The natural history, ecology, and epidemiology of
Ophidiomyces ophiodiicola and its potential impact
on free-ranging snake populations

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A B S T R A C T

Ophidiomyces ophiodiicola, the causative agent of snake fungal disease, is a serious emerging fungal pathogen of North American-endemic and captive snakes. We provide a detailed literature review, introduce new ecological and biological information and consider aspects of O. ophiodiicola that need further investigation. The current biological evidence suggests that this fungus can persist as an environmental saprobe in soil, as well as colonizing living hosts. Not unlike other emerging fungal pathogens, many fundamental questions such as the origin of O. ophiodiicola, mode of transmission, environmental influences, and effective treatment options still need to be investigated.

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Introduction

True fungal pathogens have been increasingly associated with free-ranging wildlife epidemics. Two recently studied examples include the effects of chytridiomycosis, caused by Batrachochytrium dendrobatidis, on global frog populations (Skerratt et al., 2007) and the asexual ascomycete, Pseudogymnoascus destructans, causing white-nose syndrome in North American bat populations (Blehert et al., 2009). One of the latest emerging fungal pathogens of vertebrate animal populations, Ophidiomyces ophiodiicola, was first observed in 2006 affecting populations of pitvipers in the eastern United States (Clark et al., 2011), and later confirmed in Eastern Massasaugas (Sistrurus catenatus) from Illinois in 2008.
With the current rate of decline in global biodiversity, it is apparent that wildlife diseases similar to those described above are serving as threats to population declines potentially resulting in species extinctions.

Fisher et al. (2012) described a model or concept for analysis of emerging infectious fungal diseases in animal and plant populations and ecosystem health in general. This model is useful for the study of newly emerging highly virulent agents of disease such as the snake fungal pathogen, *O. ophiodiicola* (see Table 1).

*Ophidiomyces ophiodiicola* (formerly *Chrysosporium ophidiicola*) is currently placed in the order *Onygenales* (Ascomycota) within the family *Onygenaceae* (Sigler et al., 2013). This fungus is closely related to other species of *Onygenales* within the *Chrysosporium* anamorph *Nannizzioptis vriesii* (CANV) complex that causes dermal lesions in reptiles (Sigler et al., 2013). The genus *Ophidiomyces* currently contains only one species, *O. ophiodiicola*, and it is known to infect only snakes leading to the syndrome Snake Fungal Disease (SFD) which causes widespread morbidity and mortality across the eastern United States (Fig 1) (Allender et al., 2013; Sigler et al., 2013; Sleeman, 2013).

Historically, several case reports of skin disease in snakes were confirmed (Paré and Jacobson, 2007) or suspected to be CANV or CANV-like. However, since publication of the description of the genus *Ophidiomyces* in 2013 and with the use of advanced molecular techniques, it is now thought that many of these infections may have been caused by *O. ophidiicola*. An outbreak of fungal mycosis with clinical signs consistent with SFD was seen in pigmy rattlesnakes (*S. milarius*) in Florida in the early 2000’s, but neither *Ophidiomyces* nor CANV were identified in that case (Cheatwood et al., 2003). This outbreak included 42 pigmy rattlesnakes, one garter snake (*Thamnophis* sp.), and a ribbon snake (*Thamnophis sauritus*) within a 2 yr period, and retrospectively the authors identified an additional 59 pigmy rattlesnakes with signs consistent with mycotic disease (Cheatwood et al., 2003). While these were not confirmed to be SFD at that time, it highlights the need to utilize advanced molecular techniques for accurate identification of the etiological agent during mycotic outbreaks.

Since 2008, Eastern Massasaugas from the Carlyle Lake population in Illinois have been diagnosed with SFD (Fig 5A–D) (Allender et al., 2011, 2015). Free-range infected massasaugas display skin lesions, which vary from pustules or nodules to severe swelling of the skin (Allender et al., 2011). Infections in this species have been known to invade deep muscle tissue and infect muscle and bone (Allender et al., 2011). Prior to 2014, lesions were thought to only affect the head and neck of snakes, but lesions have now been observed in the skin of the entire body in massasaugas in Michigan (Tetzlaff et al., 2015). In 2006, a population of timber rattlesnakes (*Crotalus horridus*) in New Hampshire was observed with lesions on the head, neck, and body consistent with SFD (Clark et al., 2011). The authors hypothesized that environmental conditions (increased rainfall) were likely the explanation for the increased prevalence and/or severity as no other lesions were noted in their follow-up study in 2009 (Clark et al., 2011). To date, all reports related to pitvipers have been consistently associated with dermatitis.

