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Pathogenicity of the *Chrysosporium* Anamorph of *Nannizziopsis vriesii* for veiled chameleons (*Chamaeleo calyptratus*)

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Veiled chameleons (*Chamaeleo calyptratus*) were experimentally challenged with the fungus *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV). Chameleons were exposed to conidia in their captive environment, or were inoculated by direct application of a conidial suspension inoculum on intact and on abraded skin. The CANV induced lesions in all experimental groups and was recovered from infected animals, fulfilling Koch's postulates and confirming that it may act as a primary fungal pathogen in this species of reptile. A breach in cutaneous integrity, as simulated by mild scarification, increased the risk of infection but was not required for the CANV to express pathogenicity. Initial hyphae proliferation occurred in the outer epidermal stratum corneum, with subsequent invasion of the deeper epidermal strata and dermis. A spectrum of lesions was observed ranging from liquefactive necrosis of the epidermis to granulomatous inflammation in the dermis. CANV dermatomycosis appears to be contagious and can readily spread within a reptile collection, either directly through contact with infective arthroconidia or indirectly via fomites. Dense tufts of arthroconidiating hyphae were demonstrated histologically on the skin surface of many animals that developed dermatomycosis, and these arthroconidia may act as infective propagules involved in the transfer of disease between reptiles.

Keywords *Chrysosporium* anamorph of *Nannizziopsis vriesii*, veiled chameleon, *Chamaeleo calyptratus*, dermatomycosis, mycosis, fungus, reptiles

Introduction

The *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV) has caused severe and often fatal dermatomycosis in chameleons, brown tree snakes (*Boiga irregularis*), tentacled snakes (*Erpeton tentaculatum*) and saltwater crocodiles (*Crocodylus porosus*) [1–4]. In addition, more than 20 isolates have been recovered from cutaneous or deep lesions in various reptiles, although corroborating histopathological evidence to confirm infection is not available in all cases [Sigler and Paré unpublished data]. A recent survey of the skins of healthy captive squamate reptiles from zoological and private collections revealed that the CANV is a very rare constituent of the cutaneous mycobiota of reptiles [5]. The number of cases of reptile infection caused by the CANV is high in relative contrast to reports of infections caused by fungi that are extremely common on the reptilian integument like *Aspergillus*, *Penicillium*, and *Paecilomyces lilacinus* [5–7]. The CANV challenges the assumption that mycoses in reptiles are always opportunistic and secondary to substandard husbandry or other causes of immune suppression. The objectives of this experimental challenge were to verify

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the potential for the CANV to act as a primary pathogen for reptiles, to investigate the risk associated with exposure to CANV conidia, and to examine the histological progression of the disease.

