Experimental Infection of Snakes with *Ophidiomyces ophiodiicola* Causes Pathological Changes That Typify Snake Fungal Disease

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ABSTRACT Snake fungal disease (SFD) is an emerging skin infection of wild snakes in eastern North America. The fungus *Ophidiomyces ophiodiicola* is frequently associated with the skin lesions that are characteristic of SFD, but a causal relationship between the fungus and the disease has not been established. We experimentally infected captive-bred corn snakes (*Pantherophis guttatus*) in the laboratory with pure cultures of *O. ophiodiicola*. All snakes in the infected group (*n* = 8) developed gross and microscopic lesions identical to those observed in wild snakes with SFD; snakes in the control group (*n* = 7) did not develop skin infections. Furthermore, the same strain of *O. ophiodiicola* used to inoculate snakes was recovered from lesions of all animals in the infected group, but no fungi were isolated from individuals in the control group. Monitoring progression of lesions throughout the experiment captured a range of presentations of SFD that have been described in wild snakes. The host response to the infection included marked recruitment of granulocytes to sites of fungal invasion, increased frequency of molting, and abnormal behaviors, such as anorexia and resting in conspicuous areas of enclosures. While these responses may help snakes to fight infection, they could also impact host fitness and may contribute to mortality in wild snakes with chronic *O. ophiodiicola* infection. This work provides a basis for understanding the pathogenicity of *O. ophiodiicola* and the ecology of SFD by using a model system that incorporates a host species that is easy to procure and maintain in the laboratory.

IMPORTANCE Skin infections in snakes, referred to as snake fungal disease (SFD), have been reported with increasing frequency in wild snakes in the eastern United States. While most of these infections are associated with the fungus *Ophidiomyces ophiodiicola*, there has been no conclusive evidence to implicate this fungus as a primary pathogen. Furthermore, it is not understood why the infections affect different host populations differently. Our experiment demonstrates that *O. ophiodiicola* is the causative agent of SFD and can elicit pathological changes that likely impact fitness of wild snakes. This information, and the laboratory model we describe, will be essential in addressing unresolved questions regarding disease ecology and outcomes of *O. ophiodiicola* infection and helping to conserve snake populations threatened by the disease. The SFD model of infection also offers utility for exploring larger concepts related to comparative fungal virulence, host response, and host-pathogen evolution.

Emerging diseases represent significant threats to the conservation of many wildlife species. Over the past several decades, the number of emerging fungal diseases—and the number of species extinctions and extirpations caused by those diseases— has greatly increased (1, 2). The large-scale declines and extinction events caused by the chytrid fungus *Batrachochytrium dendrobatidis* (3, 4), and the bat pathogen *Pseudogymnoascus destructans* (the cause of white-nose syndrome [5–7]) highlight the devastating impacts that fungal diseases can have on wild host populations. Due to the ability of many fungi to infect ectothermic hosts whose populations are not well-monitored, fungal diseases in cold-blooded vertebrates may go largely unnoticed until their effects become pronounced.

In 2006, the first documented occurrence of population declines associated with skin infections in snakes was reported in New Hampshire (8). Allender et al. (9) subsequently reported fungal infections in a population of massasauga rattlesnakes (*Sistru-*

rus catenatus) that threatened the viability of the species in the state of Illinois. Conservation concerns over these two imperiled snake populations brought attention to an infection that later became known as snake fungal disease (SFD). While the history of the infections in wild North American snakes remains unclear, mounting evidence indicates that SFD represents an emerging disease that poses a threat to wild snakes in the eastern United States (9–12).

Snake fungal disease has been routinely attributed to infection by the fungus *Ophidiomyces ophiodiicola* (reviewed in reference 12). *Ophidiomyces* was formerly classified as the *Chrysosporium* anamorph of *Nannizziopsis vriesii* species complex (CANV species complex), a group of fungi that are frequently associated with emerging infections in various groups of reptiles (reviewed in reference 13). Recent phylogenetic analyses have demonstrated that CANV represents a species complex that also includes fungi of the genera *Nannizziopsis* and *Paranannizziopsis* and that *Ophidiomy-*

ces is known to occur only on snakes (13–15). However, a clear cause-and-effect relationship between *O. ophiodiicola* and SFD is lacking. Specifically, it is unclear whether *O. ophiodiicola* is the cause of the skin lesions that characterize SFD or is simply an opportunistic invader of necrotic tissue. Bohuski et al. (16) reported that when sensitive detection methods were utilized (i.e., real-time PCR), *O. ophiodiicola* was occasionally found on the skin of snakes without clinical signs of SFD, suggesting that presence of the fungus is not strictly correlated with disease. Direct evidence is needed to establish *O. ophiodiicola* as a primary pathogen and to demonstrate a causal relationship between *O. ophiodiicola* and SFD.

Along with uncertainty over the cause of SFD, little is understood about progression of the disease and how it influences individual and population fitness. Reported clinical signs associated with purported *O. ophiodiicola* infections are highly variable and are often recorded at a single time point, providing little information on the affected animal's history, disease development over time, mechanisms of the host for coping with the infection, and indirect consequences on snake health and survival. Allender et al. (9) reported 100% mortality in Illinois massasauga rattlesnakes that had SFD, and Clark et al. (8) documented over a 50% decline in a population of timber rattlesnakes (*Crotalus horridus*) following the appearance of clinical signs consistent with SFD. However, the disease has been reported in other areas with apparent resolution of clinical signs and with no obvious impacts to the infected populations (17, 18). Clearly, the ecology of SFD and outcomes of the disease at individual and population levels are complex. Development of a laboratory model will be essential for studying these infections in a controlled manner to better define the intricacies of SFD dynamics.