While infections have been observed with great frequency in pitvipers (Cheatwood et al., 2003; Allender et al., 2011; Clark et al., 2011; Smith et al., 2013; Tetzlaff et al., 2015), there are numerous reports of CANV or SFD in nonvenomous colubrid snakes (Jacobson, 1980; Vissiennon et al., 1999; Nichols et al., 1999; Bertelsen et al., 2005; Rajeev et al., 2009; Dolinski et al., 2014). The manifestation of SFD in North American colubrid snakes is variable and has included pneumonia, ocular infections, and subcutaneous nodules (Rajeev et al., 2009; Sleeman, 2013; Dolinski et al., 2014). A case of *O. ophidiicola* was observed in a captive black rat snake (*Elaphe obsoleta obsoleta*) with a subcutaneous nodule (Rajeev et al., 2009). In addition, mycosis was observed in the skin as well as a more systemic invasion involving the lungs and eye (one case) and lungs and liver (one case) of garter snakes (Vissiennon et al., 1999; Dolinski et al., 2014).

While the presence of the fungus causing infections in individuals is concerning, the role that it might play in population declines is more alarming. A timber rattlesnake population in New Hampshire consisted of 40 individuals prior to 2014, lesions were thought to only affect the head and neck of snakes, but lesions have now been observed in the skin of the entire body in massasaugas in Michigan (Tetzlaff et al., 2015). In 2006, a population of timber rattlesnakes (*Crotalus horridus*) in New Hampshire was observed with lesions on the head, neck, and body consistent with SFD (Clark et al., 2011). The authors hypothesized that environmental conditions (increased rainfall) were likely the explanation for the increased prevalence and/or severity as no other lesions were noted in their follow-up study in 2009 (Clark et al., 2011). To date, all reports related to pitvipers have been consistently associated with dermatitis.

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![Fig 1 – Current known US distribution of *O. ophidiicola*. States where *O. ophidiicola* is known to occur are grey, based on previous reports (Allender et al., 2011; Sleeman et al., 2014) and unpublished data (MCA).](image-url)
While many factors are affecting the conservation of this population, the occurrence of this pathogen may serve as an additional threat to its conservation.

In this report we describe the ecology of O. ophiodiicola in the laboratory and consider aspects of this disease that require further investigation. While the true global distribution of this fungus is unknown, it is possible that snake fungal disease, if present only in North America, could be spread to other ecosystems around the world through the animal trade. A thorough understanding of this disease is of paramount importance in managing free ranging snake populations especially with rapid global climate change.

**Methods and materials**

Four Illinois O. ophiodiicola isolates (isolated in 2014) from various infection sites located on free range snakes were examined: three from infected massasaugas (12-34933: isolated from facial region, 13-42282: isolated from skin/bone, and 13-40265: isolated from caudal body) and one isolate (12-33400: isolated from lung tissue) from an infected plains garter snake (T. radix). Isolates were maintained on Difco Sabouraud's Dextrose agar (SDA) at room temperature. Unless noted, all media were sterilized at 121 °C for 15 min, and wrapped...
with Parafilm after inoculation. All assays were inoculated with actively growing mycelium, maintained in an upright position, and replicated twice at different times using two replicates each time unless otherwise noted. All carbon and nitrogen assays were incubated under 24 hr darkness at room temperature and all agar based assays contained approximately 20 ml of media. Unless noted, all chemicals utilized within this experiment were purchased through Sigma Aldrich or Fischer Scientific.

