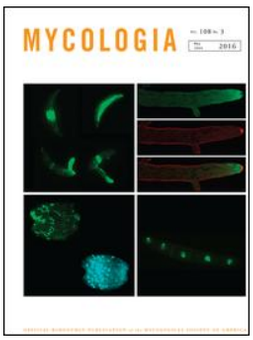

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Koch's postulates: Confirming *Nannizziopsis guarroi* as the cause of yellow fungal disease in *Pogona vitticeps*

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ABSTRACT

Nannizziopsis guarroi is an ascomycete fungus associated with a necrotizing dermatitis in captive green iguanas (*Iguana iguana*) and bearded dragons (*Pogona vitticeps*) across both Europe and North America. Clinical signs of the disease include swelling and lesion formation. Lesions develop from white raised bumps on the skin and progress into crusty, yellow, discolored scales, eventually becoming necrotic. The clinical signs are the basis of a colloquial name yellow fungal disease (YFD). However, until now, *N. guarroi* has not been confirmed as the primary agent of the disease in bearded dragons. In this experiment, we fulfill Koch's postulates criteria of disease, demonstrating *N. guarroi* as the primary agent of YFD in bearded dragons.

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Ascomycota; *Chrysosporium* anamorph of *Nannizziopsis vriesii*; emerging infectious diseases; pet trade; wildlife

INTRODUCTION

Starting in the late 1990s, case reports from veterinary clinics began associating *Nannizziopsis guarroi* with a fungal infection in bearded dragons (*Pogona vitticeps*), termed yellow fungal disease (YFD) (Sigler et al. 2013; Paré and Sigler 2016; Paré et al. 1997). Although little is known about the natural history of *N. guarroi*, an association of *N. guarroi* with the necrotic mycoses described as YFD in lizard species continues to be reported despite the direct lack of evidence for *N. guarroi* as the agent of disease. As reports of YFD in captive reptiles become common, particularly among popular species in the pet trade, concern for the potential spread of the disease among wild populations of susceptible animals is increasing.

Clinical signs associated with YFD include lesion formation and swelling: lesions begin as white raised bumps on the skin and develop into crusty, yellow, discolored scales, eventually swelling from inflammation and becoming necrotic at the sites of infection (Abarca et al. 2010; Le Donne et al. 2016; Schneider et al. 2018). However, many have attributed infections associated with *N. guarroi* to other factors compromising host health, for example, improper husbandry conditions (Le Donne et al. 2016). Case reports associating *N. guarroi* with YFD in captive reptiles dominate the literature, and reports span North America and Europe but also come from Korea and Australia (Paré et al. 1997; Bowman et al. 2007; Abarca et al. 2008, 2009, 2010; Han et al. 2010; Hedley et al. 2010; Waeyenberghe et al. 2010; Johnson et al. 2011; Schmidt-Ukaj et al. 2014, 2016; Le Donne et al. 2016).

Discovering whether a microbe is the primary agent of disease in a host or hosts is critical to elucidating disease dynamics and identifying appropriate control measures. Fulfilling Koch's postulates enables subsequent investigation into disease spread, origin, and host range. Koch's postulates are standard criteria developed to establish an organism (e.g., a fungus, bacterium, or virus) as the cause of a disease. To satisfy Koch's postulates: (i) the potential agent of disease must be found in an infected host, (ii) the agent must be isolated from the infected host and grown in pure culture, (iii) the isolated agent must cause disease when introduced into a healthy host, and (iv) the agent must be reisolated from the newly infected and diseased host while a negative control remains uninfected (Koch 1884; Isenberg 1988; Orélis-Ribeiro et al. 2010).

Koch's postulates are often used to identify primary agents of disease in wildlife. For example, *Pseudogymnoascus destructans* was confirmed as the primary agent of white-nose syndrome (WNS) in little brown bats by fulfilling Koch's postulates (Lorch et al. 2011). Similarly, Koch's postulates were used to confirm *Batrachochytrium dendrobatidis* as the primary agent of chytridiomycosis, a disease causing dramatic population declines and extinctions of numerous amphibian species (Longcore et al. 1999; Mitchell et al. 2008). Koch's postulates confirmed *Ophidiomyces ophiodiicola* as the

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primary agent of snake fungal disease (SFD; Lorch et al. 2015). SFD currently impacts multiple populations of different snake species across North America (Lorch et al. 2016; Burbrink et al. 2017).

The history of WNS, chytridiomycosis, and SFD highlights the potential for YFD to spill over into wildlife, with potentially devastating effects on naïve populations (Fisher et al. 2012). In this context, the apparently widespread distribution of *N. guarroi*, and the lack of information on its ecology and physiology, is disconcerting. Until recently, *N. guarroi* (formerly *Chrysosporium guarroi*) was considered part of the *Chrysosporium* anamorph of *Nannizziopsis vreisii* (CANV) fungal complex. It is now understood to be a separate species and was described from strains isolated from captive green iguanas (*Iguana iguana*) in Spain (Abarca et al. 2008). However, to date, no experiment has tested the assumption that *N. guarroi* is the primary agent of YFD. We aimed to fulfill Koch's postulates with *N. guarroi* by conducting a challenge experiment with captive-bred juvenile bearded dragons to determine whether the fungus is the primary agent of YFD. We are motivated in part by the potential for spillover effects from domestic populations into wildlife, particularly naïve reptile populations (Thompson et al. 2009).