To determine whether *O. ophiodiicola* is capable of serving as a primary pathogen, we attempted to fulfill Koch's postulates (19) by exposing captive-bred corn snakes (*Pantherophis guttatus*) to an isolate of the fungus cultured from a wild snake diagnosed with SFD. In addition to recreating lesions consistent with SFD, we also observed host responses that better elucidate how snakes respond to the infection and how these physiological and behavioral changes may influence disease dynamics in wild populations. Together, this work lays a solid foundation upon which future research directed toward understanding the pathogenicity of *O. ophiodiicola* and the ecology of SFD can be developed.

RESULTS

Ophidiomyces ophiodiicola **causes SFD.** Snakes exposed to *O. ophiodiicola* developed clinical signs consistent with SFD 4 to 8 days postinoculation. Initial lesions consisted of generalized regional swelling and increased pallor of scales (Fig. 1D). Swelling on the snout often resulted in occlusion of the nares and, in a couple of instances, misalignment of the upper and lower mandibles. Swelling at inoculation sites on the body was less common and more transient than that observed on the snout. Following regional swelling, individual scales became edematous as immune cells infiltrated the specific sites of infection. As the lesions progressed, scales became thickened and yellow (Fig. 1E) and eventually developed rough, brown crusts with hyperpigmentation (Fig. 1F). Lesions often began at the edges of individual scales and frequently coalesced to involve several adjacent scales. Two snakes in the infected group (25%) exhibited bouts of anorexia.

Skin lesions on infected snakes became progressively larger and more severe, until the animals shed on days 15 to 20. Several days prior to molting, fluid-filled vesicles developed that encompassed, and extended outward from, the inoculation sites (Fig. 1I); on the snout, accumulation of fluid between old and new layers of skin sometimes resulted in severe distortion of the head (Fig. 1H). Molted skins from snakes in the infected group were often bunched up with areas of the shed adhering to one another, and fragments of the molt were occasionally retained on the new skin (dysecdysis). On the molted skin of infected snakes, lesions were clearly visible as brown crusts. Lesions were grossly resolved upon completion of ecdysis; however, some previously affected scales were shrunken, deformed, or slightly depigmented. Reexposure to *O. ophiodiicola* resulted in recurrence of gross lesions.

Throughout the study, snakes in the control group remained healthy (Fig. 1A and B). Control snakes occasionally exhibited minor damage to scales at sham inoculation sites (Fig. 1C). These changes were distinct from the discolored, thickened skin that developed in infected snakes and appeared to be the result of mechanical damage due to abrasion of the skin (see Materials and Methods) or removal of the adhesive portion of the bandage. The shedding process occurred normally in the control group with no abnormalities of the molted skin and no cases of dysecdysis.

The presence of lesions indicative of SFD was significantly different between the infected and control groups ($P < 0.001$). At necropsy, all eight snakes in the infected group had gross lesions consistent with SFD. Of the 40 sites inoculated (five inoculation sites on eight snakes), 34 had gross lesions. Lesions consistent with SFD occurred more frequently at sites where the skin had been abraded (100%) prior to application of fungal conidia compared to nonabraded inoculation sites (62.5%). As the dose of conidia increased with subsequent inoculations, gross skin lesions appeared to be more likely to develop at nonabraded inoculation sites, but the dose or number of inoculations did not noticeably impact the severity of the lesion. All snakes were in good body condition with adequate fat stores at the time they were euthanized.