Methods

Animals and housing

All animals were housed in two identical adjacent experimental rooms at the University of Wisconsin Charmany Instructional Facility. Temperature in the rooms was maintained at 85°F, ±2°F. Full-spectrum fluorescent lights (ReptiSun 5.0 UVB, Zoo Med Laboratories, San Luis Obispo, CA, USA) were hung from the ceilings of the rooms. Veiled chameleons (*Chamaeleo calyptratus*) were captive-bred and purchased from a reputable breeder. Forty-eight entered the experimental fungal challenge, and an additional three animals were used in a pilot study conducted to determine an infective dose. Chameleons were around 3 wks old when purchased and ranged in weight from 8 to 15 g. All underwent a physical examination upon arrival. The skins of 10 randomly selected animals were swabbed for fungal cultures using a sterile cotton-top applicator to ascertain that they were free of the CANV. Chameleons were assigned a number and placed in their enclosure. They were housed singly in $18.5 \times 11 \times 11$ inches rectangular transparent plastic rodent cages (One Cage, Lab Products Inc., Seaford, DE, USA). Each cage top consisted of a plastic grid, lined with a felt filter (Micro-Isolator, Lab Products Inc., Seaford, DE, USA). Pieces of indoor-outdoor rug (Reptile Carpet, JustHerps, Boyertown, PA, USA) were cut to the cage floor dimension. In each cage, a single longitudinal perch consisting of assembled PVC tubes wrapped with Vetrap (3M Vetrap, 3M animal Care Products, St-Paul MN, USA) was used as a walkway by the lizards. Discarded 1 and 5-l IV fluid bags and infusion sets delivered a slow continuous tap water drip in each cage from which chameleons could drink. Excess water was collected in a plastic cup and discarded daily. A cardboard separator placed between adjacent cages precluded visual contact between animals. Chameleons were fed crickets dusted with a calcium supplement (Rep-Cal Calcium, Rep-Cal Research Labs, Los Gatos, CA, USA). Once weekly, crickets were dusted with a multiple vitamin/mineral powder (Nekton Rep, Nekton Produkte, Pforzheim Germany). Cages were spot-cleaned daily, and were completely washed weekly. Rugs and perches were cleaned weekly using 1:10 bleach dilution, followed by thorough rinsing. Plastic drip collecting cups were discarded and replaced weekly. Chameleons were allowed to acclimatize for 1 month. All experimental animals appeared healthy and weighed up to 37 g at the onset of the challenge.

CANV isolate and inoculum preparation

A freeze-dried ampoule of CANV isolate UAMH 7583, originating from the skin of a jewel chameleon (*Furcifer* (*Chamaeleo*) *lateralis*) with fungal dermatitis and pulmonary granuloma [1], was obtained from the University of Alberta Microfungus collection and Herbarium, Edmonton, AB, Canada. The content of the ampoule was reconstituted with sterile distilled water, dispensed onto a petri dish containing Mycosel agar (Beckton-Dickinson and Company, Cockeysville, MD, USA) and incubated at 28°C for 7 days until sporulation was established. The plate was then flooded with PBS-Tween 20, and gently tilted back and forth allowing for conidia to become dislodged. The PBS-Tween 20 conidial suspension was pipetted off, and centrifuged at 2500 RPM for 5 min. The supernatant was decanted and the conidial concentration determined using a hemacytometer. The suspension was aliquoted into 1 ml volumes in microcentrifuge tubes and diluted with PBS-Tween 20 to obtain a final dilution of 2.36×10^4 conidia/ml.

Experimental challenge

Animals were randomly assigned to one of six experimental groups to assess infection following exposure to conidia of the CANV in the environment or following inoculation with a conidial suspension on intact or on abraded skin. Chameleons were monitored daily for clinical signs of cutaneous infection such as focal discoloration, bullae, blisters, or crusts, and for systemic infection including weakness, anorexia, edema, or uniformly poor skin coloration over a period of up to 42 days, depending on the experimental group. If any lesion developed, it was to be biopsied under isoflurane anesthesia using a disposable 3 mm skin biopsy punch (Acu-Punch® 3 mm, Acuderm Inc., Fort Lauderdale, FL 33309, USA). Any animal showing signs of systemic disease for more than 3 days was to be humanely euthanized. Surviving animals were to be euthanized at the end of the 42-day observation period. Full necropsies were conducted on all euthanized lizards and a skin section was frozen from each animal. Infection was defined as any animal with a lesion showing histological evidence of tissue invasion with fungal elements. For these animals, fungal cultures were performed from frozen skin samples to verify that the CANV was the cause of the lesions.

Group A animals ($n=10$) tested infection from environmental exposure to conidia. Fifty microliters of inoculum were applied onto a thin strip of sterile gauze hung above the animal's walkway to allow for contact with the lateral body wall (Fig. 1). Strips were replaced daily for 1 wk, then weekly.

Group B consisted of control animals ($n=8$). A strip of sterile gauze, on which 50 mcL of PBS-Tween 20 solution was applied, was hung in each control animal cage and changed daily for 1 wk, then weekly.