MATERIALS AND METHODS

Animal husbandry.—Our experimental protocols, including the protocols for husbandry and euthanasia, were approved by the University of Wisconsin Institutional Animal Care and Use Committee (IACUC; approval number V006070IACUC). We conducted live animal experiments at the Charmany Instructional Facility in Madison, Wisconsin, USA. We acquired seven 2- to 3-month-old captive-bred bearded dragons (27–48 g) from a local vendor. At the Charmany facility, body condition, shedding, and, after inoculation, infection progression were tracked daily throughout the experiment. Each bearded dragon was housed in an individual plastic tub with a ventilated cover (FIG. 1). Water was available ad libitum; shelter and enrichment in the form of climbable sticks were also provided. We randomly designated two bearded dragons as negative controls and isolated them in a separate room while the remaining five were held in a different room for future inoculation with *N. guarroi*. Control and experimental rooms were maintained between 30 and 35 C with humidity between 30% and 40%, parameters chosen to optimize fungal growth, and which were appropriate for bearded dragon habitats; no heat gradients were provided within the enclosures. Rooms were kept on a 12 h light/dark cycle, and all animals were given 8 days

Figure 1. Animal enclosure.

to acclimate to their new environment. Research veterinary staff conducted an initial physical and wellness check of the animals and determined that all were healthy. We screened all animals for *N. guarroi* prior to the challenge study by swabbing each individual from snout tip to tail end on the dorsal and ventral sides of the body with fine-tip rayon swabs (MW113; Medical Wire & Equipment, Corsham, Wiltshire, UK) moistened with 25 µL of sterile distilled water. Each swab was plated on dermatophyte test medium (DTM) and incubated at 24 C for 20 days. All animals were found free of *N. guarroi*.

Inoculation.—As an inoculant, we used a pure culture of a strain of *N. guarroi* obtained from the UAMH Centre for Global Microfungal Biodiversity culture collection (UAMH-10352); the culture was originally isolated from a bearded dragon with YFD in 2016 in Madison, Wisconsin, USA, and was grown from a single spore. The isolate was grown on Sabouraurd dextrose agar (SDA) and incubated at 24 C for 20 days. A conidial suspension in phosphate-buffered saline containing 0.5% Tween 20 solution (84 250 conidia/µL) was prepared as described in Lorch et al. (2015). The day of inoculation, the five bearded dragons to be inoculated were gently abraded with sterile sandpaper, creating a 1-cm^2 abrasion area at four sites (snout, dorsal-side neck, ventral-side midbody, and left ventral-side leg around the femoral pits; FIG. 2) following a similar protocol described in Lorch et al. (2015); two additional sites were left unabraded (dorsal-side midbody and ventral-side neck; FIG. 2). Each treatment animal was inoculated at each of the six sites with 4 µL of the *N. guarroi* conidial suspension. Following the same abrasion protocol, the negative controls were sham-inoculated with the same, but sterile, saline solution.

