colleagues. In this study (Paré et al., 2006a) veiled chameleons (*Chamaeleo calyptratus*) were either exposed to conidia of *Nannizziopsis dermatitidis* in their environment, or directly inoculated on either intact or abraded skin. Dermatomycosis consistent with CANV was seen in all groups and *N.dermatitidis* was then again recovered from the lesions, fulfilling the postulates. A breach in the skin did increase the probability of infection, but was not required for infection. Penetrating wounds, ticks and other external parasites could therefore be important facilitating factors. The chameleons were euthanized at different times to study the various phases of infection histologically. Infection started with proliferation of hyphae in the superficial keratinous epidermal layers. Hyphal proliferation than continued invading the deeper epidermis eventually breaching the basement membrane. From there infection usually spread in the dermis, through the hypodermis into the muscle layer. An inflammatory reaction from the body was visible with dermal oedema and infiltration of heterophils, lymphocytes, plasma cells and some macrophages. Bacterial colonies were also commonly seen in the ulcerated necrotic foci.



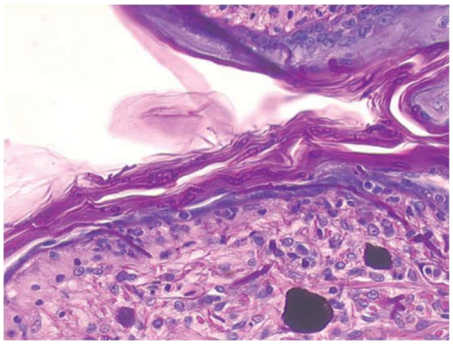


Fig 4: infection of the superficial epidermis. Fig 5: deep fungal penetration in the dermis (Paré et al., 2006a)

Infected animals also showed dense clusters of arthroconidiating hyphae on the skin surface. Late in the study the fungus was also isolated in the environment (cage top filters, environmental cultures). Both the arthoconidia and the detection of the fungus in the environment illustrate that spread through fomites in the captive environment and spread of disease between animals might be possible.

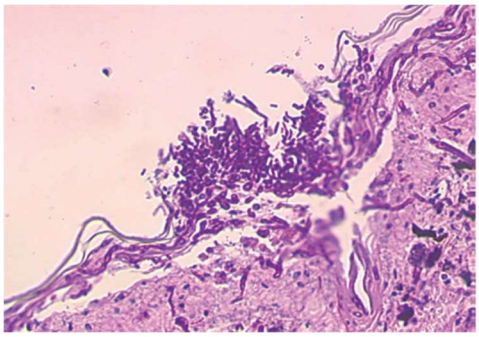


Fig 6. dense clusters of arthroconidia on the surface of the skin (Paré et al., 2006a)

As Koch's postulates have been fulfilled for veiled chameleons, it is expected that CANV can act as an pathogenic fungus in other lizard species as well.

5.2. *Ophidiomyces*

Another two studies have fulfilled Koch's postulates for *O.ophiodiicola* as a primary pathogen for snake fungal disease in cottonmouths (*Akistrodon piscivorous*)(Allender et al., 2015a) and in corn snakes (*Pantherophis guttatus*)(Lorch et al., 2015). *O.ophiodiicola* has frequently been isolated in cases of SFD, but other fungal organisms have been recovered those in cases, raising doubt whether it is really the sole primary pathogen in this disease (Allender et al., 2015c; Barber et al., 2016; Schmidt, 2015).
In the first study (Allender et al., 2015a), cottonmouths were inoculated in the nasolabial pits with a polypropylene catheter. Samples were subsequently collected twice a week using saline flushes in one nasolabial pit and using cotton-tipped swabs in the other. All inoculated snakes development lesions consistent with SFD, but only on the side where cotton-tipped swabs were used for sample collection, suggesting pathogenesis requires an abrasion of the epithelium to facilitate an infection. One of the control snakes also developed a mild lesion in the nasolabial pit sampled with a swab, but no fungal hyphae were observed. The most common clinical sign in infected snakes in this study was facial swelling, similar as in case reports of snakes with SFD. However, the severity of the clinical signs was less than in natural infection. Lesions in inoculated snakes consisted of heterophilic dermatitis with erosion or ulceration in the surrounding area of the pit. A serocellular crust covered the area. In some snakes infection spread to deeper tissues causing fasciitis, myositis, osteomyelitis and heterophilic granulomas. 13 days after inoculation the first clinical signs appeared, but only from day 31 clinical signs were consistently observed.
In the second study examining the causal relationship between *O.ophiodiicola* and SFD (Lorch et al., 2015) Kochs postulates were fulfilled as well. Isolates identical to the strain of *O.ophiodiicola* used for inoculation were recovered at necropsy.

5.3. *Paranannizziopsis*

Thus far no research has been performed concerning the role and pathogenesis of *Paranannizziopsis* as a primary pathogen. In aquatic species such as tentacled snakes integrity of the skin is extremely important as cutaneous damage could lead to passive influx of water causing oedema and osmotic imbalance ultimately leading to death. Skin lesions may also lead to secondary bacterial invasion causing sepsis and death. This was also presumed to be the cause of death in infected tentacled snakes in North America (Bertelsen et al., 2005). Those particular snakes were possibly housed in suboptimal conditions, as the pH level of the water was ≥8, whereas water in their natural habitats is acidic with pH-levels of <7. This may have predisposed the snakes for infection, and *Paranannizziopsis* may merely have acted as an opportunistic pathogen (Bertelsen et al., 2005; Rainwater et al., 2019).
Where the course of disease with *Paranannizziopsis* has been described as rapidly developing and often fatal in file snakes as well as in a coastal bearded dragon, tuataras appear to be more resistant to infection and lower fatality rates are reported, though sufficient data in this species is lacking to draw any conclusions at this stage. Differences in pathogenicity could be due to a number of factors such as environmental factors, host-specific sensitivity or difference in pathogenicity in different strains of the fungus. Body temperatures of tuataras are generally lower than those of other reptiles and can vary

strongly between seasons. It is possible that these lower temperatures lower growth rates and virulence of the fungus (Masters et al., 2016).
So in general infection with isolates of the CANV complex seem to be more frequent when the outer layer of the skin of a susceptible host species is breached. Although the abrasion facilitates infection, infections can also develop on intact skin. However, in this case higher doses of the infective stage might be needed. Koch's postulates have been fulfilled in three different species, CANV being a true pathogen of reptiles is therefor likely.

6. Epidemiology

In addition to the pathogenesis, the source of infection and epidemiology of the disease is also frequently discussed and not clear. Knowing the source of infection is important in the context of prevention and spread of the disease.