Group C ($n=10$) tested infection through direct cutaneous application of fungal conidia. Fifty microliters of inoculum were applied uniformly onto the non-adhesive 1×1 cm square center of individually wrapped sterile 7/8-in round adhesive bandages (3M™ Nexcare™ Active™ bandages, 7/8 inch Spots), and the bandage was then applied to the left flank of the chameleon (Fig. 2). Animals in Group C were euthanized in randomly selected pairs at 7, 14, 21, 28, and 35 days post-inoculation or before, if they met the criteria for humane euthanasia (as outlined above). Animals were euthanized sequentially to allow for histologic observation of the progression of the dermatomycosis, as lesions were otherwise not visible under the bandages. Nexcare bandages wet with sterile PBS-Tween 20 solution were applied to the skin of Group D control lizards ($n=5$) in a manner identical to Group C animals. One randomly-selected Group D lizard was euthanized at 7, 14, 21, 28, and 35 days post sham-inoculation.

Group E ($n=10$) chameleons were processed identically to those of Group C, but light scarification of the skin using fine grain sandpaper preceded application of the inoculum-laden Nexcare bandage. Group F ($n=5$) control animals were processed identically to Group D lizards, but light scarification of the skin preceded the sham inoculation. Animals in Groups C, D, E, and F that underwent ecdysis and lost their bandages were not re-inoculated. The number of animals that were infected at the time they died or were euthanized was compared between each experimental group and its respective control group.

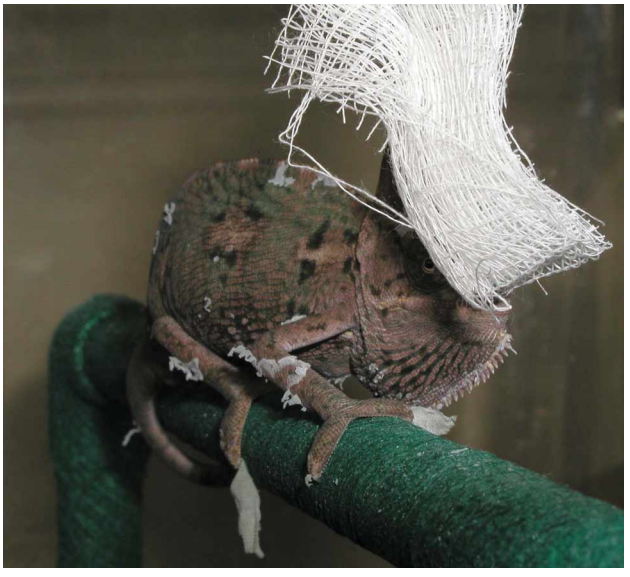

Fig. 1 Experimental Group A: Inoculum-laden gauze hanging from the cage top along the cage main walkway.

Fig. 2 Experimental Group C: Inoculum-laden adhesive bandage applied to the flank of a chameleon.

Histopathology

For animals in Groups C, D, E, and F, the whole skin area under the bandage, or the whole left flank in an animal that lost its bandage, was collected in formalin. The right flank was processed identically for comparison. For all animals, viscera and brain were collected, as well as any lesion detected at gross necropsy, and were fixed in formalin. Tissues were embedded in paraffin, sequentially sectioned and stained with H&E and PAS and examined for the presence of infection.

Pilot study

Prior to the experimental challenge, three chameleons were used in a pilot study to determine an adequate conidial inoculum dose. An inoculum-laden adhesive bandage was applied to the lateral body wall of each

chameleon, as described for chameleons of Group C. Suspensions of 2.36×10^3, 2.36×10^4, and 2.36×10^5 conidia/ml were tested on chameleons 1, 2, and 3, respectively. Chameleon 1 was humanely euthanatized 11 days post-inoculation. Chameleons 2 and 3 died at 20 and 15 days post-inoculation. All three had severe, deep, histologically-confirmed fungal dermatitis and in chameleon 2, transmural fungal infection had progressed across the body wall to cause a fungal coelomitis. An inoculum dose of 2.36×10^4 conidia/ml was selected for the experimental challenge.