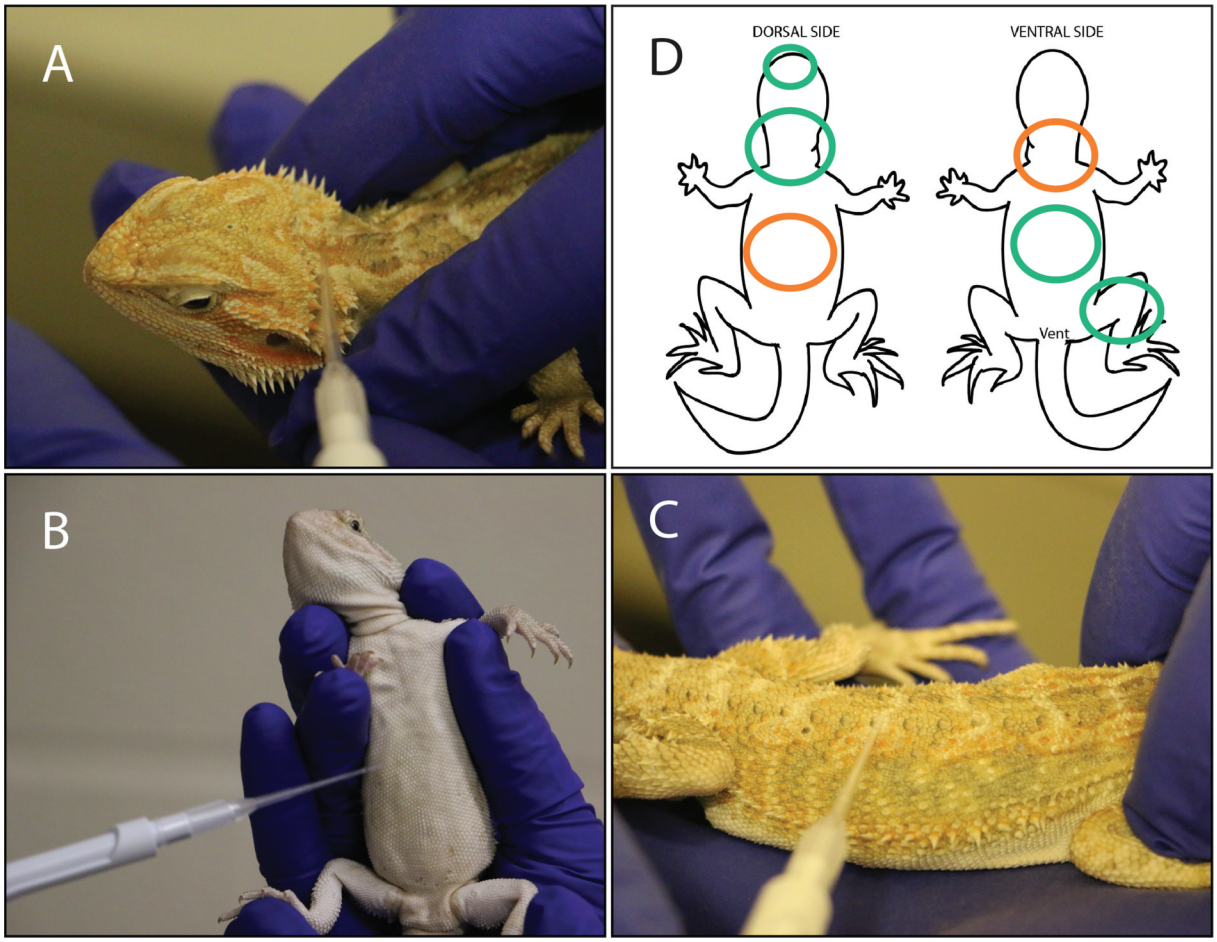

Figure 2. Inoculation of bearded dragons with *N. guarroi*. A–C. Photographs of the inoculation process using 4 µL of *N. guarroi* to inoculate sites on individual bearded dragons. D. Circles mark each of the six inoculation sites across the dorsal and ventral sides of the animal; green circles represent abraded sites, and orange circles represent sites left unabraded. The "vent" label distinguishes between the dorsal and ventral sides.

Euthanasia, necropsy, histopathology.—We euthanized animals if they developed skin lesions exceeding 1 cm in diameter, if there was a noticeable decline in body condition, or on the final day (day 52) of the experiment. Before euthanasia, each animal was anesthetized in an induction chamber using isoflurane gas. Animals were then injected intracoelomically with 0.1 mL of pentobarbital, a fatal dosage. Decapitation was then used per protocol ensuring death. Animals were necropsied by removing skin samples from each inoculated site (and from sham-inoculated sites), surrounding the 1-cm^2 abraded areas and nonabraded areas; samples were divided for DTM cultures and for histopathological analyses. Skin was removed even if there were no visible gross lesions or other presentation of clinical signs at the inoculated sites. Along with the skin samples, internal organs (heart, lung, kidney, spleen, pancreas, esophagus, stomach, and small and large intestines), decapitated heads, and inoculated legs were fixed in 10% neutral buffered formalin. After fixation, heads and legs were decalcified in saturated ethylenediaminetetraacetic acid (EDTA) solution. Tissues for histology were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E) or periodic acid–Schiff (PAS; FIG. 3) depending on tissue type: internal organs were stained with H&E and skin samples were stained with PAS. Each slide was analyzed for fungal elements (hyphae and/or arthroconidia) by a board-certified veterinary pathologist using a constructed histological scale of none, few, moderate, or many.

Sequencing.—Inoculated and sham-inoculated skin samples were cultured on DTM at 30 C for 20 days. Any fungal or bacterial growth from any skin sample was isolated and subcultured on SDA (FIG. 4). To extract fungal DNA from each subculture, a 0.5-cm-diameter plug of fungal material was scraped from the subculture using a sterile scalpel and put in a 2-mL screw-cap tube with 3-mm-diameter glass beads; tissue