Analysis

Matric potential was adjusted with polyethylene glycol 8 000 [PEG] following the equation \( \Psi = 1.29(\text{PEG})^2 - 140(\text{PEG}) + 1400 \) where [PEG] = gram of PEG 8 000 per gram of water and \( T = \text{temperature (°C)} \) (Michel, 1983), spore counts were conducted with an improved Neubauer hemocytometer, pH measurements were conducted with a Milwaukee MW102 pH/temperature meter, and microscopic evaluations were

![Fig 3 – In vitro growth of Ophiomyces ophiodiicola.](image)

(A) O. ophiodiicola demonstrating gelatinase activity. (B) Carbon enzymatic assays: PDA (control, bottom), Mn-dependent peroxidase (negative, left), chitinase (negative, top) and β-glucosidase (positive, right). (C) Carbon enzymatic assays: PDA (control, bottom), lipase using olive oil (left, positive), lipase using lard (top, positive) and lipase/esterase using Tween 80 (right, positive). (D) Keratinase assay: control left (negative) and inoculated right (positive). (E) Sole nitrogen source assays: left to right, columns 1–2 = nitrate, columns 3–4 = nitrite, columns 5–6 = ammonium, columns 7–8 = L-asparagine, columns 9–10 = control with no nitrogen source, columns 11–12 = uric acid. Rows A-D (12-34933), Rows E-H (12-33400), rows A, E (pH 5), rows B, F (pH 6), rows C, G (pH 7), rows D, H (pH 8). (F) Colony diameter of O. ophiodiicola isolates over the pH range of 5–11. Isolate colony diameters vary but growth pattern was consistent. Solid line represents mean values; dotted lines represent standard error of the mean.
conducted using an Olympus SZX12 stereo microscope. Data driven charts were generated using GraphPad Prism version 5.03 for Windows, and the evaluation of colony growth at various temperatures was conducted in a Blue M dry type bacteriological incubator and a Lab-Line Ambi-Hi-Lo-chamber. Fungal tolerance to sulfur compounds and environmental pH was determined 25 d after inoculation by measuring the diameter of each colony twice at 90° angles and averaging the two values.

All isolate identities were verified using rDNA sequence comparison of the internal transcribed spacer (ITS) region.

Fig 4 – Growth response of Ophidiomyces ophiodiicola to sulfur compounds and temperature. (A) O. ophiodiicola growth response to elevated levels of different sulfur compounds. (B) Effects of temperature on the growth of O. ophiodiicola and (C) representative colony from each temperature in Fig 3B. Left to right: 7 °C, 14 °C, 20 °C, 25 °C, 35 °C. (A, B) Bars represent mean values; error bars indicate standard error of the mean.
DNA was extracted from frozen mycelium by adding 200 μl 0.5 M NaOH. The mycelium was ground, centrifuged (14 000 RPM for 2 min), and 5 μl of the resulting supernatant was added to 495 μl 100 mM Tris–HCl (pH 8.5–8.9) (Osmundson et al., 2013). PCR was completed on a Bio-Rad PTC 200 thermal cycler. The total PCR reaction volume was 25 μl (12.5 μl GoTaq® Green Master Mix, (with 1 μl of each 10 μM primer ITS4 and ITS1F, 3 μl of the Tris-HCl-DNA extraction solution and 7.5 μl DNA free water). The thermal cycle parameters were: (1) initial denaturation at 94 °C for 2 min, (2) 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min (30 cycles), and (3) a final extension step of 72 °C for 10 min. Gel electrophoresis (1 % TBE agarose gel stained with ethidium bromide) was used to verify the presence of a PCR product prior to PCR purification [Wizard® SV Gel and PCR Clean-Up System (Promega)]. A BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems Inc.) was used to sequence the ITS in one direction using the ITS5 primer on an Applied Biosystems 3730XL high-throughput capillary sequencer. Identity was confirmed through nBLAST analysis.

**Assays**

Whole carbon source assays were conducted on autoclaved insect (freeze-dried Locusta migratoria), fish (fresh Poecilia sp.) and mushroom (Lentinula edodes) biomass. A representative sample of each was placed into glass Petri plates and inoculated with an autoclaved cotton swab that was pre-moistened.
in a 1 % NaCl solution and rubbed over ca. 5 mm × 5 mm area of a sporulating culture. In addition, a portion of fresh exoskeleton of Pleoticus muelleri was subjected to a demineralization step and another portion was subjected to a denitrogenation and deproteination step (Redde et al., 2007). The demineralization step consisted of two, 300 ml ice cold 0.25 M HCl treatments (one for 5 min and the second for 35 min) followed by neutralization with distilled water and the deproteination step incorporated three separate 100 ml (1.0 M NaOH) treatments at 95 °C; the first and second treatments were 2 hr, and the third was for 1 hr followed by neutralization with distilled water. The demineralized and deproteinated exoskeletons were placed in glass Petri plates, autoclaved, and inoculated as above. All plates were visually monitored twice a week for the presence of mycelial growth and conidiation.