Microscopically, significant skin lesions in infected snakes included discrete areas of necrosis and granulocytic inflammation in the superficial to midepidermis, with adjacent epidermal granulocytic inflammation and edema (Fig. 2D and E). Necrotic portions of the epidermis often contained a few to many 2- to 5-μm-wide, parallel-walled, occasionally septate, rarely branching, periodic acid-Schiff (PAS) stain-positive and Grocott's methenamine silver (GMS) stain-positive fungal hyphae. In one animal, rectangular arthroconidia measuring approximately 2 by 5 μm were noted on the surface of the stratum corneum (Fig. 2H). Mild to moderate dermal and intramuscular inflammation ranging from granulocytic to mononuclear was consistently present in infected snakes and was typically more severe under areas of necrosis (Fig. 2E). A few snakes exhibited dermal granulomas (Fig. 2G) which occasionally contained fungal hyphae; granulomas were most common on the head but were also noted within the neck and chin of one snake each. The microscopic lesions and morphology of the fungus were consistent with those observed in wild snakes with infections associated with *O. ophiodiicola* (Fig. 2J, K, and L) (11, 20, 21) and corresponded to the presence of gross lesions observed at necropsy. Small gaps in the stratum corneum of both control and infected animals were almost exclusively present on areas of skin that had been abraded. In snakes exposed to *O. ophiodiicola*, breaks in the stratum corneum were more com-
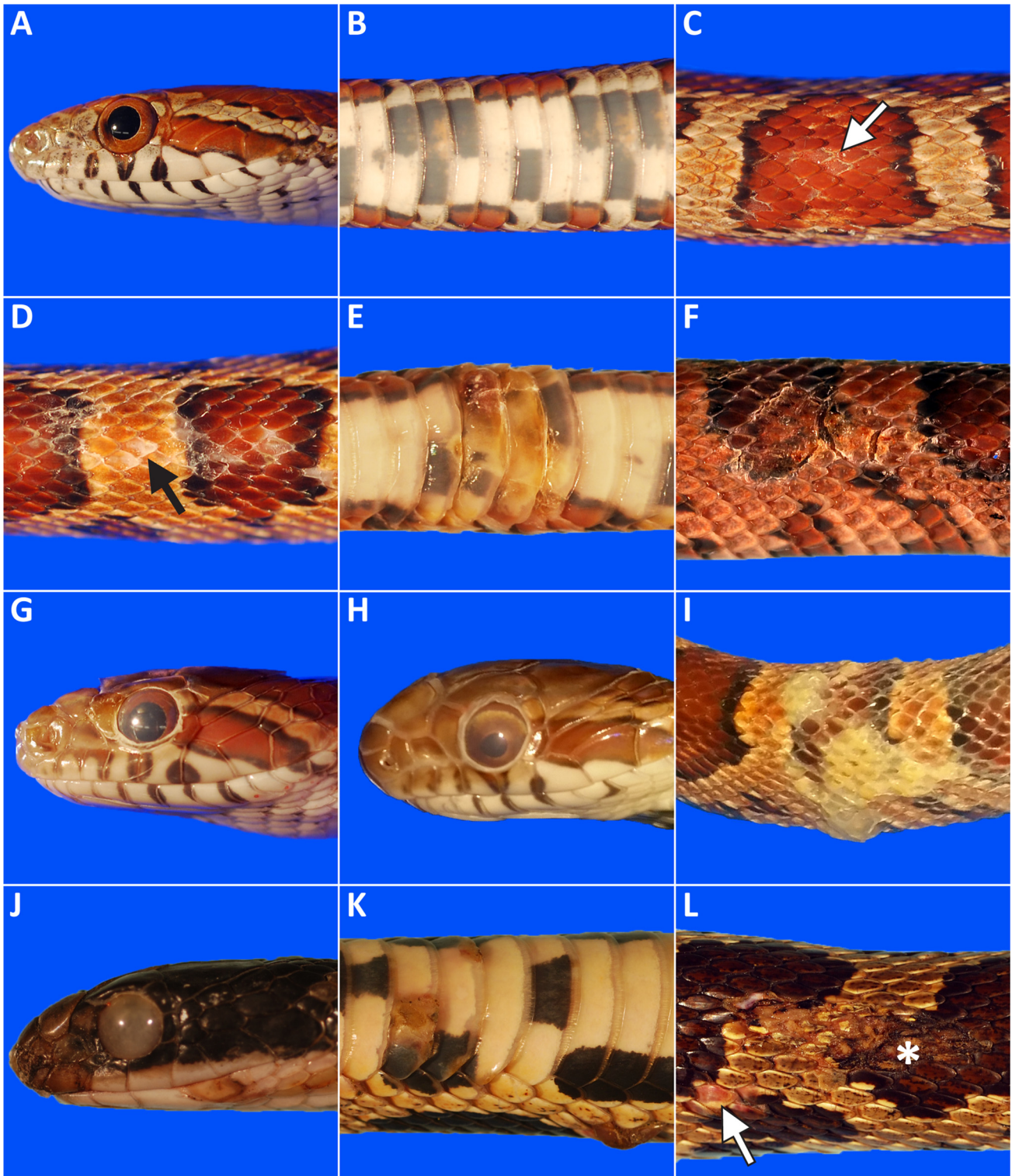
FIG 1 Clinical signs of SFD in snakes experimentally challenged with *Ophidiomyces ophiodiicola*. (A to C) Sham-inoculated sites of snakes in the control group did not develop gross lesions characteristic of SFD. However, subtle damage to the scales (arrow) caused by the abrasion process was visible at the dorsal midbody site. In contrast, snakes exposed to *O. ophiodiicola* developed a range of clinical signs as the disease progressed. (D) Initially, individually infected scales were swollen and whitened (arrow). (E and F) Infected scales later became thickened and turned yellow to brown (E), eventually forming crusts of necrotic skin (F). (G) Infected skin on the snout became similarly thickened and yellow-brown. (H and I) Immediately prior to shedding, fluid accumulated between the old and new layers of skin, causing distortion of the head (H) and vesicle formation at inoculation sites on the body (I). (J to L) The presentations observed in experimentally infected snakes were consistent with those observed in wild snakes diagnosed with SFD at the U.S. Geological Survey National Wildlife Health Center, which often included thickened, yellow-brown areas of skin on the head (J) and ventral scales (K) and edematous scales (arrow) and crusting (asterisk) of the skin (L).
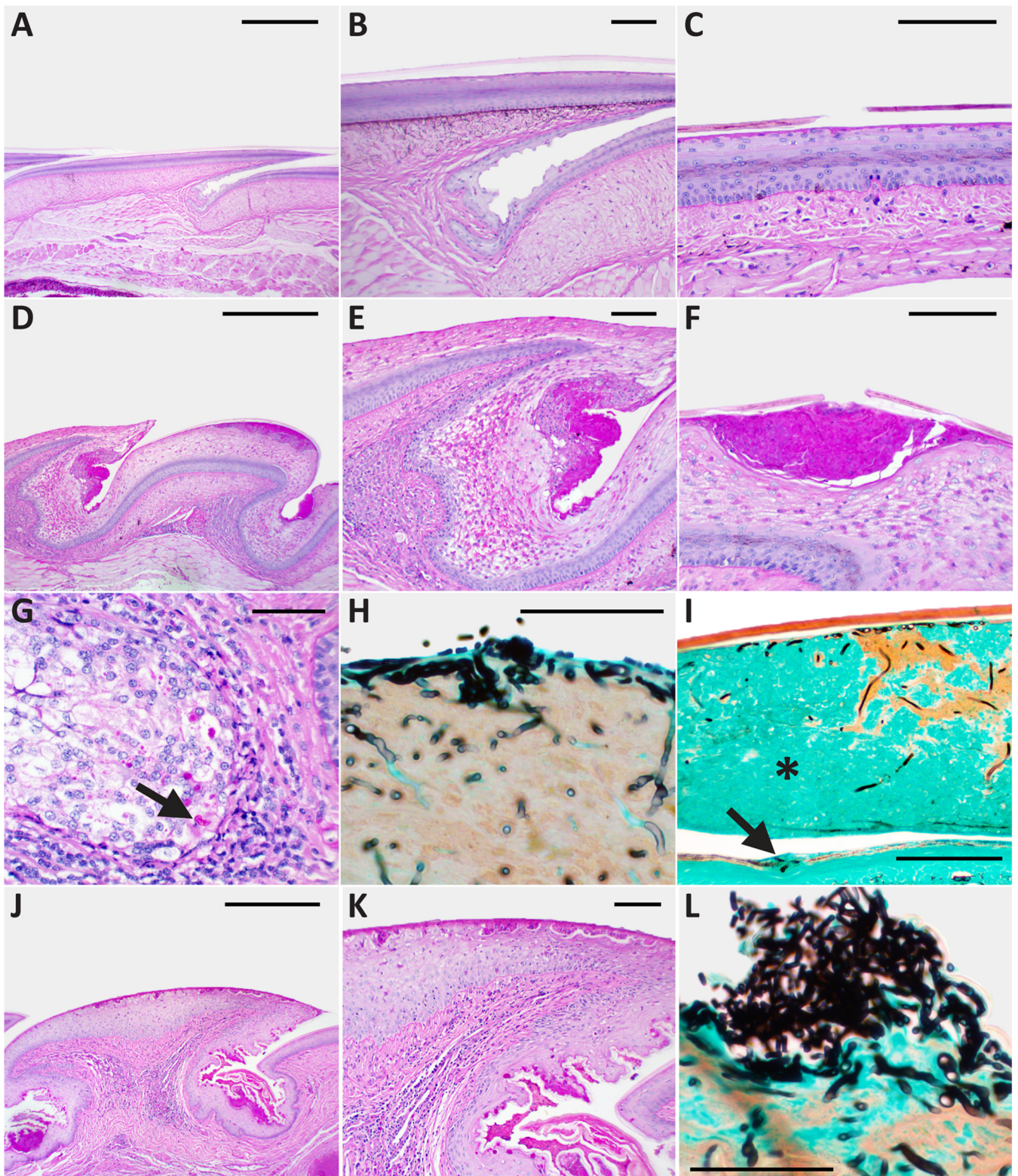
FIG 2 Microscopic lesions of SFD in snakes experimentally challenged with *Ophidiomyces ophiodiicola*. (A and B) Skin samples from sham-inoculated snakes were within normal limits (PAS stain). Bar, 500 μm (A) or 100 μm (B). (C) Skin samples from sham-inoculated snakes exhibited focal breaks in the stratum corneum attributed to mechanical damage from abrasion; the underlying epidermis was generally within normal limits (PAS stain). Bar, 100 μm. (D and E) Skin samples from snakes exposed to *O. ophiodiicola* developed multifocal superficial epidermal necrosis with extensive epidermal edema (D) and heterophil infiltration and mononuclear to granulocytic dermal inflammation (E) (PAS stain). Bar, 500 μm (D) or 100 μm (E). (F) Breaks in the stratum corneum in infected snakes were most common over areas of epidermal necrosis and granulocytic inflammation, suggesting that infection may be facilitated by preexisting damage to the skin surface (PAS stain). Bar, 100 μm. (G) Some infected snakes developed granulomas consisting of fungal hyphae (arrow) and epithelioid