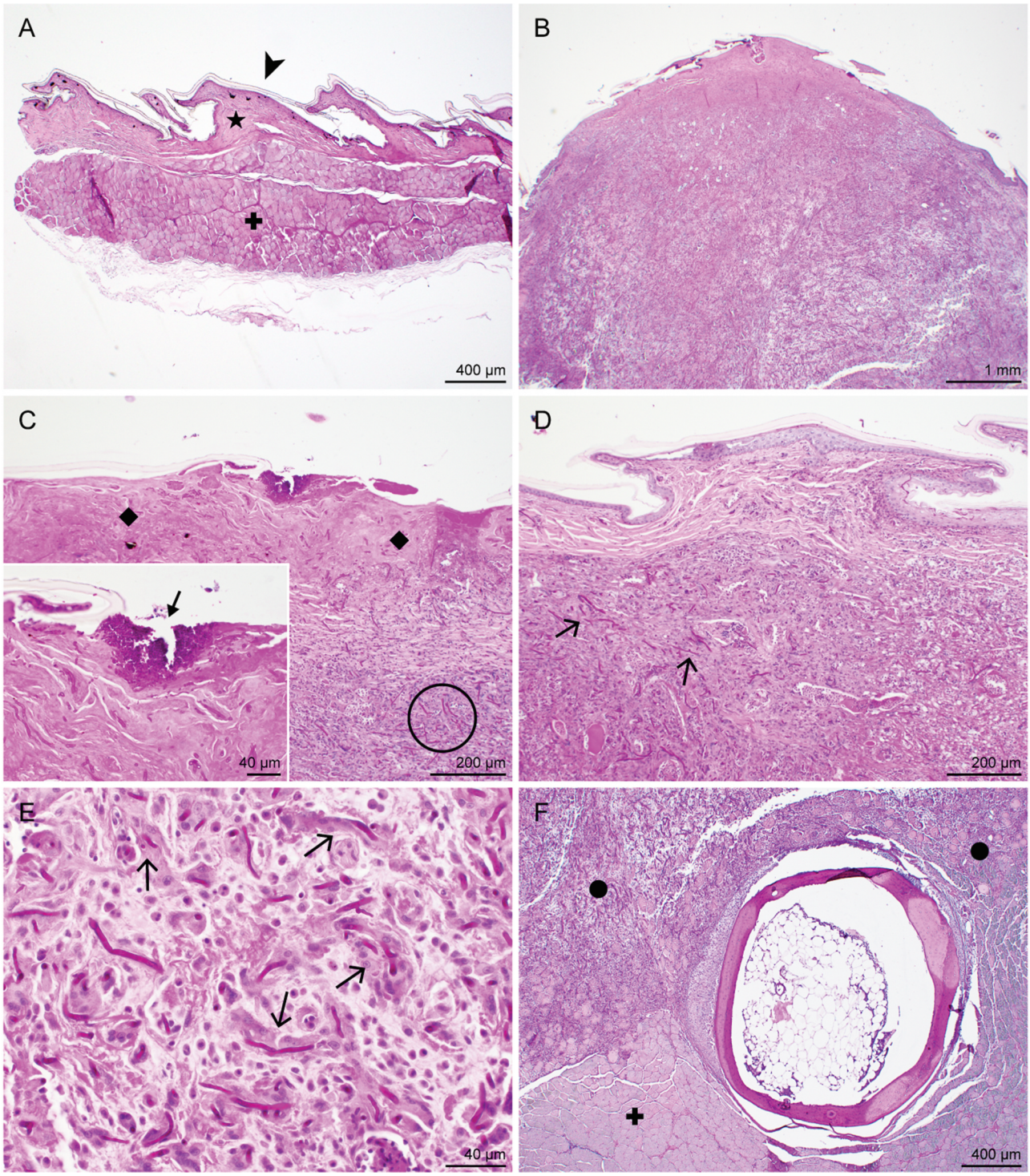

Figure 3. Histological images of tissue samples from control (A) and inoculated (B–F) sites stained with periodic acid–Schiff stain. A. Dorsal neck, BD1NC, negative control. A thin epidermis and stratum corneum (arrowhead) overlie a sparsely cellular dermis (star) and thick muscular layer (cross). B. Leg, BD12N. The epidermis, dermis, and muscular layers are obscured and replaced by widespread necrosis and inflammation. C. Leg, BD10N. There is marked epidermal and dermal necrosis (diamonds) and granulomatous dermatitis with intralesional fungal hyphae (circle) and conidia; inset: a cluster of conidia (thick arrow) fills an ulcer. D. Ventral body, BD12N. There is deep granulomatous dermatitis with intralesional fungal hyphae (arrows). E. Ventral body, BD12N. Fungal hyphae within the dermis are surrounded by macrophages (arrows). F. Leg, BD12N. There is marked necrotizing myositis (solid circles) extending to the femur. Only a small portion of unaffected muscle remains (cross).