6.1 Presence of CANV on healthy reptile skin

The first possibility is that the fungus is a common constituent of the skin of reptiles and can lead to infection when the immune system is compromised or that asymptomatic carriers spread the fungus to susceptible individuals. There are a couple of studies that suggest that CANV is not merely an opportunistic infection only taking advantage of necrotic tissue. First of all the amount of cases of dermatomycoses caused by CANV are relatively high compared to reports of infections with fungi frequently found on reptilian skin (Paré et al., 2006a). One study (Paré et al., 2003) set out to evaluate common constituents of the mycobiota of healthy squamate reptiles. Shed skin of 36 lizards and 91 snakes was collected and placed on fungal culture and identified by microscopic observation and colonial features. An average of 4.2 genera of fungi were cultured per healthy reptile, *Aspergillus spp., Penicillium spp.* and *Paecilomyces lilactinus* were most frequently isolated. All of these species are common environmental fungi found on the reptile integument and have previously been described in literature as occasional causes of dermatomycosis. CANV was only isolated from only one animal, a African rock python (*Python sebae*), suggesting that there is a low prevalence of CANV on healthy squamate reptile skin. This suggests that exposure to CANV often leads to infection. However, the shed skins were sent through the mail, thus making it possible that environmental factors such as temperature, humidity or competition with other microflora could have affected the results of the study (Mitchell and Walden, 2013). Moreover when a more sensitive detection method was used (TaqMan real-time polymerase chain reaction) to search for *Ophidiomyces ophiodiicola* on snakes showing symptoms of dermatomycosis as well as healthy asymptomatic snakes, low levels of the fungus were detected on approximately 6% of the asymptomatic snakes, suggesting that presence of the fungus is not strictly correlated with disease (Bohuski et al., 2015). More studies are needed to further evaluate the presence of CANV on the skin of healthy reptiles and determine more likely sources of exposure.

6.2. Spread of infection between individuals

Important to note is that one study (Tetzlaff et al., 2017) showed that infected free-ranging massasaugas all overwintered in a condensed area as opposed to uninfected individuals. Some infected individuals even shared the same burrow. It cannot be excluded that the disease is spread between individuals in such communal burrowing sites. Anecdotal reports of Yellow fungus disease spreading between individuals may support that this is a potent way for the disease to spread (Paré et al., 2006a; Paré and Sigler, 2016). It is likely infection with all three genera of CANV are contagious, as almost all species produce arthroconidia in culture as well as in infected cutaneous tissues. Arthroconidia are thought to be the primary propagules for spread of the disease between individuals (Sigler et al., 2013). It is unclear where exactly the pathogen initially emerged from. As infections with *Nannizziopsis* have thus far only been reported in captive reptiles, the worldwide pet trade may be important in the spread of the disease. *N.guarroi* was first isolated from captive green Iguana's in Spain, this case coincided with the first reports of YFD in inland bearded dragons in the rest of Europe. Subsequently the fungus has also been isolated from pet inland bearded dragons with YFD in North America. It is possible that infection spilled over to/from green iguana's to/from bearded dragons through the pet trade (Sigler et al., 2013). More research is needed focussing on the relationship between spread of the disease between individuals.

For *Ophidiomyces* it is possible that even though most reports of this disease are quite recent, the fungus has been around in North America for longer. Over the last couple of decades field biologists have reported a condition referred to 'hibernation blisters' or 'hibernation sores' in snakes emerging from hibernation (Branson and Baker, 1974; Clark et al., 2011; Fitch, 1963; Jacobson, 1980; Page, 1966). This condition consist of dermatitis and has rarely been investigated properly. When examined more closely, one study showed that 41% of snakes had these lesions when coming out of hibernation, 74% of these snakes tested positive for SFD and histological findings were compatible with SFD as well. Although this does not prove *O.ophiodiicola* is the causative agent for these previously reported

'hibernation blisters' it does raise the question whether the fungus has been present for longer than initially suspected (Lorch et al., 2016).

6.3. Environmental transmission

Indirect infection and transmission through the environment is another possibility. Occurrence of SFD over the years has been at different times and in different places, direct transmission is there for not necessary (Allender et al., 2011). It is already established that abrasion of the skin facilitates infection (Lorch et al., 2015). Recovery of *N.dermatitidis* from settle plates and cage materials in experimental evaluation of pathogenicity in veiled chameleons, supports the probability that CANV isolates can disseminate fast in a captive environment (Paré et al., 2006a). Overwintering in condensed location in wild snakes may give a high environmental load increasing the risk of infection. Propagules on the skin or shed of sick animals could result in contamination of the soil or objects in touches (Paré et al., 2006a; Paré and Sigler, 2016; Tetzlaff et al., 2017). Environmental sampling in future research cases might be helpful to further clarify the distribution of the disease. A study surrounding the properties of *O.ophiodiicola* found several factors that make it likely that the fungus occurs in the environment. It's ability to utilize multiple complex carbon and nitrogen sources, to tolerate a wide pH-range and most naturally occurring sulphur compounds and to tolerate low matric potentials occurring in soil all support this possibility. The typical distribution of lesions on snakes around the head also point to substrate as a source of infection. Substrate in reptiles housing therefore probably plays an important role (Allender et al., 2015c; Mitchell and Walden, 2013).

An anthropogenic base as source for infection and dissemination is possible. *Chrysosporium* spp. have been associated with disease only in humans with underlying immunosuppression. Molecular data confirmed that none of the human isolates include *Nannizziopsis* species compromising the reptile isolates. Zoonotic potential of these fungi is therefore not a concern (Mitchell and Walden, 2013).. Human behaviours such as hiking and sampling for monitoring health in reptiles could however alter disease transmission. Therefore handling and sampling techniques should be done with care, as to not further the spread of the disease (Allender et al., 2015c; Cheatwood et al., 2003).

6.4. Pathogen-strains

To understand the epidemiology of SFD establishing the range of this pathogen is important in addressing research questions related to origins of the fungus and potential differences in virulence between strains (Franklinos et al., 2017). At the moment snake fungal disease is mainly present in snakes in eastern and Midwestern USA (Sutherland et al., 2014). SFD has been isolated from snakes in captivity for a longer period, but only as of 2006 it has frequently been reported in wild-ranging populations of snakes. The first report was in a population of pit vipers in eastern USA and later in a population of Eastern Massasaugas in Illinois in 2008 (Allender et al., 2015c). Although the global distribution *Ophidiomyces* has not yet been completely determined, most reports of infection with *O.ophiodiicola* have been in the USA. SFD had previously been reported in captive snakes outside North America, but up until recently there were no case reports of SFD in wild snakes elsewhere. A survey preformed 107 on moulted skin and snake carcasses from Great Britain and the Czech Republic done in 2017 revealed that the fungus is indeed present in wild snakes in Europe. Skin lesions were present in 23.8% of the examined samples and *O.ophiodiicola* was isolated through real-time PCR in 31% of these skin lesions. A positive sample from Czech Repbulic confirms that the fungus is also present in mainland Europe. In a later study the fungus was also isolated from free ranging grass snakes with obvious clinical lesions in Switzerland further corroborating this (Meier et al., 2018). One snake without macroscopic lesions (an adder (*Vipera berus*)) also tested positive, although it cannot be excluded that small lesions were missed upon examination. When the fungus was cultured, the European strain appeared to grow more slowly than those strains isolated in North America and a genetic distinction could be made between the two clades. This indicates that the European strain is different than that associated with SFD in North America and suggests that spread from North America to Europe through animal trade is not the cause for SFD emerging in Europe. It also makes it unlikely that Europe was the source of introduction to North America. The lesions in European snakes were mild to severe and were likely the indirect or direct cause of death for these snakes, this demonstrates that SFD is a threat to European wild snakes as well, though further surveillance and research is required to evaluate it's true

impact (Franklinos et al., 2017). As wildlife disease does not stop at borders it is realistic to expect the disease to be spread through the rest of Europe.