(Continued)

mon over infected areas of skin (Fig. 2F), while breaks in control snakes were equally common over normal and minimally necrotic skin and were attributed to the abrasion process (i.e., not associated with an etiological agent) (Fig. 2C). All control snakes that were undergoing molt at the time of necropsy (*n* = 5) also had low numbers of granulocytes in the upper layers of the epidermis in the interscalar space. This finding was considered normal, as heterophils migrate through the epidermis during the molting process (22). Microscopic lesions of internal organs included mild chronic lymphoplasmacytic to lymphohistiocytic inflammation in the liver, lungs, heart, stomach, and colon. Three snakes had granulomas within the coelomic mesentery or spleen; bacteria were noted within the mesenteric granulomas. Two snakes had mild focal to multifocal epithelial necrosis in the esophagus and colon. These lesions were present in both control and infected animals. Six of the eight infected and three of the seven control snakes were PCR positive for one or more of the snake adenoviruses at the end of the trial, and positive detections generally corresponded to mild lesions in internal organs. There was no evidence of disseminated fungal infection, even in snakes that developed SFD.

Ophiodiomyces ophiodiicola was not detected by real-time PCR on the skin of snakes prior to inoculation. However, *O. ophiodiicola* was isolated from skin collected at necropsy for all snakes in the infected group. All recovered isolates of *O. ophiodiicola* for which portions of the intergenic spacer (IGS) region of the rRNA gene complex were sequenced were 100% identical to the isolate used for the initial inoculation. *Ophiodiomyces ophiodiicola* was the only fungus cultured from snakes in the infected group. The ability to culture *O. ophiodiicola* from snakes in the infected and control groups was significantly different (*P* < 0.001), with no fungi being recovered from snakes in the control group.