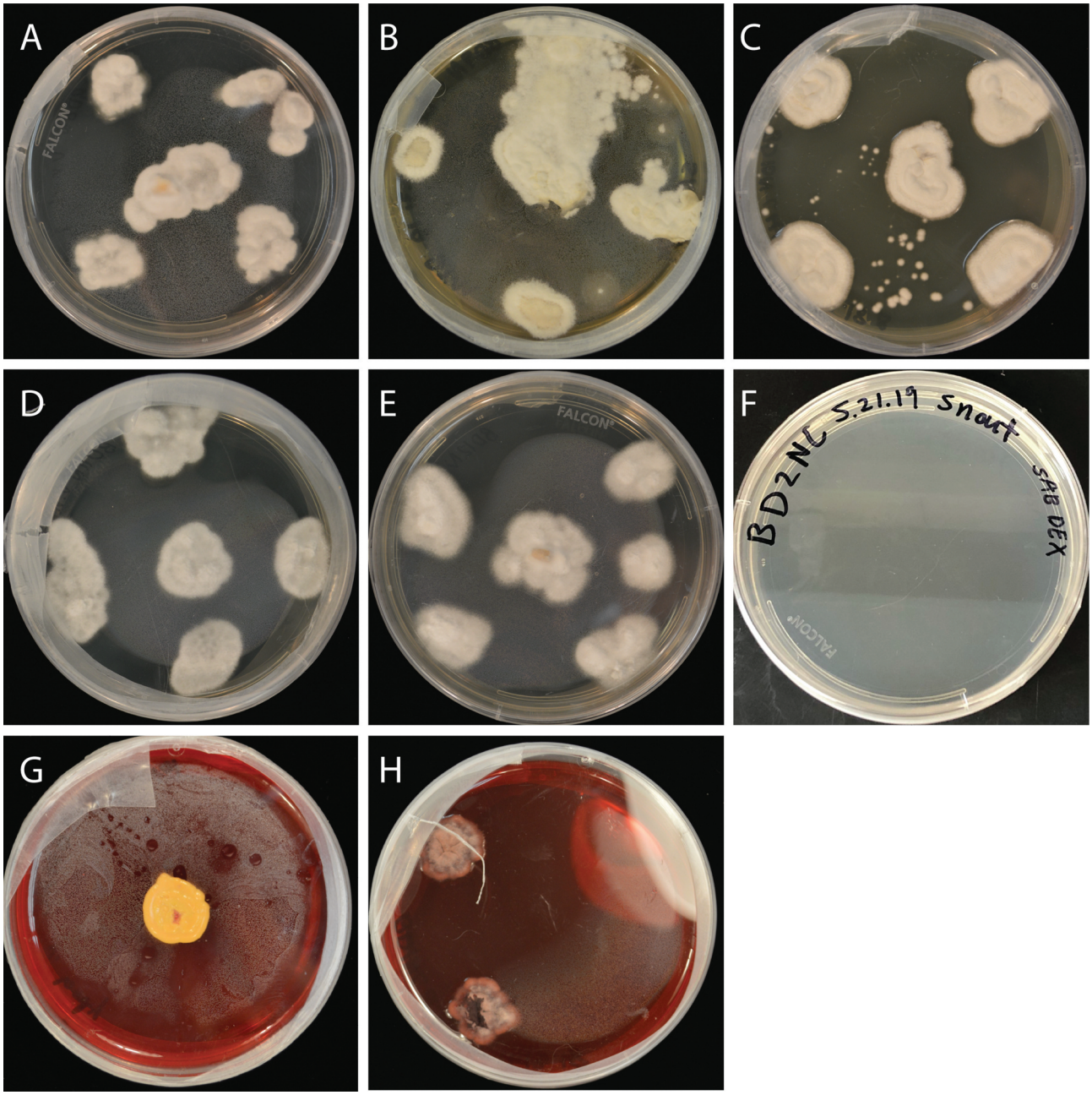

Figure 4. Culture results from each of the seven bearded dragons. A–E. Cultures of *N. guarroi* maintained on SDA isolated from diseased bearded dragons. F. Plate of SDA with no growth isolated from a negative control, BD2NC. G, H. Cultures of the *Kocuria* sp. and *C. globosum*, respectively, on DTM, isolated from the negative control, BD1NC.

was macerated using a mini-beadbeater (product number: 112011; BioSpec Products, Bartlesville, Oklahoma). Macerated tissues were subsequently placed in 600 µL of cetyltrimethylammonium bromide (CTAB) buffer and incubated at 65 C for 1 h. We then added 600 µL of chloroform isoamyl alcohol to each tube, mixing it with the CTAB and then centrifuging at 13 000 rpm for 10 min. Supernatants were pipetted off and placed in new, sterile 2-mL Eppendorf tubes; we added 600 µL chloroform isoamyl alcohol to each tube and gently rocked tubes on a tipping tray for 10 min. Next, tubes were centrifuged a second time. Following centrifugation, iced 96% ethanol (EtOH) was added at 2× the total sample volume to wash the extracted DNA. We washed each DNA pellet twice and then dried the pellets in a DNA SpeedVac system (Thermo Fisher Scientific, Asheville, North Carolina, USA) for 30 min before adding 400 µL of Tris-EDTA (TE) buffer and storing extracted DNA in the −20 C freezer to be used for downstream polymerase chain reaction (PCR).

Bacterial DNA was extracted using the Qiagen DNeasy PowerSoil Kit (Germantown, Maryland) following the manufacturer's protocol.

We conducted PCR for Sanger sequencing on all extracted DNA. For fungi, we amplified the internal transcribed spacer (ITS) region in fungal samples using standard fungal primers, ITS1-F and ITS4 (Manter and Vivanco 2007), EconoTaq PLUS 2× Master Mix (Lucigen, Radnor, Pennsylvania, USA), and distilled water. PCR reactions used the following parameters: 2 min at 95 C, 15 s at 95 C (30 cycles), 15 s at 49 C, 1 min at 68 C, and 5 min at 68 C. For bacteria, we amplified the 16S ribosomal region using primers rp2 and fD1 (Weisburg et al. 1991) and PCR reactions used the following parameters: 5 min at 95 C, 1 min at 94 C (25 cycles), 1 min at 59 C, 2 min at 72 C, and 5 min at 72 C.

We submitted the ITS and 16S amplicons to a third-party vendor for Sanger sequencing (Functional Biosciences, Madison, Wisconsin), receiving the ab1 files and then visually inspecting and trimming the chromatographs to verify the quality of each of our samples. To determine the species of fungi and bacteria we cultured, we next used BLAST (Basic Local Alignment Search Tool; Altschul et al. 1990) to compare our sequences with sequences stored in GenBank (Benson et al. 2016). Subsequently, we deposited four sequences into GenBank (accession nos. MT503283, MT503284, MT503285, and MT503223), one of which (MT503283) represents our *N. guarroi* cultures (every culture yielded an identical ITS sequence); sequences are described in more detail in Results.