7. Ecology and conservation implications

CANV fungi causing infections in individual reptiles is concerning, but the effect on population level is just as alarming. As *O.ophiodiicola* and possibly *P.australasiensis* can be found, and cause disease in wild animals, this is cause for alarm as an emerging disease could further threaten sensitive species. Especially in light of the recent free-ranging wildlife epidemics associated with true fungal pathogens causing significant population decline in amphibians (chytridiomycosis) and bats (white nose syndrome), extra concern is warranted (Sutherland et al., 2014). Ecology and epidemiology of SFD on population levels appear to be complex and even though knowledge about CANV isolates ecology has increased over the years, long-term effects of SFD on snake populations remains largely unknown.

7.1. Disease dynamics

Dynamics of fungal emerging disease are driven by biotic and/or abiotic drivers, which often interact. The first category, biotic drivers as cause of emerging fungal disease comprise the ability of the fungus to adapt to its host or environment. This includes factors pertaining to the fungus such as virulence, reservoir, host-specificity and others. Factors pertaining to other organisms influencing the fungal pathogen are also part of the biotic drivers. Fungi possess the ability to respond rapidly to selection posed by challenging environments due to flexible genetic architectures, which allows them to emerge as disease in new hosts and environments. Fungi are often opportunistic and generalist pathogens with long-lived environmental stages which can lead to the ability to infect a broad spectrum of hosts (Fisher et al., 2016). This is clear in the case of SFD, as up till this day the fungus has been isolated from over 35 different snake species. Some of these species may act as amplifiers, vectors and/or reservoirs of infection (Fisher et al., 2016). A study (Allender et al., 2015c) demonstrated that *Ophidiomyces* is active at a wide range of pH (5-11) as well as being active at a wide range of temperatures. For all isolates growth inhibition happened at 7^{o}C and significant growth reduction took place at 14^{o}C. Optimal growth rates occurred at 25^{o}C and significant growth reduction was shown at 35^{o}C. Besides this wide pH and temperature range ability to utilize complex carbon, nitrogen and sulphur resources was shown. Due to these characteristics numerous ecosystems and species can be affected. The broad geographic and taxonomic distribution are worrying. Sensitive and quick diagnostic tests are important to discover reservoirs and are therefore essential in monitoring the spread of this fungus.

The second category, the abiotic drivers of emerging fungal infections (Fisher et al., 2016) include factors such as temperature, rainfall and pH. Climate change is one of the factors often considered of importance for the rise of SFD (Clark et al., 2011). The response of a host to a stressor, such as physiological responses to extreme climates can have a negative effect on other host responses such as immune-reactions. As changes in climates become more extreme the conflict between such responses to biotic and abiotic variables will become more pronounced (Fisher et al., 2016). Studies in bats and amphibians have shown that microclimates and their fungal loads in particular are a key determinant in developing disease. The presence of hibernation blisters and the higher prevalence of disease in snakes that hibernate in the same burrow is a manifestation of these abiotic drivers (Lorch et al., 2016). Slight raises in temperatures during brumation season and moist conditions due to increased rainfall could lead to *O.ophiodiicola* growing at faster rates and thus establish more severe infections (Allender et al., 2015c).

Changing environmental conditions influence different host species in different ways. Development of disease amongst different host species and different locations may not be explicable as being facilitated by just one specific set of environmental parameters. Environmental changes must therefore be considered at all sites, including microclimates at hibernation dens (Lorch et al., 2016). Habitat fragmentation increasing snake densities and inbreeding might also be plausible as abiotic drivers causing decline in population size (Clark et al., 2011; Lorch et al., 2016). These abiotic drivers could diminish overall health of snakes and exacerbate the effects of SFD (Lorch et al., 2016). Determining what the exact abiotic and biotic drivers are and their influence on SFD and its population-dynamics is difficult and for now can only be hypothesized about.

7.2. Effect on wild populations

The severity of clinical signs associated with *O.ophiodiicola* differ greatly between species. The main species at risk of being affected by *O.Ophiodiicola* are Eastern massasaugas and timber rattlesnakes as these species are already considered endangered species in several North American states. However, only one study (Clark et al., 2011) till date has indicated a significant decline in number of timber rattlesnakes in one population. A population of timber rattlesnakes in New Hampshire of around 40 snakes was reduced to 19 individuals. Even though fungal infection was present, decline was attributed to a combination of genetic isolation, inbreeding and weather conditions rendering the snakes susceptible to the fungus. Eastern Massasaugas are a sensitive population as well, symptoms appear to be severe in these snakes. Several studies described a 100% mortality rate in clinically infected animals in these species in a population in Illinois (Allender et al., 2011; Tetzlaff et al., 2015). Nonetheless infection has been reported in other areas, where symptoms resolved with no obvious impact on the population (Glorioso et al., 2016; Wright et al., 1999). However it is important to note that baseline data of overall population health is often not available prior to these documented outbreaks, thus reports on the influence of SFD should be interpreted with caution (Lorch et al., 2016).

The combination of habitat loss, fragmentation and infectious diseases such as SFD could very well lead to problems in the future. Long term health monitoring will become increasingly important in forming conservation goals and developing recovery strategies to minimize disease threats. To date most efforts to manage SFD have consisted of rehabilitating individual snakes. This technique can be feasible for greatly endangered species, where survival of each individual is significant. However such an approach is resource intensive and impractical when dealing with larger populations and it does not prevent reinfection (Lorch et al., 2016). It is also important to make sure snakes are truly free of *O.ophiodiicola* before reintroducing them into the wild, as resolution of clinical signs does not mean infection has been eliminated (Lorch et al., 2015).

The management of fungal infections in natural surroundings, as opposed to in (temporary) captivity, gives rise to more discussion. For chytridiomycosis in amphibians techniques for mitigation of the disease have been developed such as bio augmentation, pesticides, augmented evolution, vaccination and environmental manipulation. However, all of these strategies have significant shortcomings and no real success has been made. Striving to ensure a long-term host-pathogen coexistence may be the most sustainable for conservation of biodiversity (Fisher et al., 2016). No efforts have been made yet to try such techniques for SFD.

7.3. *Paranannizziopsis* in wild tuatara

The threat to sensitive populations is also demonstrated in Tuatara. Tuatara are reptiles native to New Zealand and are the only remaining representative of the *Rhynchocephalia*. This species survived mainly in small numbers on offshore islands as a result of human colonization and associated predators. The past decades conservational affords have been made, successfully restoring numbers and reintroducing them on islands where they previously had disappeared. A Department of Conservation recovery plan includes a breed for release program with Auckland Zoo as an active participant successfully breeding tuatara for this purpose (Masters et al., 2016). Therefore the detection of *P.australasiensis* in these captive tuatara has significant implications for conservation and management of not only this species, but possibly other native New Zealand reptiles as well. Up until this time no active surveillance of wild populations to detect *P.australasiensis* has been performed. As tuataras are translocated from captivity to the wild, the impact of a novel emerging fungal disease on a naïve population should not be underestimated (Humphrey et al., 2016).