Infection by *O. ophiodiicola* causes host physiological and behavioral changes. Snakes exposed to *O. ophiodiicola* molted much more frequently than animals in the control group (*t* = 5.627, *P* < 0.001). Specifically, mean shed intervals (time between skin molts) prior to experimental treatments were 39.0 (±5.3 [standard deviation]) days for the control group and 42.9 (±11.9) days for the infected group. Post-sham inoculation, the mean shed interval for the control group was 28.8 (±5.0) days. After exposure to *O. ophiodiicola*, the mean shed interval for the infected group was 15.6 (±3.9) days. The two snakes in the infected group that exhibited intermittent periods of anorexia demonstrated 2.0- and 2.7-fold reductions in relative growth rate after 5 weeks of exposure to *O. ophiodiicola*. However, there was no statistical difference in the relative growth rate for snakes in the infected group for the 5 weeks before and the 5 weeks after exposure to the fungus (*t* = 0.340, *P* = 0.746). There was also no significant difference detected in relative growth rates between the control and infected groups over the 5-week period that snakes were exposed to the

fungus or vehicle solution (*t* = 1.120, *P* = 0.285). Snakes in the infected group were significantly more likely to be encountered in conspicuous areas of their enclosures during daily checks (*t* = 3.295, *P* = 0.006). Specifically, infected snakes were noted in exposed areas of their cages on 42.9% (±10.0%) of checks, while snakes in the control group were found outside hiding areas on only 20.6% (±14.8%) of checks.

DISCUSSION

Ophiodiomyces ophiodiicola is frequently associated with emerging cases of SFD, but its exact role in these skin infections had not been previously established. Our work demonstrates a direct casual link between the fungus and SFD. Specifically, all snakes experimentally challenged with pure cultures of *O. ophiodiicola* developed clinical signs and histopathologic lesions characteristic of SFD, while those in the control group did not develop the disease. Furthermore, *O. ophiodiicola* was isolated from the lesions of all snakes in the infected group (but not from snakes in the control group), fulfilling Koch's postulates to identify the causative agent of a disease (19). While other fungi can likely cause skin infections in snakes, the strong associations reported between *O. ophiodiicola* and SFD in the literature (9, 11, 12, 18) indicate that this pathogen is the most consistent cause of such infections. Due to its importance in both veterinary medicine and in snake conservation, we propose that the term SFD refer specifically to infections caused by *O. ophiodiicola*.

A variety of clinical signs have been described in association with SFD (9, 11, 20, 21, 23). However, most published accounts of this disease represent diagnoses made at a single time point with no explanation for why clinical signs are so variable. In this experiment, we documented the progression (and regression) of lesions over time and found that variations of clinical signs were explained by the stage of infection (Fig. 1). Within days of exposure to *O. ophiodiicola*, snakes develop swelling in the general area of infection. The response quickly becomes more localized, with individually infected scales becoming edematous. Based on histopathologic examination, this discoloration of scales appears to be correlated with granulocytic infiltration to the site of infection. Early in the infection process, *O. ophiodiicola* breaches the stratum corneum and colonizes the immediately subjacent epidermis. The fungus is seemingly partial to the margins and tips of scales, as indicated both by the gross discoloration pattern and the necrosis pattern seen in histopathology. As the infection progresses, hyphae penetrate deeper into the epidermis, causing necrosis. The fungus proliferates in the necrotic tissue and expands outward and downward into adjacent tissue, individual lesions coalesce, and the epidermis becomes markedly thickened by a combination of edema, inflammation, and necrosis. Grossly, these changes result in lesions turning yellow to brown and the eventual development of crusts. Immediately prior to molting, excessive accumulation of

Figure Legend Continued

macrophages surrounded by lymphocytes and plasma cells (PAS stain; bar, 50 μm). (H) Areas of epidermal necrosis in snakes exposed to *O. ophiodiicola* often contained 2- to 5-μm-diameter, parallel-walled, septate, branching, fungal hyphae and ~2- by 5-μm superficial rectangular arthroconidia (GMS stain). Bar, 50 μm. (I) In a snake preparing to undergo ecdysis, the new stratum corneum can be seen beneath the necrotic epidermis (asterisk) of a lesion. Most fungal hyphae (stained black) are within the older epidermis that will be shed; however, hyphae that have invaded the new epidermis (arrow) may persist after the molt (GMS stain.) Bar, 100 μm. (J to L) Skin samples from free-ranging wild snakes diagnosed with SFD at the U.S. Geological Survey National Wildlife Health Center exhibited lesions similar to those that developed in experimentally infected snakes, including multifocal epidermal necrosis, granulocytic inflammation, and edema (PAS; bar, 500 μm) (J), mixed dermal inflammation (PAS; bar, 100 μm) (K), and fungal hyphae and arthroconidia morphologically consistent with *O. ophiodiicola* (GMS; bar, 50 μm) (L).

presumptive lymphatic fluid between the new and old layers of skin at the sites of infection results in the formation of "blisters." Because fungi are often confined to the superficial layers of the epidermis when the molting cycle is initiated, lesions largely resolve following a molt, as the affected epidermis is shed. However, a few fungal hyphae may remain in the underlying epidermis (Fig. 2I), allowing for eventual reinfection. Previously infected scales may be misshapen and have the appearance of scarring. Granulomas form when *O. ophiodiicola* penetrates the epidermal basement membrane and enters the dermis, and these granulomas are the likely cause of the nodules associated with SFD (9, 11, 20, 23). A combination of regional swelling, epidermal thickening, and granuloma formation on the head may result in the "facial disfiguration" that has occasionally been described in the most extreme cases of SFD (9).