RESULTS

By day 52 post inoculation, all lizards exposed to *N. guarroi* had developed clinical signs of infection; the negative-control animals developed no clinical signs of infection. The initial clinical signs appeared between 15 and 31 days post inoculation (FIG. 5). Initial clinical signs were discolored, yellow scales at inoculated sites, often on the ventral midbody and around the femoral pits. Affected areas of skin eventually became swollen, thickened, and darker in coloration. Following are the observed onset of clinical signs for each bearded dragon inoculated with *N. guarroi* summarized from our daily logs; individual animals are identified as BD8N, BD9N, BD10N, BD12N, and BD11N. Animal BD8N developed a single lesion on the ventral side of the neck, 31 days after inoculation. The lesion did not swell or become necrotic by the end of the experiment. Animal BD9N also developed a lesion on the ventral side of the neck 31 days after inoculation that did not swell or become necrotic by the end of the experiment, similar to BD8N. However, BD9N developed an additional lesion on the tail, which was not a site we abraded or inoculated. Animals BD10N and BD12N developed lesions on the ventral midbody and femoral pits 20 days after inoculation, both of which progressed into swelling and necrosis at the inoculated sites by the end of the experiment. Animal BD11N developed necrotic lesions on the femoral pits and across the snout 15 days after inoculation, which progressed into the eye, causing swelling and a thickening of the scales around the eye. Due to its rapidly declining body condition, BD11N was euthanized 22 days after inoculation and did not reach the end of the experiment. Among the five treated bearded dragons, there was no consistent pattern between abraded and nonabraded sites, as lesions formed in both kinds of (but not all) sites. Ecdysis (shedding of the skin) was infrequent during the experiment. Some animals completed a full shed within 2–3 days, whereas others shed but retained skin remnants for up to 14 days. However, regardless of whether shedding events were complete or incomplete, clinical signs persisted, even after shedding.

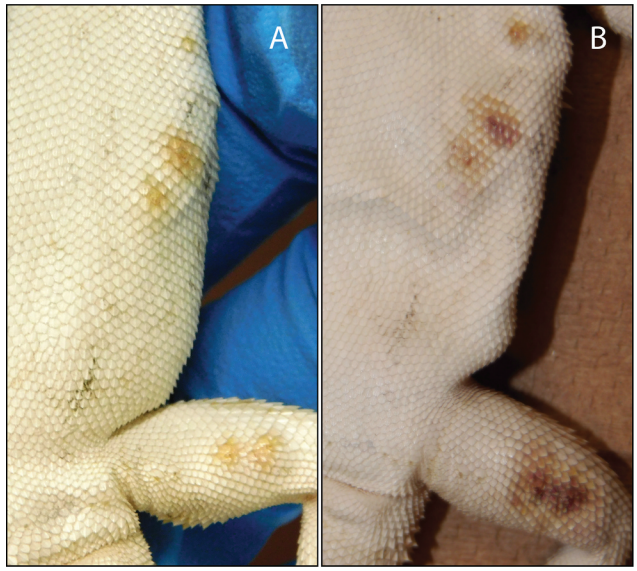

Figure 5. Disease progression on an individual bearded dragon photographed on different days. A, B. Manifestation of clinical signs of infection 20 (A) and 35 (B) days post inoculation, respectively.

Histological analyses confirmed a positive infection in four of the five treated bearded dragons. Fungal elements (hyphae and/or arthroconidia) were present in epidermal, dermal, and/or muscle tissue (FIGS. 3, FIGS. 5). Microscopically, skin lesions were consistent with those previously reported for YFD in bearded dragons (Bowman et al. 2007; Paré and Jacobson 2007; Hedley et al. 2010). Lesions were characterized by epidermal ulceration, epidermal and dermal necrosis, and

histiocytic to granulomatous dermatitis, panniculitis, and myositis. Lesions contained moderate to large numbers of 3–5-µm-diameter, parallel-walled, septate, rarely branching fungal hyphae extending from the epidermis to the underlying muscular layer. Rarely, fungal hyphae were found within blood vessels of the dermis and subcutis. Cylindrical 2 × 3 µm conidia were occasionally present in clusters on the skin surface. In some areas, infection was limited to the subcutis and muscle, with minimal involvement of the epidermis and dermis. Mature granulomas as described in previous reports were not seen, probably because of the relatively short duration of the infection trial. In the five treated bearded dragons, none of the internal organ samples had fungal elements, indicating no sign of systemic infection; the negative controls had no fungal elements present in any tissue samples.