All in all it is not only the question whether CANV isolates can act as primary pathogens that is important (the biotic driver), but also the question what role abiotic drivers such as climate change and population density increase due to habitat loss play in the epidemiology of this disease. Epidemiological models could be made to project the risk of SFD and other CANV isolates in wild populations in the future, however more information and research is needed to fully understand all relevant drivers.

8. Diagnostics

The lesions seen in CANV infections are often evident though generally non-specific, other differentials important to consider are primary bacterial infections (especially *Devriesea agamarum*), other fungal or bacterial infections due to mite ectoparasitosis or inadequate husbandry conditions and primary viral infections (primarily ranavirus) (Schmidt, 2015). Fungi are ubiquitous on reptile skin therefore merely isolating a fungus in culture without doing a (semi)quantitative determination of species is clinically irrelevant. Isolating a large number of clinically relevant fungi can however be diagnostic. Good diagnostic techniques and support of the presence of fungal elements in tissue sections morphologically consistent with CANV are often preferred. Multiple biopsies should be taken so samples can be used for histopathology as well as for culture or molecular techniques. Taking biopsies is preferred to taking swabs as swabs are less effective for culturing the fungus (Paré and Sigler, 2016). Concurrent infections of *D.agamarum* and *N.guarroi* have been described in bearded dragons, further underlining the importance of mycological cultures and histopathological examination (Schmidt-Ukaj et al., 2014). Fungal and bacterial lesions are often clinically indistinguishable from one another, isolation of bacteria from a lesion does not rule out primary or secondary fungal involvement and may further mislead the clinician in correctly diagnosing the infection (Paré et al., 2006b). Misidentification of pathogen has previously occurred. Several case reports of different *Chrysosporium* species causing cutaneous and systemic mycoses have been published (Nichols et al., 1999; Vissiennon et al., 1999), but many have later on proven to be misidentified (Abarca et al., 2009; Nichols et al., 1999; Vissiennon et al., 1999). Furthermore in case reports of mycoses the aetiological agent is often never identified because fungal disease was initially not suspected (Paré et al., 2006b). To gain a better understanding of fungal disease in reptiles identification of the pathological agent is ideally done to the species level. Isolating the same fungus species from multiple individuals merits more value than isolating fungi from the same genus.

8.1 Culture and morphology

Isolation of fungi from samples can be challenging. Even when the fungal nature of the lesion is clear on histology, inadequate processing of the material, treatment of the animal with antifungals or bacterial contamination can lead to false negatives (Paré et al., 2006b). Culture mediums containing cyclohexamide and an antibiotic such as chloramphenicol are recommended for initial plating off the sample (Paré and Sigler, 2016). Since contaminants such as aspergilla and zygomycetes are fast growing, they can easily overgrow slower growing fungi, cyclohexamide makes selective recovery of dermatophytes and other onygenalean fungi possible as it completely inhibits or severely restricts growth of contaminants (Paré et al., 2006b; Paré and Sigler, 2016). All CANV fungi grow under aerobic conditions on a potato dextrose agar (PDA) within 3 to 5 days, though plating a specimen on a second plate is recommended to isolate any other possible fungi involved in infection (Paré and Sigler, 2016; Schmidt, 2015). Bacterial contamination of the samples can be heavy, so culturing fungi can be facilitated by immersing the sample in enrofloxacin or an acidified broth for a couple of minutes reducing contamination (Paré et al., 2006b; Paré and Sigler, 2016). Commercially available sabouraud agar with gentamycin and chloramphenicol can also be used when high bacterial contamination is suspected, however growth of fungi is slower on this agar and incubation for at least 10 days is recommended (Schmidt, 2015). The CANV show varying thermotolerance, most reptile-associated species are either strongly inhibited or completely unable to grow at temperatures higher than 35^{o}C. However, growth of *N.chlamydospora, N.guarroi, N.pluriseptata* and *N.vriessii* is only moderately inhibited at 35^{o}C (Sigler et al., 2013; Stchigel et al., 2013). Incubation temperatures in diagnostic laboratories often vary from 25 to 35^{o}C and for certain species such as bearded dragons, higher temperatures are regularly used. Communicating incubator temperatures with the laboratory is therefore important to prevent false-negative results from occurring (Mitchell and Walden, 2013). Incubation at 30^{o}C with culture times of up to three weeks is recommended. During these three weeks regular monitoring for growth of the characteristic white colonies should take place along with secondary early subcultures on to fresh culture medium to increase the chance of isolation and avoid bacterial and fungal contamination (Paré and Sigler, 2016).

When grown in culture fungi can develop different morphologies depending on the growth conditions, making it challenging to determine whether these distinct presentations represent the same or different species (Paré et al., 2006b). Medical laboratories therefor sometimes have difficulty identifying or

speciating a fungal isolate, sometimes even leading to misdiagnosis(Paré et al., 2006b). In the past, CANV-infections have been under-diagnosed or misidentified as *Trichophyton, Geotrichum, Malbranchea* or a specific *Chrysosporium* species (Paré et al., 2006b; Paré, 2014). Similarities in microscopic presentation, such as the solitary-single-celled conidia resembling the microconidia of dermatophites and arthroconidia formed by fragmentation, promote confusion with other species of *Chrysosporium*, *Geotrichum* or *Trichophyton* (Paré et al., 2006b). Morphological and physiological descriptions for CANV isolated have only recently been available along with their molecular data. All isolates produce whitish, dense colonies when grown in culture, some young cultures may appear moist. Single-celled aleurioconidia (solitary conidia released by lytic dehiscence (Sigler et al., 2013)) originating at the sides of hyphae or on short stalks are produced by all species. The aleuroconidia are clavate or pyriform with truncate bases and are mostly single-celled, although two-celled aleuroconidia can be present. Conidia are thin and smooth walled (Stchigel et al., 2013) Other distinctive traits in most of these species include undulate hyphal branches and arthroconidia produced in adjacent chains. Fungi such as *Fusarium spp, aspergillus spp,* or *Paecilomyces lilacinus* may produce similar findings in lesions, but isolating a white, powdery culture exhibiting both aleurioconidia and cylindrical arthroconidia make it possible to rule out these fungi as causative agents (Schmidt, 2015). Within the CANV conidia vary in size and length between species, though these are insufficient for a reliable identification on species level, so molecular data through DNA sequencing is still needed to determine the exact species (Paré et al., 1997; Sigler et al., 2013). All species are tolerant of cycloheximide and keratinolytic and therefore can be grown on dermatophyte media. Budding arthroconidia are sometimes demonstrated in moist, yeast-like colonies (Sigler et al., 2013).
In addition to aiding the clinician in diagnosing infection with CANV isolates, culture is also necessary to provide a sample for anti-fungal sensitivity testing (Mitchell and Walden, 2013).