Infection by *O. ophiodiicola* appears to be facilitated by scarification of the stratum corneum. Paré et al. (24) reported a similar observation with a related fungal pathogen of lizards. In our study, breaks in the stratum corneum caused by the abrasion process were apparent in histologic sections of the skin, and fungus-induced lesions in infected snakes were frequently located directly below these breaks (Fig. 1F). When the surface of the skin was compromised, even low-inoculum doses resulted in infection. In wild snakes, superficial damage to the outer layers of the epidermis (e.g., scrapes, scratches, and nicks) may serve as the primary route by which the fungus gains entry into the skin. In areas where SFD is considered a threat to snake populations, researchers should exercise caution when using techniques that compromise the surface of the skin (e.g., scale clipping and cauterization for marking individuals, tissue and blood collection, etc.). Although infections were more easily established when the skin was scarified, lesions also developed at inoculation sites that were not abraded. This was especially true when high concentrations of conidia were applied to the skin. Wild snakes residing in heavily infested environments may be susceptible to *O. ophiodiicola* infections even when the stratum corneum is intact. Using bandages to hold the inoculum in place did not appear to influence infection, except that removal of the adhesive perimeter frequently damaged the outer layer of the epidermis and may have provided an entry point for the pathogen.

Molting is considered an important immunological defense in snakes (22, 25). The shed interval decreased for snakes in the control group after sham inoculation, likely due to damage caused by the scarification process (histopathology supported the presence of breaks in the stratum corneum and minor epidermal necrosis on sections of skin that had been abraded with sandpaper). However, even with this increase in molting frequency, snakes in the infected group still shed nearly twice as often as animals in the control group. Molting appeared to be largely effective at reducing or eliminating clinical signs of disease. Microscopically, the majority of fungal hyphae were present in the outer layers of the epidermis that would be shed during the molting process (Fig. 2I). Immediately before shedding, there was marked infiltration of presumed lymphatic fluid between the old and new layers of skin at sites of infection. This may be a host response aimed at eliminating fungal hyphae and conidia residing on the surface of new epidermis and preventing reinfection.

Snakes in our trial did not develop gross lesions postmolt until they were reinfected with *O. ophiodiicola*. At the time of reinoculation, many snakes in the infected group were already showing signs of an impending second shed. Histopathology performed at the end of the experiment revealed occasional hyphae within the new epidermal layer of snakes that were about to shed; these hyphae would persist after ecdysis and could cause disease recurrence. Furthermore, some snakes in the infected group exhibited dysecdysis, in which portions of the old skin overlying the lesion adhered to the new epidermis, potentially exposing the new skin to a fungal inoculum. Molting events in rapid succession can likely remove residual fungi in the superficial epidermis (or trapped beneath retained skin) before they have the opportunity to penetrate into the deeper portions of the skin which cannot be cast off during ecdysis. This would explain why repeated inoculations were required to reestablish gross lesions in our study. The ideal husbandry conditions (e.g., constant warm temperatures and frequent feedings) and good body condition of snakes in this experiment likely facilitated the short shed intervals that were observed. In a natural setting, snakes can be expected to have longer shed intervals because they are subjected to other conditions that influence body condition and molt frequency (e.g., sporadic food availability, cooler temperatures, etc.). For this reason, wild snakes may, under some circumstances, be more prone to developing chronic and severe presentations of SFD.

In our experiment, two of the eight (25%) snakes in the infected group exhibited bouts of anorexia after developing skin lesions. A third snake was tentative about accepting food. In contrast, snakes in the control group never refused food and were always eager to eat. Abnormal feeding behaviors in some of the infected snakes appeared to be linked to facial lesions; normal feeding behaviors generally resumed after the lesions regressed (postmolt). The two anorexic animals had 2.0- and 2.7-fold decreases in relative growth rate over the course of the trial. However, both snakes were in good body condition when examined at necropsy, and there was no significant difference in relative growth rates in the infected group before and after exposure to *O. ophiodiicola* or between the control and infected groups postinoculation. This may have been an artifact of the relatively short duration of the infection trial and captive conditions that allowed the snakes regular access to food. For wild snakes, decreased appetite and apprehension to attack prey may have greater impacts on overall health, since prey items are less frequently encountered and more difficult to capture. Furthermore, infections caused by *O. ophiodiicola* are energetically costly, requiring a continual response by the immune system, healing of damaged skin, and frequent molting. Thus, chronic *O. ophiodiicola* infections could have significant impacts on host energy balance and body condition. Failure of infected wild snakes to procure sufficient food could result in a feedback loop that reduces host defenses, facilitating more severe infections that further compromise a snake's ability to obtain prey.

In addition to physiological responses, infected snakes also exhibited behavioral changes. Specifically, infected snakes would frequently lie exposed in their cages instead of remaining concealed inside or under the provided shelters. Similar responses have been reported in wild snakes with SFD (11). The reason for these behaviors is unclear but may be related to fighting infection. Although snakes were housed under stable environmental conditions, spending more time in conspicuous areas of the cage may represent attempts to seek out conditions less conducive to development of fungal infections. Specifically, some snakes are known to induce fever by basking (26). Alternatively, snakes may have

been avoiding microhabitats where environmental fungal loads are typically highest (e.g., in the moister and cooler areas under cover objects where they would spend most of their time in the wild) or devoting more time to hunting to compensate for the energy expended as a consequence of infection. Regardless of the mechanism, wild snakes that spend more time out in the open are at a greatly increased risk of predation and, at certain times of year, may be exposed to potentially lethal temperatures. Thus, SFD may also be an important contributor to indirect forms of mortality in wild infected snakes.