Nannizziopsis guarroi was reisolated in culture from 24 of the 30 skin samples taken from the 30 inoculated sites of the five bearded dragons exposed to the fungus (FIG. 4; TABLE 1). Sequencing data confirmed that every culture of *N. guarroi* was identical to the initial strain used during inoculation. Six of the 30 skin samples (1 from BD8N, 2 from BD9N, 1 from BD11N, and 2 from BD12N) did not grow fungi when cultured (TABLE 2). *Neosetophoma guiyangensis* was cultured from a single skin sample of one bearded dragon (BD8N; TABLE 2) from which *N. guarroi* was also cultured; we deposited the *N. guiyangensis* sequence into GenBank (MT503284). The *Neosetophoma* are a genus of fungal endophytes and saprobes associated with plant hosts and are not known to be pathogenic to animals (Karunarathna et al. 2017; Hyde et al. 2018). The skin samples from the negative controls never generated *N. guarroi* in culture. However, *Chaetomium globosum* and a *Kocuria* sp. were isolated in culture from one negative-control animal (BD1NC; TABLE 2); we deposited sequences of both cultures into GenBank (MT503285 and MT503223, respectively). Neither organism is a known pathogen of bearded dragons; *C. globosum* is a cosmopolitan fungal endophyte and *Kocuria* sp. is a Gram-positive bacterium that occurs on the skin of humans and animals without inciting disease (Kandi et al. 2016; Wang et al. 2016). The negative-control samples containing *C. globosum* and the *Kocuria* sp. did not present any internal or external signs of infection or abnormal clinical signs that would suggest disease from those specific microbes; no histopathological evidence of any of these microbes was seen.

DISCUSSION

By fulfilling Koch's postulates, our work definitively demonstrates *N. guarroi* as the primary agent of YFD in bearded dragons: all five treatment animals developed clinical signs of YFD and *N. guarroi* was reisolated from each animal.

Nannizziopsis guarroi persisted at the inoculated sites throughout the experiment and was reisolated from 80% of inoculated sites. However, results of the

Table 1. Clinical signs, histopathology, and reisolation results of *N. guarroi*.

Animal ID	Treatment	Clinical signs	Histopathology	Reisolation	Clinical signs	Histopathology	Reisolation
				Inoculated sites			
			Snout*			Dorsal neck*	
BD1NC	Neg. control	–	–	–	–	–	–
BD2NC	Neg. control	–	–	–	–	–	–
BD8N	*N. guarroi*	–	–	+	–	–	+
BD9N	*N. guarroi*	–	–	+	–	+	+
BD10N	*N. guarroi*	–	–	+	–	–	+
BD11N	*N. guarroi*	+	–	+	–	–	+
BD12N	*N. guarroi*	–	–	–	–	–	+
			Ventral neck			Dorsal midbody	
BD1NC	Neg. control	–	–	–	–	–	–
BD2NC	Neg. control	–	–	–	–	–	–
BD8N	*N. guarroi*	+	–	+	–	–	+
BD9N	*N. guarroi*	+	+	+	–	+	–
BD10N	*N. guarroi*	–	–	+	–	–	+
BD11N	*N. guarroi*	–	–	+	–	–	–
BD12N	*N. guarroi*	–	–	–	–	–	+
			Ventral midbody*			Ventral hindleg*	
BD1NC	Neg. control	–	–	–	–	–	–
BD2NC	Neg. control	–	–	–	–	–	–
BD8N	*N. guarroi*	–	–	+	–	–	–
BD9N	*N. guarroi*	–	+	+	–	–	–
BD10N	*N. guarroi*	+	+	+	+	+	+
BD11N	*N. guarroi*	+	+	+	+	–	+
BD12N	*N. guarroi*	+	+	+	+	+	+

*Inoculated site that was abraded before inoculation. (–) None observed. (+) Positive observation.

Table 2. Cultured fungi and bacteria isolated from inoculated sites.

		Inoculated sites					
		Snout	Dorsal neck	Ventral neck	Dorsal midbody	Ventral midbody	Ventral hindleg
Animal ID	Treatment	Cultured fungi and bacteria					
BD1NC	Negative Control	—	—	—	*Chaetomium globosum*	*Kocuria* sp.	—
BD2NC	Negative Control	—	—	—	—	—	—
BD8N	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Neosetophoma guiyangensis* *Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	—
BD9N*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	—	*Nannizziopsis guarroi*	—
BD10N	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*
BD11N*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	—	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*
BD12N	*Nannizziopsis guarroi*	—	*Nannizziopsis guarroi*	—	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*	*Nannizziopsis guarroi*

*BD9N also developed a lesion on its tail from which a culture was taken, resulting in a positive identification of *N. guarroi*. BD11N developed a lesion near the eye and part of that was also taken for culture and identified as *N. guarroi*.

histopathological analyses and reisolation were not uniform across each treatment animal or individual inoculation site (TABLE 1). For example, a bearded dragon sometimes did not present histological evidence of infection at an inoculation site from which *N. guarroi* was cultured. Conversely, a bearded dragon sometimes presented histological evidence at a site from which *N. guarroi* was not reisolated. In the case of one bearded dragon, a lesion formed on the tail, a location that was neither abraded nor inoculated. Our results highlight the variability in clinical signs of YFD: three animals developed swelling and lesions at inoculated sites 20 days after inoculation, whereas two remained clinically normal for 41 days post inoculation, 4 days before euthanasia. The most likely explanation for both the variability among sites and the two bearded dragons with a late onset of clinical signs is early-stage infection. Had the experiment continued, the early-stage infections associated with a relatively late onset of clinical signs would likely have progressed into a more severe disease.