Monitoring of experimental rooms for contamination

During the experiment, open Mycosel agar plates were placed in various locations within the experimental rooms. These settle plates were covered after 24 h, incubated at 28°C, and monitored for fungal growth. Additionally, 1×1 cm sections from the cage top filter of 8 randomly selected challenged animals (Groups A, C, and E) were cultured once weekly. The filters were then replaced with new ones. Lastly, samples of skin from animals that were actively shedding in Groups A (3 wks post-inoculation), and Groups C and E (5 wks post-inoculation) were opportunistically collected and plated onto Mycosel agar plates.

Fungal cultures

Tissue sections and material submitted for culture were planted onto Mycosel agar and incubated at 28°C. Plates were examined daily for 3 wks for the presence of white, powdery colonies suggestive of the CANV, but all fungi were identified to genus based on morphology.

Statistics

The numbers of animals infected between exposed and control groups in each exposure condition were compared using Fisher's Exact Test to test for statistically significant differences.

Results

Nine of 10 veiled chameleons exposed to conidia in the environment (Group A) survived the 42 d observation period. One died 13 days post-exposure, with no premonitory signs. There was no evidence of fungal disease grossly or histologically. One surviving chameleon developed a histologically confirmed deep fungal pododermatitis, with superficial epidermal ulceration and dermal necrosis. The left front foot and wrist were visibly swollen in this lizard. Hyphae penetrating through the skin and into underlying muscles were demonstrated histologically. Seven control chameleons (Group B) survived the 42 d observation period and were free of detectable fungal lesions grossly and histologically when euthanized. One chameleon died 20 days post sham exposure, of non-infectious cause. In this chameleon, there were foci of mineralization in the myocardium and in the kidneys, and no evidence of fungal disease.

Four of 10 chameleons in Group C that were exposed to the fungus through prolonged direct application of conidia to the skin developed histologically confirmed fungal lesions. Two of these animals were euthanized 5 wks post exposure and the other two were euthanized at 2 and 4 wks post-inoculation. One of the five control chameleons subjected to a sham cutaneous contact exposure (Group D) was euthanized at 5 wks post-sham inoculation and was found to have a single, grossly undetectable, fungal epidermal lesion that was identified histologically on one section of skin.

Eight of 10 chameleons subjected to cutaneous scarification prior to direct spore application (Group E) developed histologically confirmed fungal lesions. One died 29 days post-inoculation with deep dermatomycosis. The two chameleons that did not develop disease were the first chameleons to be euthanized, 1 wk post-inoculation. No scarified control chameleons (Group F) developed fungal disease. Only in this exposure mode was a statistically significant difference demonstrated, with a p-value of 0.0070.

Therefore, 13 challenged chameleons and one control chameleon, for a total of 14 lizards, were diagnosed with histopathologically confirmed fungal infection. The CANV was cultured from frozen skin samples of five of these 14 chameleons. Growth was profuse over and around the whole skin sample circumference. Four samples yielded no growth. Five yielded scant colonies of *Aspergillus*, *Penicillium*, and *Mucor* species, usually away from the skin sample, and all were deemed to be contaminants.

Environmental cultures were negative for the CANV until late in the challenge. Cage top filter sections were negative until week 4, when the CANV was isolated from the cages of one animal from Group A, one from Group C, and one from Group E. One settle plate, which had been left on top of a cage, yielded the CANV at week 5. None of the 47 other settle plates cultured throughout the challenge yielded the CANV but other fungi isolated and identified included *Aspergillus, Paecilomyces, Cladosporium, Alternaria, Scopulariopsis, Mucor,* and *Cunninghamella* species. The CANV was isolated only twice from shed skin. These samples were collected from the two remaining Group C

chameleons, just before they were euthanized 5 wks post-inoculation.