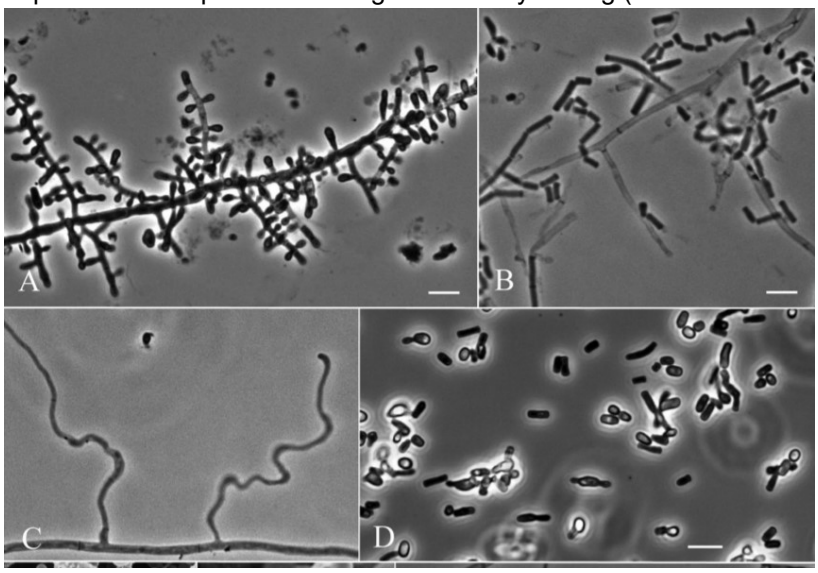


Fig. 7 Microscopic morphology of Nannizziopsis dermatidis. A; aleuroconidia.. B: fission arthroconidia. C: undulate hyphae D: arthroconidia and budding cells produced on PDA (Mitchell and Walden, 2013).

8.2 Histopathology

Histopathology can be used in combination with culture or other diagnostic techniques in getting a correct diagnosis. A causal relationship between fungus and disease cannot be assumed when using only culture or molecular techniques without (semi)quantitative determinations (Paré and Sigler, 2016). When high numbers of a clinically relevant fungus are isolated, histopathology may not be necessary. Samples for histopathology should be taken of a representative, preferably early lesion taken from either a live, anesthetized animal or on necropsy. To confirm the diagnosis the microscopic features of the infiltrating fungus on histology should be consistent with the features of the fungus isolated in culture, especially when multiple species have been recovered from culture (Paré et al., 2006b). When culture comes back positive for a fungus, but no fungal elements are found in the histopathology sample, interpretation should be done with caution and a repeat sample can be necessary. Sample collection should be done at the edge of the lesion. To best demonstrate the tissue response to the infiltrating

fungi a hematoxylin and eosin (H&E) stain is recommended, though this only stains the fungus very lightly (Toplon et al., 2013). The fungus stains well with periodic acid-Schiff (Mitchell and Walden, 2013; Paré et al., 2006b; Toplon et al., 2013). Lesions often have a mixture of inflammatory cells including macrophages and heterophils and the infiltrating fungus (Mitchell and Walden, 2013). The formation of granulomas is also common, these granulomas contain dense aggregates of degenerate and necrotic heterophils surrounded by numerous fungal hyphae (Bowman et al., 2007; Mitchell and Walden, 2013; Toplon et al., 2013). Hyphae of CANV fungi are hyaline, 3 to 6 µm wide, septate and parallel-walled with sporadic branching (Paré and Sigler, 2016). Arthroconidia are often seen and arthroconidial tufts (massive amounts of arthroconidia at the skin surface) are considered pathognomonic for infection with a CANV fungus. Deeper in the dermis and in granuloma's yeast-like elements are occasionally seen (Paré and Sigler, 2016).

8.3 Molecular techniques

Even though differences between species in morphological presentation in culture have been described, if specification on a species level is desired molecular sequencing is necessary (Le Donne et al., 2016; Paré et al., 2006b). Molecular techniques can also be helpful in entry control and in follow-up of treatment. Additionally, when yielding the fungus in culture proves difficult PCR assays can be used. Besides conventional PCR assays there are increasingly more refined molecular techniques available for detecting fungal DNA in samples (Allender et al., 2015b; Paré and Sigler, 2016). CANV isolates can be identified using DNA sequencing of the internal transcribed spacer (ITS1-5.8S-ITS2) region. Sequences of these rRNA gene sequences are available in the GenBank for different species (Sigler et al., 2013; Stchigel et al., 2013). The D1-D2 domains of the 28S rDNA and the small subunit (SSU) regions of the nuclear rRNA gene have also been used in determining species and phylogeny (Le Donne et al., 2016; Sigler et al., 2013; Stchigel et al., 2013). Bohuski and colleagues as well as Allender and colleagues developed real-time PCR (qPCR) methods to rapidly identify *O.ophiodiicola* in clinical samples (Allender et al., 2015b; Bohuski et al., 2015). For diagnostic work the ITS assay is the most reliable option since ITS is of a more conserved nature than others and as a result is more likely to be able to detect different strains or genetic variants of the fungus. The quick turnaround time of real-time PCR can be useful in clinical setting to rapidly start treating an animal suspected of infection (Bohuski et al., 2015). Culture appears to have a lower sensitivity rate for detecting fungi, especially when the fungus is present only in small quantities (Bohuski et al., 2015; Paré et al., 2003). Since real time-PCR has a higher sensitivity it may be particularly useful in researching the relationship between the pathogen and disease, prevalence of fungus on asymptomatic animals and the importance of pathogen load on developing disease. In addition PCR could provide an important role in prevention and containment of the disease a sensitive method to screen environmental samples for contamination or possibly detect asymptomatic carriers of the fungus during quarantining or importing animals into a collection. However, it is important to note that is has not been proven yet that the PCR assay does not cross-react with yet to be characterized environmental fungi genetically similar to CANV, so caution should be provided when interpreting results from environmental samples (Bohuski et al., 2015).

Taking biopsies is the most sensitive method for obtaining a DNA sample, however this can prove to be impractical in field settings. When taking a sample for DNA sequencing a swab can be used as well, and is preferred over saline-flush samples as more useable DNA is obtained using this technique (Allender et al., 2015a). However, even with a sensitive method as PCR, false negatives still occur (Allender et al., 2015a; Hileman et al., 2018). The presence of clinical signs is not consistently reflected in the DNA results. Even when taking biopsies a false negative rate of 2% has been reported (Bohuski et al., 2015). Swabbing lesions appears to yield good results up to two months after inoculation. False negatives are likely not due to the PCR techniques, but rather to swabbing methods and stage of infection. (Allender et al., 2015a; Bohuski et al., 2015). It is possible that once the pathogen has invaded the epithelium and is only present in deeper tissues false negatives are more likely. One study (Hileman et al., 2018) showed a high probability of obtaining a false negative for snakes without clinical signs (97%) as well as for snakes with clinical signs (73%) when only one swab was used. This probability can be decreased significantly for snakes with clinical signs to <5% when using 5 swabs for re-sampling. 41. However for snakes without any clinical signs, re-sampling has to be done extensively (93 times) to achieve the same threshold of <5% false-negatives.