Disseminated infections caused by *O. ophiodiicola* (23, 27) are rare, and we saw no evidence that the fungus invaded deep tissues. Mild inflammatory lesions observed in internal organs were present in both infected and control snakes and were likely unrelated to SFD. Instead, these findings were thought to be the result of prior or mildly active snake adenovirus infections. There was no indication that these viral infections influenced the skin infections caused by *O. ophiodiicola*. Specifically, snakes in the infected group without adenovirus developed SFD lesions that were identical to those observed in snakes that were PCR positive for the virus. Furthermore, recent work indicated that exposure to and infection by adenoviruses in clinically healthy snakes are quite common (28). Instead, snake adenoviruses may cause significant disease only in certain circumstances. All snakes were clinically healthy for at least 6 weeks prior to initiating the infection trial and did not demonstrate "classic" clinical signs of adenovirus infection (29) during the experiment. While adenovirus did not appear to influence the ability of *O. ophiodiicola* to cause disease in this study, the role that coinfections might play in contributing to host response, disease progression, and mortality warrants further investigation, especially since wild snakes likely harbor a variety of viruses and parasites.

The mechanisms by which SFD causes death in wild snakes are likely multifactorial. Most accounts of the disease do not provide detailed pathological findings other than those directly related to skin lesions (9, 11), making it difficult to ascertain proximate causes of death. Although no snakes died in our trial, we did not let the disease progress to a stage that was life-threatening. Regardless, we observed changes that may contribute to mortality in wild snakes. The full ecological complexities of SFD are not replicated by the standard methods we used to house snakes. For example, regular access to food, water, and stable environmental conditions represents a situation that may be more conducive to snakes surviving infections caused by *O. ophiodiicola*. Nonetheless, a laboratory model for studying SFD is essential in investigating host responses, pathogenicity of *O. ophiodiicola*, and the role of the environment in disease outcome. Corn snakes make for an ideal model host organism because captive-bred animals are widely available, easy to maintain, and develop infections that are identical to those observed in wild snakes with SFD. Corn snakes can also tolerate broad ranges in environmental conditions (e.g., temperature and humidity) and physiological processes (e.g., hibernation) that could be altered in the laboratory to study how these factors influence pathogenesis.

Snake fungal disease has highlighted how little we know about reptiles and their associated pathogens. This study fills some of the fundamental knowledge gaps surrounding SFD by demonstrating that *O. ophiodiicola* is a primary pathogen, documenting the progression of the disease, and revealing host responses to the infection. Furthermore, establishment of *O. ophiodiicola* as the caus-

ative agent of SFD paves the way for future research aimed at understanding the epidemiology of this disease, elucidating reasons for SFD emergence, informing management strategies to mitigate its impacts on imperiled snake populations, and perhaps providing a broader understanding of the evolution of fungal virulence. *Ophidiomyces ophiodiicola* is a member of a group of fungi that are frequently associated with disease in reptiles (14, 15). Many of these fungi are specialized to particular reptile taxa, and pathogenic and nonpathogenic fungal species are intermixed on the phylogenetic tree (14), a situation that parallels that of the medically important dermatophytes. Hence, *O. ophiodiicola* and its relatives could serve as important organisms for studying the genetic underpinnings that facilitate the evolution of fungal pathogens and their adaptations to certain host species.

MATERIALS AND METHODS

Animal husbandry. This study was approved by the U.S. Geological Survey National Wildlife Health Center (NWHC) Institutional Animal Care and Use Committee (protocol ep131223). Eight- to 10-month-old (8- to 26-g) captive-bred corn snakes that originated from a captive colony with no history of grossly visible skin infections were housed individually in clear plastic totes with buckled locking lids (product number sk-130E; IRIS USA, Inc., Pleasant Prairie, WI). Five-millimeter-diameter ventilation holes were created throughout the sides and lid, and silicone caulk was used to plug gaps between the lid and container. Each enclosure was lined with a paper cage liner (Diamond Tek, Harlan, Indianapolis, IN) and contained hiding areas in the form of an inverted plant saucer (product number 410-7012; KECO, Oklahoma City, OK) and a Ziploc container (15.5-cm length by 8-cm width by 5-cm height; S.C. Johnson and Son, Inc., Racine, WI) in which holes were created to allow the animals access. Water was available *ad libitum*, and snakes were offered ~2.7 g of thawed (previously frozen) pinky mice (Big Cheese Rodent Factory, Azel, TX) once per week. The enclosures were maintained within environmental chambers (Percival Scientific, Perry, IA) at 24°C ($\pm$0.15°C; 24°C is near the growth optimum for *O. ophiodiicola* [12]) and 66.5% ($\pm$8.9%) relative humidity, on a 12-h light cycle. Snakes were randomly assigned to one of two treatment groups that were balanced by individual body mass, and they were acclimated to the conditions for a period of at least 10 days. Body mass was measured once per week, and dates of ecdysis (i.e., shedding or molting of the skin) were recorded.

Experimental infection. A pure culture of *Ophidiomyces ophiodiicola* originally isolated from a wild water snake (*Nerodia sipedon*) that died of SFD was used for the infection trial. Conidia were harvested from cultures grown at 24°C for 15 days on Sabouraud's dextrose agar containing chloramphenicol and gentamicin by flooding the plates with phosphate-buffered saline containing 0.5% Tween 20 (PBST). The liquid was collected and spun at 3,500 $\times$ *g* for 10 min to pellet the cells. Conidia were washed once with PBST, enumerated using a hemocytometer, and resuspended at the concentrations described below.