Histopathology

Multiple skin sections from both body walls were examined microscopically. Animals from Group E that underwent scarification prior to fungal exposure developed the most severe lesions. The lesions consisted for the most part of coagulative epidermal necrosis with dermal edema and marked mixed inflammation made up of heterophils, lymphocytes, plasma cells and some macrophages. In PAS stained tissue sections, hyphae were present in the keratin layer, the epidermis, dermis, and sometimes extended past the deep dermis into the skeletal muscle (Figs. 3–5). Hyphae were narrow and rather uniform in width (2–3 μm), septate, with occasional branching. In many animals, dense tufts of aerial arthroconidiating hyphae were demonstrated at the surface of the skin (Figs. 6 and 7). Bacterial colonies were common in ulcerated necrotic foci. Multinucleated giant cells were occasionally observed around fungal elements in the deep dermis. Group E animals that were euthanized early had less severe or no fungal lesions. One animal euthanized 3 wks post-inoculation had a single focus of fungal proliferation with very mild heterophilic infiltrate. Neither of the two animals euthanized 1 wk post-inoculation had hyphae present in the scarified areas, although multifocal severe epidermal necrosis with heterophilic inflammation was present in one animal and mixed subepidermal inflammation in the other. In the Group F control chameleon euthanized 1 wk post-sham inoculation, skin lesions consisted of mild to moderate infiltration of the superficial dermis with mixed inflammatory cells, and there was one foculal pustule; however, no hyphae were found to be associated with the lesions.

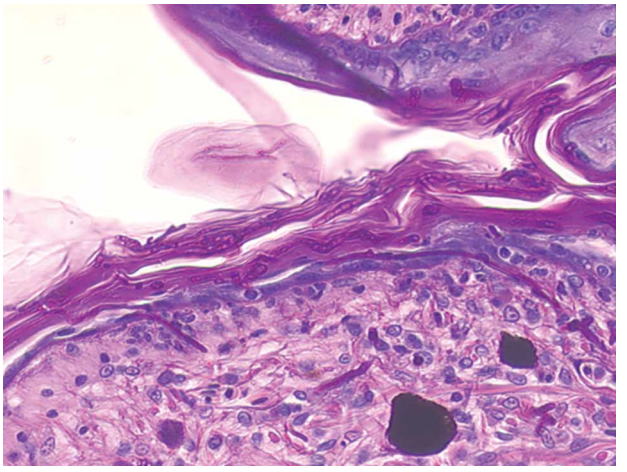

Fig. 4 Downward dermal penetration of fungal hyphae, with moderate heterophilic inflammatory infiltrate. PAS 400×.

Four of ten animals in Group C developed lesions with hyphae on the contact side. One of these animals also had a lesion on the non-contact side, but devoid of hyphae. In two of these animals, the lesions were severe, with ulceration and hyphae invading into the deep dermis and skeletal muscle. The remainder had moderate epidermal and dermal infiltrates of variable numbers of heterophils, lymphocytes and some plasma cells centered on fungal hyphae. In one Group D control animal, a mild dermatitis was present and there was a single focus of hyphae in the stratum corneum.

Three of the environmental challenge Group A animals had skin lesions that ranged from mild

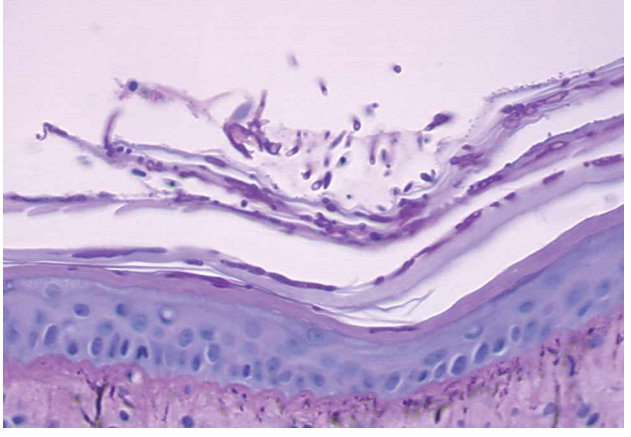

Fig. 3 Fungal proliferation in the epidermal corneum of a veiled chameleon experimentally challenged with the CANV. PAS 200×.