Proteinase activity was determined using gelatin as the sole carbon and nitrogen source. The medium consisted of distilled water containing 3 % (pH 6.0 ± 0.1) or 6 % (adjusted to pH 7.0 ± 0.1 with KOH) gelatin (Knox original unflavored) and 0.002 % phenol red as the pH indicator (Raudabaugh and Miller, 2013). Each sterilized gelatin medium (150 μl) was pipetted into a separate UV-sterilized 96-well standard microplate and each test well was inoculated with a BB size pellet of scraped mycelium. The 96-well plates were covered with microtiter plate sealing film, incubated, and monitored daily for gelatin degradation (liquefaction) and pH change (color change from yellow below pH 6.8 to red/pink above pH 8.2).

Keratinase activity was determined using the following medium: 0.1 % glucose, 0.2 % yeast extract, 0.1 % KH₂PO₄, 0.02 % MgSO₄·7H₂O, 1 % Na₂CO₃ (autoclaved separately and added to achieve pH 8) and 1.5 % agar 1⁻¹ distilled water (Ito et al., 2005). Keratin azure was cut into small ca. 3 mm length sections, autoclaved separately in a dry beaker and a portion of the cooled (55 °C) basal medium was added to the keratin azure to produce a 1 % keratin azure overlay medium. Using sterile techniques, 5 ml of basal medium was added to 10 ml autoclaved glass test tubes. After the basal medium solidified, 1 ml of the 1 % azure keratin overlay medium was pipetted into the glass test tubes. Each test tube was inoculated with a BB size pellet of scraped mycelium. Test tubes were sealed with Parafilm and visually monitored weekly for dye release.

Chitinase activity was determined using colloidal chitin obtained from crab shell (Murthy and Bleakley, 2012). Colloidal chitin was obtained by grinding 20 g of crab shells into a powder followed by acid digestion for 1 hr, at room temperature, in 150 μl of 12 M HCl under constant stirring. The chitin was precipitated in ice cold water followed by vacuum filtration (coffee filter: two layers). Neutralization was conducted by wrapping the precipitate in multiple layers of coffee filters and floating the wrapped precipitate in two changes of distilled water (500 ml) at room temperature for a total of 24 hr. The colloidal chitin was unwrapped, autoclaved, and stored in a sealed container at 10 °C until needed. Colloidal chitin basal medium consisted of 0.7 g K₂HPO₄, 0.3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 0.5 g FeSO₄, 0.2 mg ZnSO₄, 0.1 mg MnSO₄ and 2 % agar 1⁻¹ distilled water. Filter sterilized (0.20 μm) thiamine chloride and biotin solution was added (providing a final concentration of 5 μg and 100 μg, respectively) to liquid medium cooled to 55 °C and poured into Petri plates. Colloidal chitin was added to another portion of the colloidal chitin basal medium to produce a 2 % colloidal chitin overlay medium of which 2 ml was pipetted on top of the solidified basal medium. The overlay was inoculated with a 5 mm agar plug, and examined twice per week for the presence of a clearing zone around the colony.

Cellulase and manganese-dependent peroxidase activities were assayed with esculin plus iron agar and Difco Potato Dextrose Agar (PDA) supplemented with MnSO₄ 1⁻¹, respectively (Pointing, 1999; Overton et al., 2006). Esculin plus iron agar consisted of 5 g L-tartaric acid diammonium salt, 0.5 g MgSO₄·7H₂O, 1 g K₂HPO₄, 0.1 g yeast extract, 0.001 g CaCl₂, 2.5 g esculin sesquihydrate and 8 g agar 1⁻¹ distilled water. The medium was adjusted to pH 7.0 ± 0.1 with 3 % KOH prior to sterilization. Filter sterilized (0.20 μm) aqueous ferric sulphate (2 %) was added to 55 °C medium at a ratio of 1 ml per 100 ml of media. The medium was then poured into Petri plates, inoculated with a 5 mm diameter agar plug and evaluated twice per week for the appearance of a brownish black precipitate. Manganese-dependent peroxidase was assayed by supplementing 39 g PDA with 100 mg 1⁻¹ and 200 mg 1⁻¹ MnSO₄. The medium was sterilized, inoculated as above, and examined twice per week for a black precipitate.