8.4 MALDI-TOF-MS

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) is an already established, diagnostic tool to identify fungi and bacteria in clinical microbiology. It is considered time-saving, accurate, affordable and thus a feasible tool in diagnostics. A study in 2018 showed that this technique can be accurately used for diagnosing YFD caused by *N.guarroi*. The database containing different strains and fungi should however be expanded so that in the future use of this proteomic analysis can become more widely used (Schneider et al., 2018).

8.5 Further diagnostic work-up.

Other diagnostic techniques such as bloodwork and medical imaging are routinely used in mammals to evaluate their health status. Interpretation of such tests in reptiles are not as well defined and the ectotherm nature of reptiles further complicates interpretation (Allender et al., 2016). They can however be used to give a general health assessment and give an insight in the general physiologic state of the animal . Reptiles presenting with lesions which raise the suspicion of CANV infection should go through a thorough diagnostic work-up to assess the overall health and physiological state of the individual (Mitchell and Walden, 2013). A further diagnostic work-up is not only useful for an individual, but can also be favourable to determine overall population health and subsequently pinpoint possible mechanisms for the emergence of a fungal disease (Allender et al., 2016). Upon presentation severely affected animals tend to be dehydrated. On haematology regenerative anaemia associated with chronic disease and an inflammatory leukogram can often be found. On biochemistry an increased packed cell volume, total protein, albumin, sodium, and chloride levels are common. Hyperglobulinemia and an inverse albumin/globulin ratio can also be observed (Mitchell and Walden, 2013). Biochemistry and haematology can also be useful in evaluating possible toxicity of antifungal treatment, especially markers for liver function are important to follow up on(Van Waeyenberghe et al., 2010). Besides bloodwork, survey radiographs, echography and other imaging modalities can be used to further investigate the extent of soft tissue and/or bone involvement, or locate lesions for biopsy and culture (Paré et al., 2006b; Schmidt, 2015).

9. Treatment and prevention

9.1. Treatment

For the treatment of CANV infections in reptiles there are many therapy recommendations available, though in many cases treatment either has none or a limited effect and often lesions reoccur and euthanasia or natural death are no exception (Hellebuyck and Martel, 2012). Successfully treating CANV infections appears to be a considerable challenge. Generally both topical and systemic preparations with an antifungal activity are used (Mitchell and Walden, 2013; Paré, 2004).
Besides treatment with antifungals ,surgical debridement or debulking of lesions is advised. Dead skin in lesions likely contains large amounts of infective conidia out of reach of systemically administered antifungals (Paré and Sigler, 2016). During debridement is also an ideal time to take biopsies of the lesions for diagnostic purposes.

9.1.1. Topical treatment

For topical treatment several options are available and have been used with varied success, though no research has been done yet to evaluate the effectiveness of these topical treatments. Silver sulfadiazine (SSD) cream applied every 12 hours has been used to treat bearded dragons with yellow fungus disease. Although there are no studies yet on the effect of SSD on CANV, it has been proven to be effective against other fungi as well as being effective against bacteria (Bowman et al., 2007; Johnson, 2004; Masters et al., 2016; Wright et al., 1999). Topical treatment was only part of the treatment plan, but both cases were unsuccessful and the animals were euthanized (Bowman et al., 2007; Johnson, 2004). Topical treatment with a chlorhexidine solution has also been recommended (Bowman et al., 2007; Schmidt, 2015), but a study exploring the effectivity of disinfectants against *O.ophiodiicola* showed to only decrease fungal growth, not prevent it (Rzadkowska et al., 2016). Chlorhexidine is frequently used in veterinary settings because of its broad spectrum and even though it is not effective against the fungus, it might be effective in treating secondary bacterial involvement in infection (Hileman et al., 2018). Daily baths in dilute povidone-iodine solutions are topical application of an iodine solution has been reported as well (Bertelsen et al., 2005; Bowman et al., 2007; Masters et al., 2016; Thomas et al., 2002). Iodine in combination with formalin has been used successfully in the treatment of CANV infection in crocodiles (Thomas et al., 2002), and iodine appears to have slowed down progression of disease in tentacled snakes (Bertelsen et al., 2005). Although there has been some success with treatment with iodine-based solutions, not all have been successful (Bowman et al., 2007; Nichols et al., 1999), so concurrent systemic treatment is recommended. Topical treatments containing miconazole, nystatin, enilconazole or tolnaftate have been used with varying success (Bowman et al., 2007; Hellebuyck et al., 2010; Paré et al., 2006b). In tuataras topical treatment or complete surgical excision alone, in some cases was enough to completely resolve the infection (Masters et al., 2016). When choosing the type of topical treatment, it might be useful to select an antifungal with a different mode of action than the systemic antifungal to perhaps create a higher efficiency in treating due to a complementary action of the drugs (Paré, 2004).

9.1.2. Systemic antifungals

Even though topical treatment and surgical debridement might be helpful in treating the infection, on their own they are often insufficient to lead to a full recovery and systemic antifungal drugs are indicated (Paré and Sigler, 2016). The azoles are most commonly equipped in the treatment of CANV infections, all with variable success (Schmidt, 2015). Their broad spectrum and low toxicity compared to other antifungals makes them the evident choice (Paré et al., 2006b). They inhibit ergosterol biosynthesis binding to fungal cytochrome P-450 enzymes (Abarca et al., 2008). However a thorough evaluation of the antifungal drugs available have only been sparsely done in just as a small number of reptile species (Paré et al., 2006b). Ketoconazole, fluconazole, itraconazole and more recently voriconazole are the drugs most readily available.

7.1.2.1. Ketoconazole

Of the available azoles fluconazole has a limited spectrum and has been proven to not be active against filamentous fungi, and therefore cannot be used to treat CANV. Ketoconazole does have activity against filamentous fungi, but has a higher toxicity and therefor is also not the best option(Paré, 2004). However

one case study reported improvement of the lesions on two green iguanas diagnosed with a CANV fungus after with treatment with ketoconazole (20 mg/kg/24h PO) in combination with 2%chlorhexidine and terbinafine administered topically (Abarca et al., 2008). No side effects of the treatment were reported in this study. Ketoconazole was chosen in this case after an antifungal susceptibility test using the disk diffusion method which showed ketoconazole to be highly active against the two isolates in vitro. In South-Korea 3 Iguanas were diagnosed with *C.guarroi* and subsequently treated in the same manner. However infection in these cases had progressed to be more severe and deeper when therapy was initiated and unfortunately in these cases did not prove successful (Han et al., 2010). Antifungal susceptibility tests are not yet standardized, there are some guidelines available, but for *Chrysosporium* sp. there are none available. Treatment with antifungals is generally recommended for at least 2-4 weeks.