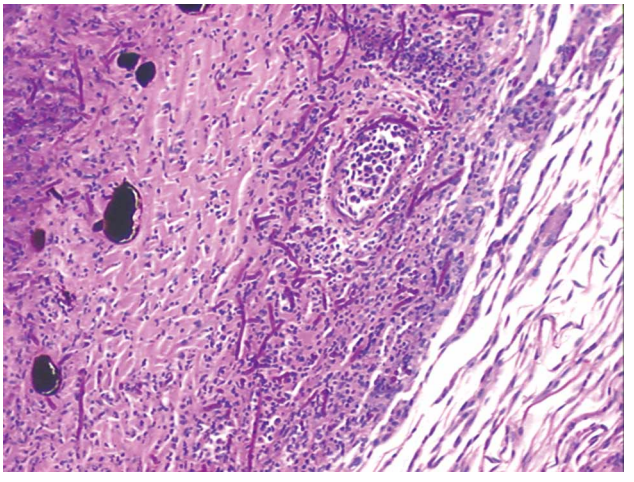

Fig. 5 Proliferation of fungal hyphae in the deep dermis of a veiled chameleon experimentally challenged with the CANV. PAS 200×.

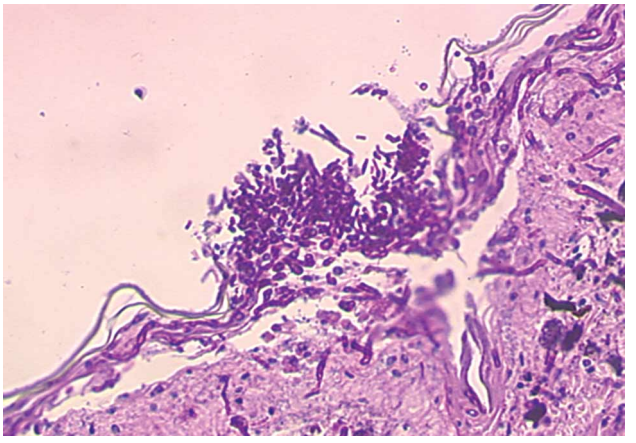

Fig. 6 Dense tufts of arthroconidiating hyphae at the epidermal surface in chameleons experimentally infected with the CANV. PAS 200×.

lymphoid dermatitis to severe necrotizing heterophilic dermatitis with bacterial invasion of subepidermal tissues. Only in the animal with pododermatitis were hyphae present.

Multiple animals had mild to moderate multifocal mineralization in the myocardium; representatives of each study group were included among these.