Observed variability may also be explained by the texture, topography, and coloration of bearded dragon skin, which can make it difficult to observe abnormalities, including lesions, especially when gross lesions are <1 cm in diameter. Because of the relatively small sizes of some lesions, it was difficult to divide samples and ensure that a portion of the lesion was analyzed both histologically and by culture. Moreover, capturing the infection within any lesion histologically can be difficult because the thin sections viewed microscopically represent a very small portion of sampled tissue.

Nannizziopsis guarroi was able to be reisolated even after the bearded dragons underwent ecdysis (shedding of the skin). In some animals, shed remnants adhered to the new epidermis for as long as 14 days, and this time may have allowed the pathogen to establish into deeper tissues or enabled reinfection of the new epidermis.

Epidermal abrasion does not appear to be a requirement for infection. Paré et al. (2006) demonstrated that breakage of the epidermis was an important variable for the development of infection using an isolate of a *Nannizziopsis* sp. on chameleons (*Chamaeleo calyptratus*). In our study, infection also appeared to involve epidermal breakage, as both visible gross lesions and histopathological evidence of disease were predominantly seen on abraded areas. However, there was also evidence of fungal elements in nonabraded areas, implying that an intact epidermis is not a completely effective deterrent to infection. Although epidermal breakage may facilitate infection and can serve as a primary route for invasion, fungal presence in nonabraded areas highlights the ability of *N. guarroi* to colonize even unabraded skin, similar to *O. ophiodiicola* (Lorch et al. 2015). Had the experiment

continued, we hypothesize that disease would have appeared and progressed at all inoculated sites on every individual animal, abraded and unabraded. The dorsal side of the epidermis of bearded dragons is more heavily keratinized (it is thicker) than the ventral side, a feature that makes abrasions more likely to occur on the abdomen. The thickness of the dorsal side of the epidermis may slow the rate of fungal invasion, which may explain why we found fungal elements within dorsal tissue but did not see visible clinical signs of infection in that area.

The ecology of *N.* guarroi is poorly understood. Apart from optimal growth temperatures, little is known (Abarca et al. 2008, 2009). Closely related pathogenic fungi, including *O. ophiodiicola*, are thought to persist in environments outside of host animals, often establishing in soil, which acts as a reservoir of infection (Guthrie et al. 2016). Reservoirs of potentially infectious material may facilitate consistent exposure to a host, continual reinfection, and potentially a more systemic and fatal disease. *Nannizziopsis guarroi* can generally tolerate warmer temperatures than such pathogens as *O. ophiodiicola* and *B. dendrobatidis* or *Batrachochytrium salamandrivorans* (Allender et al. 2015; Blooi et al. 2015), which may allow it to persist for longer periods of time and in a greater range of environments in the absence of a host. Research into *N. guarroi*'s native geographic range, natural history, and ecology is critically needed to enable an understanding of the transmission and epidemiology of YFD.

Fungal pathogens often appear opportunistic in nature, primarily causing infections in hosts with preexisting health problems and, in the case of *N. guarroi*, also in animals kept under poor husbandry conditions (Cabañes et al. 2014; Le Donne et al. 2016). Although declining body conditions and suboptimal environments will certainly influence the onset of infection, the former CANV (*Chrysosporium* anamorph of *Nannizziopsis vriesii*) fungi (e.g., *O. ophiodiicola*) have increasingly been demonstrated to function as primary agents of diseases in various reptile species (Han et al. 2010; Cabañes et al. 2014). As we demonstrate here, *N. guarroi* also appears to be a primary agent of disease.

Nannizziopsis guarroi has never been discovered in a wild host and is only known from captive animals. Case reports of *N. guarroi* associated with fatal infections and implicating the fungus as a primary pathogen span 1997–2018, and disease has been described from multiple species of lizards in captivity across North America, Europe, Korea, and Australia, but without any discussion of how dispersal might occur or whether there is potential for the fungus to spill over and establish as an invasive pathogen in ecosystems (Fisher et al. 2020). If the fungus were to spill over into wild populations, it has the potential to cause significant morbidity and mortality to naïve populations of other reptile species. Although degrees of resistance have been documented for animals exposed to other fungal pathogens (e.g., Archey's frog, *Lepiopelma archeyi*, is less susceptible to *B. dendrobatidis* than other frog species, and the big brown bat, *Eptesicus fuscus*, is resistant to infection by *P. destructans* (Savage and Zamudio 2011; Frank et al. 2014; Palmer et al. 2018), whether different reptile species vary in susceptibility to *N. guarroi* is unknown. However, the methods presented here could be used to establish differential susceptibility experimentally; the true risk that YFD poses to wild reptile populations requires further study.

Species known to be susceptible to *N. guarroi* are popular species commonly found in the pet trade (i.e., chameleons, green iguanas, and bearded dragons), and trade provides opportunities for the pathogen to be moved long distances and to jump to new hosts. Roughly 350 million live animals are imported and exported across the globe annually; many are poached from the wild (Warwick 2014). The potential spillover of YFD into naïve populations is an imminent threat. Our findings are a first step toward illustrating the importance of *N. guarroi* as a primary pathogen and emphasize the need for additional research to better understand the ecology and impact of the fungus on reptile populations worldwide.

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