7.1.2.2. Itraconazole

Itraconazole has a wide spectrum, it is active against yeast, dimorphic fungi, aspergilla and other moulds and against dermatophytes. Additionally is also accumulates in the corneum and can therefore be especially useful in treating dermatomycoses (Paré, 2004). Up until this point there has been some experience with using itraconazole in treatment of CANV in bearded dragons and chameleons, all with varying success (Bowman et al., 2007; Hedley et al., 2010; Johnson et al., 2011; Paré et al., 1997). In the chameleons a dose of 10 mg/kg/24h PO during 21 days was used, lesions in one of the two chameleons improved and disappeared, but the chameleon became anorexic and a general loss of condition was noted. The other chameleon died 6 days after initiation of therapy with evidence on necropsy of systemic mycosis. Two bearded dragons were treated with the same dose of 10 mg/kg/24h PO, progressive anorexia combined with significant weight loss was observed. Hepatotoxicity is a well-known side effect of the azoles in mammals thus hepatic enzymes were monitored, but no changes were observed after three weeks of antifungal therapy. The third bearded dragon in this case study was treated with 5 mg/kg/24h PO in the hope to avoid the anorexia as seen in the other bearded dragons, no adverse effects were noticed with this dosage (Bowman et al., 2007). All bearded dragons showed some improvement after treatment and the last patient fully recovered, though the only affected area which was the right forelimb, was amputated which alone may have been sufficient for a full recovery. In general itraconazole seems to be moderately effective in treating *Chrysosporium* isolates, reflected in low MIC values found in several studies (Paré, 2004; Van Waeyenberghe et al., 2010). However, toxicity, especially hepatotoxicity remains a concern. This has not been visible on histopathology on necropsy, however it is suggested based on blood chemistry in some cases. The hepatotoxicity clinically often presents as anorexia and depression, on blood examination aspartate aminotransferase (AST), gamma glutamyltransaminase (GGT) and bile acids are most commonly used to assess hepatic disease secondary to drug toxicity. Liver biopsies can be helpful to determine whether accumulation of the drug is reaching unsafe levels (Mitchell and Walden, 2013). Pulse therapy with itraconazole in reptiles has been suggested as an alternative, but this has yet to be applied successfully. Lower doses of itraconazole given at a less frequent interval may also reduce the risk of toxicity, but further research is needed (Mitchell and Walden, 2013; Van Waeyenberghe et al., 2010). Furthermore a study showed the distribution of the minimal inhibitory concentration (MIC) value of itraconazole is bimodal, suggesting acquired resistance in one isolate in the higher MIC range. More research is needed to investigate if more CANV strains show resistance to itraconazole and what mechanisms are at its base.

7.1.2.3. Voriconazole

Newer on the market is voriconazole which has been shown to be a safer option in treatment of dermatomycosis (Paré, 2004). Voriconazole has a broader spectrum and the *in vitro* activity against filamentous fungi exceeds that of itraconazole (Van Waeyenberghe et al., 2010). The incidence of side effects in use of voriconazole also appears to be lower compared to other antifungals (Mitchell and Walden, 2013). Based on pharmacokinetic and therapeutic information about voriconazole and susceptibility testing a dose of 10 mg/kg/24h PO is recommended (Hellebuyck et al., 2010; Hellebuyck and Martel, 2012). Voriconazole has been successfully used and appeared to be well tolerated in a girdled lizard (*Smaug giganteus*) and in bearded dragons. Clinical cure and apparent elimination of the fungus was achieved in these animals (Hellebuyck et al., 2010; Van Waeyenberghe et al., 2010). During therapy with voriconazole some bearded dragons still showed significant increase in AST levels, this suggests hepatotoxicity is still a concern in treating reptiles with voriconazole. An inter-individual variability of plasma concentrations of voriconazole was also observed. However the survival rate was

higher for voriconazole versus itraconazole, indicating voriconazole to be the preferred method of treatment (Van Waeyenberghe et al., 2010).

9.1.3. Treatment of snake fungal disease

There are even less reports on the effectiveness and safety of treating snakes with SFD. Voriconazole has been used to treat timber rattlesnakes diagnosed with SFD (10 mg/kg per cloaca three times a week for 4 weeks), but no follow up information was provided nor were drug concentrations measured. Additionally lesions regressed in snakes not receiving treatment, so possibly infection may be self-limiting in these species (McBride et al., 2015). One study concerning voriconazole and itraconazole by Lindemann and colleagues is available done in cottonmouths and massasauga rattlesnakes (Lindemann et al., 2017). Based on this study there appeared to be no clear correlation between voriconazole dose administered (5mg/kg SC vs 10 mg/kg SC) and adverse correlation and death. Adverse effects included depression, lethargy, loss of righting reflex, torticollis and eventually death. Snakes that died after treatment with subcutaneous voriconazole showed regions of acute degenerative myopathy. An osmotic-pump subcutaneous delivery of voriconazole was chosen to safely treat venomous snakes exhibiting signs of SFD. However this only resulted in sufficient therapeutic plasma concentrations in one timber rattle snake, not in the massasauga rattlesnakes. The massasauga rattlesnakes most likely passed away due to the severe stage of the disease. Lesions in the timber rattle snake regressed significantly, though no conclusions can be drawn from this one case as lesions are known to regress on their own in timber rattlesnakes. Itraconazole administered per cloaca in cottonmouths did not reach therapeutic voriconazole plasma or tissue levels. More research is needed to find safe and effective methods to treat snake with *O.ophiodiicola* infections.

9.1.4. Supportive care

Evidently, nonspecific supportive therapy, such as fluid therapy, thermal and nutritional support are almost always indicated alongside a critical assessment of the captive conditions to identify and correct inadequacies (Paré et al., 2006b; Paré and Sigler, 2016). Because infections with CANV strains appear contagious among reptiles proper biosecurity measures should be taken to prevent spread to other animals (Paré and Sigler, 2016). Besides the fact that the reptiles immune response and metabolism are heat dependant and necessary for correct husbandry (Cabañes et al., 2014), raising and maintaining the environmental temperature in the upper zone of the species preferred optimal temperature range might be additionally helpful in treating infection given the limited thermotolerance of *Chrysosporium* species (Paré et al., 2006b; Paré et al., 1997). Antibiotics are often given alongside antifungals when secondary or concurrent bacterial disease is expected (Paré et al., 2006b).
Ravuconazole, Posaconazole, echinocandins and chitin synthase inhibitors are other novel antifungals that in the future may provide another save option for treatment, however up till date no research of the use of these antifungals have been done in reptiles (Paré et al., 2006b; Paré, 2004).
Duration of therapy varies and is often determined empirically and can take weeks or months. As there is no set time period for therapy available, the effectiveness and duration of antifungal therapy should ideally be evaluated by repeated sampling of the dermal lesions weekly until two consecutive samples are negative for fungal culture (Hellebuyck et al., 2012; Van Waeyenberghe et al., 2010). Important to keep in mind is the reserved prognosis for systemic mycoses with CANV, even with systemic antifungal treatment (Hellebuyck and Martel, 2012).

9.2. Prevention

Especially because treatment of the CANV isolates can be so challenging and prognosis is not favourable, prevention is key in managing this disease. The first major factor in preventing CANV and many other reptilian diseases is limiting stress. Factors such as crowding, suboptimal husbandry, temperature and lighting, handling, transport, trauma and others could cause immunosuppression.. Other underlying comorbidities should be taken into account as well (Cabañes et al., 2014; Schneider et al., 2018). In many cases (Abarca et al., 2008; Schneider et al., 2018; Thomas et al., 2002) husbandry was suboptimal, perhaps making the animals more susceptible to disease, however in others husbandry appeared to be adequate for the species (Bowman et al., 2007; Hellebuyck et al., 2010; Schmidt-Ukaj et al., 2014).