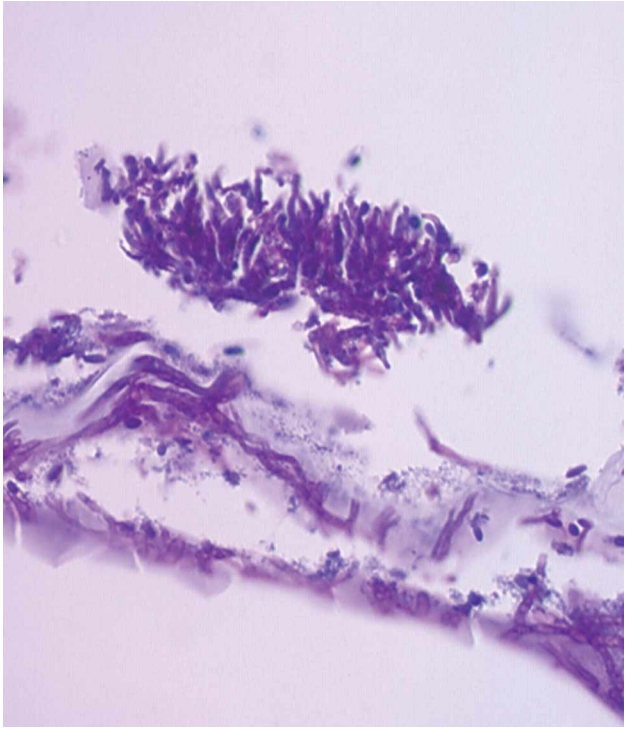

Fig. 7 Dense tufts of arthroconidiating hyphae at the epidermal surface in chameleons experimentally infected with the CANV. PAS 400×.

Additionally, several animals showed varying signs of renal disease including epithelial cell mineralization, distended tubular profiles, mild to moderate interstitial fibrosis and tubular urate tophi.

Discussion

The CANV induced histologically confirmed fungal lesions in each of the experimentally challenged groups and the fungus was recovered from several animals with well-developed lesions, fulfilling the 3rd and 4th Koch's postulates (disease, isolation) and supporting a primary pathogen role of the CANV for veiled chameleons. In all, 14 chameleons, one control and 13 challenged animals, were diagnosed with CANV infection. Following challenge, 13 of 30 animals (43%) became infected. When conidia were applied directly to the skin (Groups C and E), this ratio increased to 12 of 20 animals (60%). All Group C and E chameleons that survived or were allowed to survive to 5 wks post-inoculation had severe fungal disease, suggesting the rate of infection might have been higher if the experimental design had been different and all cutaneously exposed animals allowed to survive 5 wks or longer. In the scarified group, the only two lizards that failed to develop fungal infection were those euthanized 1 wk post-inoculation. Although the CANV was recovered in culture from only 5 of 14 chameleons with lesions, the morphology of hyphal elements in tissue sections was identical in all infected animals.

Based on published reports [1–4] and other accounts [Paré and Sigler, unpublished data], CANV dermatomycosis appears to be contagious and can readily spread within a reptile collection, either directly through contact or indirectly via fomites. In this study, dense tufts of arthroconidiating hyphae were demonstrated histologically on the skin surface of many animals that developed dermatomycosis. Arthroconidia may act as infective propagules involved in the transfer of disease between reptiles. The isolation of the fungus from cage top filters and from a settle plate located on top of a cage illustrates the potential for dissemination of the CANV in the captive environment. Airborne CANV propagules may have accounted for the Group D control animal developing an epidermal fungal lesion.

Exposure through casual contact with conidia present in the environment was assessed with Group A and resulted in infection in one of ten animals. Many chameleons used the suspended gauze to climb onto the cage ceiling's plastic grid, and the pododermatitis in the one infected animal may reflect this habit.

Although it was limited to the foot, infection was severe, deep, and active.

Animals were euthanized sequentially to observe various stages of infection histologically. Hyphal proliferation initially occurred within superficial dead keratinous epidermal layers. From there, hyphae penetrated downward within the epidermis and through the basement membrane. Fungal invasion usually progressed to the deeper dermal layer beyond the dermal papillae, and often through the thin hypodermis into the subjacent muscular layer. The inflammatory reaction paralleled that seen histologically in mammals with fungal infection. Bacterial colonies were often scattered through the necrotic epithelium, and ulceration often extensive. Fungal proliferation in the dermis in many animals suggest that progression to disseminated infection or invasion of the coelom through the body wall, as occurred in one of the chameleons in the pilot trial, could have ensued if animals had not been euthanized.

This experimental model of fungal infection documents the sequence of events from epidermal proliferation to dermal penetration and establishes that the CANV can act as a primary pathogen of veiled chameleons. Further research is needed to determine the ecological niche of this fungus, the range of susceptible reptile species, and to investigate prophylactic and therapeutic options.

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