To confirm that each snake to be used in the experiment was free of *O. ophiodiicola*, shed skin from the molt immediately preceding the inoculation was screened for the presence of *O. ophiodiicola* by using real-time PCR (16). Snakes were exposed to either *O. ophiodiicola* (infected group; *n* = 8) or vehicle solution (sterile PBST without fungal conidia; control group; *n* = 7) at five locations: snout, chin, ventral neck skin, ventral body skin, and dorsal body skin. Each application site was approximately 1 cm by 1 cm in size. In a previous study, abrasion of the skin was demonstrated to be an important factor in the development of fungal skin infections in lizards (24). To determine if this was also true of *O. ophiodiicola* infection, the skin on the snout, ventral neck, and dorsal body sites was abraded by gently stroking a piece of sterile fine grain (P150) sandpaper five times with and five times across the grain of the scales. Chin and ventral body skin locations were not abraded. Fifty microliters of conidial suspension was applied to the absorbent pad of a 22-mm by 26-mm sterile Nexcare

waterproof bandage (3M Consumer Health Care, St. Paul, MN) and affixed to the inoculation site. Bandages placed on the ventral neck skin and ventral body skin were inoculated with 10^5 conidia; the bandage placed on the dorsal body skin was inoculated with 10^4 conidia. The snout and chin were inoculated with 2-μl aliquots of suspension containing 10^5 and 10^4 conidia, respectively. Since placing bandages on the snout and chin was not feasible, the suspension was spread evenly across the surface of the skin at these sites. Animals in the control group were treated identically except that PBST (without fungal inoculum) was used. Bandages remaining in place after 3 days were removed. Animals were checked daily for development of clinical signs consistent with SFD (9, 11, 20, 21, 23).

Reinfection and euthanasia. Molting complicated the disease process by making clinical signs of SFD unapparent. Specifically, snakes initiated a second molt without recurrence of clinical signs. Therefore, snakes were reinfected with *O. ophiodiicola* after each molt. One animal developed lesions that met euthanasia criteria (see below) after the second inoculation, but the remaining seven snakes underwent additional molt cycles and received a third (*n* = 6) or fourth (*n* = 1) exposure to *O. ophiodiicola*. To determine whether a higher dose of conidia would increase the severity of the lesions, five snakes were exposed to higher doses during the third challenge: three snakes were inoculated with 10^5 conidia at all inoculation sites, and two snakes were inoculated with 10^6 conidia at all inoculation sites. The snake that received four inoculations was exposed to 10^6 conidia at each inoculation site (it had previously been inoculated three times with the initial dosage). Prior to each reinoculation, skin was freshly abraded at sites that had been abraded initially. Snakes in the control group were similarly treated (with vehicle solution) each time they molted.

Snakes were euthanized if brown, crusty skin lesions exceeding 1 cm in diameter were present for 5 days without resolution or at the end of the study (47 to 58 days postinoculation). One animal developed lesions that met euthanasia criteria, and this snake was euthanized 14 days after the second exposure. The remaining infected snakes were euthanized 47 to 57 days after initial infection. One control snake was euthanized on day 54 because it was at the same point in the molt cycle as some of the infected snakes and thus provided an opportunity to better compare histopathology between control and infected groups. The remaining snakes in the control group were euthanized 57 to 58 days after the initial sham inoculation. Euthanasia was performed by first anesthetizing the snakes with isoflurane gas and then injecting 50% (vol/vol) tricaine methanesulfonate (MS-222) into the coelomic cavity, as described by Conroy et al. (30). Death was confirmed by decapitation.

Histopathology and culture analyses. Skin from all five inoculated/sham-inoculated sites, select internal organs (heart, lung, liver, kidney, spleen, pancreas, esophagus, stomach, and small and large intestine), and the entire head were fixed in 10% neutral buffered formalin, decalcified in a saturated ethylenediaminetetraacetic acid (EDTA) solution (skin and head samples only), trimmed, embedded in paraffin, and sectioned at 5-μm thickness. For culture analysis, skin samples from each inoculated or sham-inoculated site were placed onto dermatophyte test medium (31) and incubated at 30°C for 20 days. Fungi were isolated and identified by sequencing the internal transcribed spacer region (ITS) of the rRNA gene, using the methods of Lorch et al. (32). To ensure that isolates of *O. ophiodiicola* recovered from snakes at the end of this study were identical to the strain used for inoculation, both the 3′ and 5′ ends of the IGS were sequenced for one isolate per snake, as described by Bohuski et al. (16). To determine if adenovirus infection might be responsible for some of the minor pathology observed in internal organs of some of the snakes, portions of the esophagus, stomach, intestine, and liver were pooled and tested for adenovirus by PCR (33) at the University of Florida College of Veterinary Medicine Diagnostic Laboratories.

Statistical analyses. Statistical analyses were performed using Sigma-Plot 11.0 (Systat Software, Inc., San Jose, CA). Differences in the recovery of *O. ophiodiicola* from sampled skin and the presence of gross and histopathologic lesions consistent with SFD were compared between the control and infected groups using Fisher's exact test. A *t* test was used to compare shed intervals (i.e., time between the first and second skin molting events posttreatment), the proportion of encounters during which snakes were exposed in their cages (days with missing data were excluded, as was the snake from the infected group that was euthanized on day 38), and relative growth rates (i.e., change in body mass per day relative to starting body mass) between the infected and control groups over the 5-week trial. Relative growth rates for snakes in the infected group were also compared between the 5-week period leading up to inoculation and the 5 weeks following experimental infection by using a paired *t* test to account for individual variation.

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