Secondly the fungus appears to be persistent in the environment (Hellebuyck and Martel, 2012; Thomas et al., 2002). Thorough disinfection of materials, surfaces and the terrarium is recommended to prevent spread and reinfection. Taking early diagnostic samples of each individual presenting with dermatological lesions can also aid in preventing spread of the infection (Hellebuyck and Martel, 2012). Wearing medical clothes during examination and treatment can also help to prevent spread to other reptiles (Schmidt, 2015). Several disinfectants were tested for their activity against *O.ophiodiicola* (Rzadkowska et al., 2016). 3% bleach appeared to be effective at inactivating the fungus and can be used to disinfect equipment between individuals. 2-, 5- and 10-minute contact times were effective. Bleach can also be used in shoe-washing stations in high risk areas to prevent spread. Quaternary ammonia products (NPD) were as effective as bleach when 10-minute contact times were used. NPD are less corrosive and less irritating to the skin compared to bleach, but any treated surfaces should be thoroughly rinsed and aired out to prevent any direct contact with the reptile (Pasmans et al., 2008; Rzadkowska et al., 2016). Chlorhexidine did not appear to be effective against *O.ophipdiicola*. Despite decreasing fungal growth, it did not prevent it completely. 70% ethanol for at least 2 minutes was proven to be effective. Lysol products and benzalkonium chloride also inhibited growth when exposure was at least 10 minutes (Rzadkowska et al., 2016).
Chemical disinfectants should be applied only to empty cages to decrease organic debris and to ensure adequate exposure to the product and the manufacturers' instructions should always be followed (Pasmans et al., 2008; Rzadkowska et al., 2016).
This also applies when handling wild reptiles in preventing risk of epidemics (Meier et al., 2018). Appropriate biosecurity procedures, dedication of gear and work spaces for wild versus captive reptiles and frequent disinfection of tools and hands may provide additional disease control (Lorch et al., 2016). Additionally the use of radio-transmitters in studies should carefully be assessed. A study showed that prevalence of *O.ophiodiicola* was higher in snakes implanted with radio-transmitters (35%) as opposed to snakes without radio-transmitters (13%). Immune-suppression post-surgery, creation of a skin defect following implantation or iatrogenically introducing the fungus due to non-sterile techniques may be the cause. The use of radio-transmitters ideally should be avoided in populations with high incidence of SFD, unless proper placement protocols can be assured and benefits to the population outweigh the risk to the individual (Hileman et al., 2018).
As transportation, suboptimal housing, crowding can cause immunosuppression and therefore are associated with disease outbreaks quarantine of newly purchased reptiles is essential in preventing disease and introducing CANV in a collection (Schmidt, 2015).
The possibility of use of probiotics, antifungal bacteria or their metabolites has also been researched (Hill et al., 2018). The cutaneous microbiome is known as a first line defence against pathogens in several animal species and augmentation of the normal biome has previously successfully been used in marine and freshwater aquaculture. In this study one isolate was found, *Morgeanella morganii*, that showed potent anti-*O.ophiodiicola* defences. This isolate is a known natural symbiont of snakes and might be a candidate for the use in probiotics for therapeutic trials. While this anti-Oo effect has been proven in vitro, it still has to be researched if it can inhibit the pathogen in vivo, while continue persisting on the skin along with other microbial symbionts and not causing any disruptions in the existing microbiome. Further research could investigate the possibility of implementing this technique in treating affected animals or possibly as a preventative measure.
When introducing a new reptile in a collection a high risk of disease introduction exists. Fungi belonging to the CANV complex further emphasise the importance of entry control for infectious agents, disinfection and quarantining. Environmental spread has been demonstrated (Paré et al., 2006a) indicating that transmission between terraria is possible. Newly acquired animals should be kept in quarantine in a separate room from the existing collection. Instruments used in husbandry should be kept separate between the rooms and be thoroughly cleaned and disinfected after use. The length of quarantine is generally set at anywhere from 90 days to 6 months (Pasmans et al., 2008). Molecular techniques such as PCR are ideal to use for entry control due to their high sensitivity and the possibility to detect asymptomatic carriers.

10. Discussion

Al in all there is a lot yet to be discovered about fungal infections in reptiles. Even though the last decade there has seen an increasing amount of research been performed on CANV-fungi, knowledge is still limited. A lot of the research is based on case reports in a lot of different species, so coming to a general consensus regarding these infections is particularly challenging.

The first challenge lies in defining the host range and most susceptible species. CANV infections seem to be common in a whole range of reptile species. Most case reports of *Nannizziopsis* species seem to be in bearded dragons, chameleons and Iguanas. However these reptiles are also the most popular species to be kept as pets. Additionally commercial breeding could cause stress and high population densities are more likely to occur under these circumstances making spread of the disease easier. The host range is likely more broad as not all cases get reported. *Paranannizziopsis* seems to have a smaller host range, but case reports are continuously being published about infection in new species. *Ophidiomyces* has an extremely wide host range and almost all snake species seem to be susceptible to some form of infection.

The pathogenesis is one of the areas where knowledge is severely lacking. How infection occurs exactly and what factors play a role in infection are not clear. Learning more about the pathogenesis and knowing how infections takes place could be helpful in prevention of the disease. This is particularly important as prognosis is not great for infected animals. A clear start has been made in researching the pathogenesis, but more research is needed. Especially the way the fungus spreads between animals and in the environment needs to be explored further. An in depth study showcasing the dissemination of the fungus could be of real added value.

There are also some contradicting results in determining whether CANV can be present on a healthy reptile skin without causing infection. If subclinical carriers are possible and exist, entry-control using sensitive techniques such as PCR become even more important and should be emphasized when introducing a new reptile in an existing collection.

It also has become clear that there appear to be at least two different strains (North-American and European) of *Ophidiomyces ophiodiicola*, with different virulence. Perhaps these different strains also play a role in the difference in pathogenicity between different species. This would also explain the difference described in case reports on impact of the disease on different populations in North-America. A change in virulence could also explain why SFD appears to have become more prominent the last decades. However this could also be explained through increasing awareness and correct diagnostic techniques. Determining the abiotic and/or biotic drivers and the true influence on population dynamics can help learn us if the claim that SFD is as much of a threat to sensitive snake populations as chytridiomycosis is to amphibians holds any merit. The only way to determine this is to start a project monitoring sensitive populations over a couple of years where samples are taken using a sensitive method for detecting *O.ophiodiicola*.

Contradictions in literature can be found are regarding the correct diagnosis of CANV infections. Some authors claim that histopathology is absolutely essential for diagnosis. However in practice a culture or (semi)quantitative PCR determination with high numbers of a clinically important fungus is seen as sufficient for a diagnosis. Only when there is no clear result from culture or PCR or there are doubts about co-infections, histopathology is truly necessary.

Treatment options are available, but there is only limited information about safety and efficacy of different products. As fungal infections are becoming more common it would be wise to further determine safe and effective concentrations for different products in each species. There is especially limited data in treatment of SFD in snakes.

All things considered a decent start has been made in investigating this fungus, but a lot of information is still lacking. Future research will give us the data that is needed.

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