Lipase and esterase activity were assayed using Rhodamine B agar (8 g tryptone, 4 g NaCl, 0.001 % wt./vol. rhodamine B, 31.25 ml of sterile lipid (lard or olive oil) and 10 g agar 1⁻¹ distilled water) and Victoria blue B agar (10 g tryptone, 5 g NaCl, 0.1 g CaCl₂, 0.01 % wt./vol. Victoria blue B, 10 g sterile Tween 80 and 15 g agar 1⁻¹ distilled water) (Carissimi et al., 2007; Rajan et al., 2011). Both media were adjusted to pH 7.0 ± 0.1 with 3 % KOH prior to sterilization. The dye rhodamine B was filter sterilized (0.20 μm) separately, while all lipid sources (lard, olive oil and Tween 80) were autoclaved separately, and both dye and lipid source were added to the appropriate basal medium cooled to 55 °C. Each medium was shaken vigorously for 1 min to emulsify the lipid, poured into Petri plates, inoculated with a 5 mm diameter agar plug, and monitored twice a week for a positive reaction. A positive reaction for Rhodamine B agar was the appearance of orange fluorescence when exposed to UV light at 365 nm, while a positive reaction for Victoria blue B agar was the presence of insoluble long chain fatty acid calcium soap crystals.

Assays for sole nitrogen source were conducted for ammonium, l-asparagine (amino acid), nitrate, nitrite, urea and uric acid. Stuart’s urea broth base (0.1 g yeast extract, 9.1 g KH₂PO₄, 9.5 g Na₂HPO₄, 0.012 g phenol red 1⁻¹ distilled water (pH 6.8 ± 0.2)) and modified Christensen’s urea broth base (1.0 Tryptone, 2 g KH₂PO₄, 1 g dextrose, 5 g NaCl, 0.012 g phenol red 1⁻¹ distilled water (pH 6.8 ± 0.2)) without urea were used as control media. Filter sterilized (0.22 μm) urea (20 g 1⁻¹) was added to a portion of each cooled medium above to test for urease activity. An aliquot of each medium (150 μl) with and without urea was pipetted into a separate UV-sterilized well in a 96-well standard microplate. For control purposes, only half of each of the medium types were inoculated with a BB size pellet of scraped mycelium. The 96-well plate was covered with microtiter plate sealing film and was monitored daily (visually) for a change in color (yellow to pink) in both the
inoculated and control wells. Ammonium, l-asparagine, nitrate, nitrite, and uric acid assays were conducted using the following basal media (Hacksaylo et al., 1954): 20 g n-glucose, 2 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g FeSO₄, 0.2 mg ZnSO₄, 0.1 mg MnSO₄, 5 µg thiamine chloride, and 100 µg biotin (0.2 µm filter sterilized and added to 55 °C media) l⁻¹ distilled water and one of the following five nitrogen sources: (1) 3.07 g KNO₃, (2) 2.0 g (NH₄)₂SO₄, (3) 2.58 g KNO₂, (4) 2.28 g asparagine monohydrate, or (5) 1.275 g C₅H₄N₄O₃ l⁻¹. After sterilization and addition of the micronutrient solution, each medium pH was adjusted using a saturated solution of NaOH and 12 M HCl. Assays were conducted at pH 5, 6, 7, and 8 for basal media containing nitrate, nitrite, l-asparagine, ammonium, or no added nitrogen source (control) and at pH 8 for uric acid. An aliquot of each medium was pipetted into a separate UV-sterilized 96-well standard microplate well and inoculated as stated above. The 96-well plate was covered with microtiter plate sealing film and monitored daily for mycelial growth.

Three tolerance assays were utilized to determine the tolerance of O. ophiodiicola to pH, water availability (matric potential) and environmental sulfur compounds. The pH tolerance assay consisted of 9 g Malt broth, 2.25 g tryptone, and 18 g agar per 900 ml of distilled water (Nagai et al., 1998). One of the following buffer solutions (100 ml) was added after sterilization to achieve the following pH levels: pH 5 (6.9 g NaH₂PO₄·H₂O), pH 7 (3.904 g NaH₂PO₄·H₂O and 3.105 g Na₂HPO₄), pH 9 (0.318 g Na₂CO₃ and 3.948 g NaHCO₃), and pH 11 (5.3 g Na₂CO₃). After sterilization and addition of buffer, the Petri plates were inoculated with one 5 mm diameter agar plug and assayed for tolerance as described above. The matric potential assay medium consisted of 0.2 g sucrose, 0.2 g d-glucose, 1 l-asparagine, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl l⁻¹ distilled water and adjusted to pH 7 with NaOH (Raudabaugh and Miller, 2013). The medium was amended with PEG 8 000 as previously stated to generate the following matric potentials at 20 °C: −0.07 MPa, −1 MPa, −2.5 MPa, and −5 MPa. After sterilization, 40 ml of each medium was inoculated with a 500 µl spore suspension in water (average spore count = 4.0 × 10⁶ per 500 µl), sealed with parafilm, and placed on shake culture at 100 rpm for 25 d at 20 °C. Tolerance was qualitatively assayed as either visible growth or no visible growth after 25 d. The nitrogen source (KNO₃) from Raudabaugh and Miller (2013) was replaced with l-asparagine because O. ophiodiicola lacked robust growth when nitrate was the sole nitrogen source (see Results). The environmental sulfur compound tolerance assays consisted of 39 g PDA medium supplemented with 0.2 g FeSO₄ and one of the following: Na₂S₂O₃·5H₂O, Na₂SO₃, or 97% l-cysteine at 100 mg, 300 mg, 500 mg, and 700 mg l⁻¹ distilled water. After sterilization, Petri plates were inoculated with one 5 mm diameter agar plug and assayed for tolerance as described above. To determine the temperature range in which O. ophiodiicola could grow, each isolate was grown on SDA at 7 °C, 14 °C, 20 °C, 25 °C, 30 °C and 35 °C. Each isolate was assayed in sets of four replicates, with three temporal replications. The diameter of each colony was measured 3 d post inoculation (spore inoculation) and the linear extension of each colony was measured every 2–3 d until 15 d post inoculation. The linear growth for each set of replicates was averaged to determine the average growth of each isolate, and the average accumulative linear growth of all the replicates at 15 d post inoculation was reported.

Results

In vitro, O. ophiodiicola isolates produced powder to zonate yellowish-white colonies, with a reverse side that appeared white to pale yellow (Fig 2A). All isolates of O. ophiodiicola reproduced asexually by arthroconidia (schizolytic dehiscence) via sessile to stalked, hyaline to pale yellow aereioconidia with rhexolytic dehiscence (Fig 2B–C respectively). In vitro, O. ophiodiicola demonstrated robust growth on dead fish, dead insect, dead mushroom tissue (Fig 2D–F) and demineralized shrimp exoskeletons (Fig 2G), while sparse growth occurred on demineralized-deproteinated exoskeletons (Fig 2H). Ophidiomyces ophiodiicola isolates were positive for gelatinase activity (with subsequent medium alkalinization), β-glucosidase, lipase, lipase/esterase, and keratinase activity (Fig 3A–D respectively), while no positive results were obtained for Mn-dependent peroxidase and chitinase activity (Fig 3B). Assays for sole nitrogen source demonstrated that O. ophiodiicola produced robust growth on ammonium sulfate and l-asparagine monohydrate under neutral to alkaline conditions (pH7 and pH8) compared to growth under the same conditions on nitrate or nitrite (Fig 3E). The urease assay was positive for all isolates, while mycelial growth was sparse on uric acid (Fig 3E). Ophidiomyces ophiodiicola isolates grew over the entire pH range 5–11, with the largest colony diameter at pH 9 (Fig 3F), was tolerant of matric induced water stress (−5 MPa), and all isolates grew at elevated levels of l-cysteine, sulfate, sulfite and thiosulfate (Fig 4A). For all isolates, growth inhibition occurred at 7 °C, significant growth reduction occurred at 14 °C, greatest growth occurred at 25 °C, followed by a significant reduction of growth at 35 °C (Fig 4B and C).

Discussion

The effects of infectious diseases on wildlife populations are of increasing concern, especially for endangered species already persisting at small population sizes (Daszak et al., 2000). Furthermore, fungal pathogens of at least one species, the sharp-snouted day frog (Taudactylus acutirostris), have led to an extinction event due to chytridiomycosis (Schloegel et al., 2006). The impact Ophidiomyces has on snake populations is unknown, but understanding the ecology is the first step to determining the approach to management and characterizing the epidemiology.

While the sexual stage of the life cycle has not yet been encountered (Rajeev et al., 2009), O. ophiodiicola has been shown to reproduce asexually by arthroconidia with schizolytic dehiscence (Sigler et al., 2013) and asexually via sessile to stalked, hyaline to pale yellow aereioconidia with rhexolytic dehiscence (Rajeev et al., 2009) (Fig 2B–C respectively). Several factors support O. ophiodiicola occurring as an environmental saprobe: (1) its ability to utilize multiple complex carbon and nitrogen sources, (2) its ability to tolerate pH and most naturally occurring sulfur compounds, and (3) its ability to tolerate low matric potentials occurring in soil. In vitro, O. ophiodiicola
grew on a variety of dead substrata and had a broad complement of enzymes for saprotrophically utilizing many environmental carbon and nitrogen sources. Also, it tested positive for urease activity, which has been proposed as a dual use virulence factor in other fungal pathogens (Casadevall et al., 2003). Taken together, the data suggest that *O. ophiodiicola* most likely infects snakes in an opportunistic manner. The data also suggest that the environmental presence of ammonium would be more beneficial for growth of *O. ophiodiicola* than the presence of nitrate or nitrite.

All isolates grew over a wide pH range (pH 5–11), and elevated levels of sulfur compounds found in soils and reptile skin did not inhibit the growth of *O. ophiodiicola*. Substratum pH and the presence of elevated sulfur compounds are unlikely to inhibit its persistence in the environment. Surprisingly, *O. ophiodiicola* demonstrated tolerance to matric induced water stress, which is one of the most limiting factors for soil fungi (Marin et al., 1995). In general, soil fungi are typically intolerant of matric induced water stress below ~3 MPa (Magan and Lynch, 1986; Deacon, 2006); however *O. ophiodiicola* demonstrated growth down to ~5 MPa indicating that soil matric potential will also be unlikely to inhibit its persistence in most soils.

Several studies have indicated that temperature is a significant factor affecting the growth of *O. ophiodiicola* in natural ecosystems, with inhibition occurring at 37 °C (Rajeev et al., 2009; Sigler et al., 2013). Our data suggest that snake populations that hibernate in the lower part of the thermal range of 0 °C–10 °C (Macartney et al., 1989) should have less infection during the spring than snake populations that hibernate in the upper part of the thermal range of 0 °C–10 °C. In addition, our data also suggest that with increasing global temperatures, snake populations will be more vulnerable to *O. ophiodiicola* infection in regions experiencing frequent mild winter conditions.

### Future perspectives

**The spread of disease by introduced species**

Because snakes make excellent pets, they are valuable commodities in the worldwide animal trade. Containers used to breed and house animals for the worldwide trade are often good incubators for reproduction of animal pathogens especially fungi. These issues were discussed recently by Ashley et al. (2014). Animal control agents and customs agents need molecular methods to test for infectious diseases. Present procedures for containing infectious disease are insufficient in general. It is also possible for many fungal diseases to jump between species or persist as saprobes within the environment. The spread of snake fungal disease needs to be monitored frequently by epidemiologists. With the recent description of a rapid, quantitative molecular assay for SFD, this may be possible (Allender et al., 2015).

**Treatment and transmission of snake fungal disease**

To date, treatment with antifungal compounds has been unsuccessful in the Eastern Massasauga (Allender et al., 2011; Tetzlaff et al., 2015), so the continued search for an effective treatment is of paramount importance. In addition, the mode of transmission and the influence of environmental triggers on prevalence of this disease are not understood. Further research in these areas is necessary for a more thorough understanding of the dynamics of this disease.

### Conclusions

The fungus *O. ophiodiicola* is acting alone or in conjunction with other pathogens to cause snake fungal disease (Allender et al., 2015; Sleeman, 2013). The threat to free-ranging snakes needs to be determined. *Ophidiomyces* was observed to be active at a range of temperatures and pH, in addition to its ability to utilize complex carbon, nitrogen, and sulfur resources. These characteristics indicate this pathogen may be present in numerous ecosystems, and thus many snakes may be exposed to it. Future and current epidemiological surveys and ecological experiments that determine the extent of disease, species range, fungal characteristics, prevention strategies, and therapeutic options are greatly needed.

### References


