AN OUTBREAK OF FUNGAL DERMATITIS AND STOMATITIS
IN A WILD POPULATION OF PIGMY RATTLESNAKES,
SISTRURUS MILIARIUS BARBOURI, IN FLORIDA: DESCRIPTION, FACTORS,
CYCLICITY, AND PREVENTION

By

JOSEPH LATON CHEATWOOD

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
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For my wife, Amy Pazzalia Cheatwood. You have supported me unconditionally since the first day. I am truly blessed. The best it yet to be.

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Major Department: Veterinary Medicine

        This study consists of three sections. First, a fungal disease in a wild population

of pigmy rattlesnakes, *Sistrurus miliarius barbouri*, was studied over a 20-month period

in 1998 and 1999. Weekly searches were conducted for infected animals in the study

population. Lesions found on infected snakes were biopsied and prepared for both

histology and fungal culture. Fungi successfully isolated from lesions included

Sporothrix schenkii (two snakes with severe facial lesions), an unidentified *Paecilomyces*

sp. (one snake with subdermal granulomas), *Pestalotia pezizoides* (one snake with

subdermal granulomas), and *Geotrichum candidum* (=*Galactomyces geotrichum*) (one

snake with subdermal granulomas). Fungi were also isolated from leather gloves used by

members of an ongoing ecological study in the population to restrain the snakes. Two

fungi were identified from the gloves: *Cladosporium sphaerospermum* and *Pestalotia*

pezizoides. Neither of these fungi has been previously identified as pathogenic organisms in reptiles, though both are pathogens of plants.

Environmental data spanning the length of the ecological studies being conducted by research group in this population were analyzed to determine which environmental factors, if any, were correlated with an increase in the number of new cases of fungal dermatitis and stomatitis. Factors analyzed included habitat water level, temperature, and a calculated value used to represent the combined effect of the two. Simple and multiple linear regressions did not indicate a statistically significant direct correlation between any of the factors and the incidence of disease in the population at a given time.

Significant differences were shown to exist between the numbers of new cases found per year. Years were placed into three groups based on the yearly incidence of disease (new cases divided by total snakes captured). The eight years covered by the data can be clearly divided into four groups: A, B, C, and D. The 1997-1998 outbreak is the only member of group D, however, and is significantly different from all of the other years covered by the study. Time series analyses show that there are significant seasonal and cyclical patterns to the disease outbreaks. These repeating patterns could be due in part to many factors including environmental conditions, even though a direct relationship is not evident from the regression models.

While conducting the studies, concern arose about a possible anthropogenic component of the disease. Though a relationship between research methods and disease was never proven, the possibility prompted the construction of a pamphlet containing recommended handling and sampling protocols for conducting research with wild reptiles.

CHAPTER 1
REVIEW OF FUNGAL DISEASE AND METHODS LITERATURE

Introduction

Many different mycotic diseases have been reported in captive chelonians, crocodilians, and squamates. No reports describing mycotic disease in the tuatara (*Sphenodon punctatus*), a member of the order Rhynchocephalia, could be found in a Medline search. Relatively few mycotic diseases have been seen in free ranging reptiles. As in other vertebrates, fungal pathogens in reptiles may be primary or secondary invaders. Mycotic disease may be associated with predisposing factors including high humidity, overcrowding, and debris accumulation in the animal's environment. The system affected may vary between the major groups of reptiles. For instance, whereas mycotic pneumonia is uncommon in snakes, it is commonly seen in captive chelonians. Compared to mammals, systemic mycotic diseases such as histoplasmosis, coccidiodomycosis, and cryptococcosis are rarely seen in reptiles. Similarly, the dermatophytes *Trichophyton* and *Microsporum* are rarely reported in reptiles. In contrast, fungi that are seldom reported as significant problems in birds and mammals are common in reptiles. Beginning with an overview of fungal taxonomy, this chapter will review the available literature on fungal infections of reptiles.

Fungal Taxonomy

Many different fungi have been identified in the tissues of humans (and other mammals), reptiles, and birds. Almost any fungus can be a facultative pathogen, moving into preexisting lesions and preying on soft tissues of immunocompromised patients. However, some fungi are primary pathogens, causing damage to healthy tissue without the aid of other organisms. These primary pathogens are considered medically important.

All fungi are members of the Kingdom Fungi (Myceteae). Most pathogenic fungi are classified in the Division Amastigomycota. Within this division, there are seven classes containing medically important fungi. Table 1 shows the taxonomic breakdown in greater detail, listing genera in each family that are known human pathogens.

Hundreds of species of fungi have been shown to be of medical importance. Since appropriate therapies differ for various pathogens, it is important to rapidly and accurately identify fungi. Morphological characteristics, such as width of hyphae, presence or absence of septae, type and size of reproductive structures, colony morphology (including size, shape, rate of growth, and color), and optimum incubation temperature are used to identify and classify fungi. These characteristics are explained in greater detail in many medical and clinical mycology reference books (Kwon-Chung and Bennett, 1992; Fisher and Cook, 1998). It is important to consult the most recently published reference books to ensure that the most current techniques and products are used for fungal identification and that the most widely accepted names are used.

Table 1 - Taxonomic Tree of Medically Important Fungi in Humans and Animals

Kingdom Fungi (Myceteae)
Division: Amstigomycota

        Class: Zygomycetes
            Order: Mucorales
                Genera:
                      Absidia
                      Mucor
                      Rhizomucor
                      Rhizopus
                      Syncephalastrum

        Class: Ascomycetes
            Order: Endomycetales
                Genera:
                      Endomyces
                      Hansenula
                      Saccharomyces

        Class: Loculoascomycetes
            Order: Myriangales
                Genus: *Piedraia*

        Class: Plectomycetes
            Order: Microascales
                Genus: *Psuedallescheria*

        Class: Basidiomycetes
            Order: Ustilagenales
                Genus: *Filobasidiella* (Teleomorph of *Cryptococcus*)

        Class: Blastomycetes
            Order: Cryptococcaceae
                Genera:
                      Candida
                      Cryptococcus
                      Malassezia
                      Phaeoannellomyces
                      Phaeococcomyces
                      Rhodotorula
                      Torulopsis
                      Trichosporon

Table 1—continued

Class: Hyphomycetes
Family: Moniliaceae
Genera:

Acremonium
Apergillus
Blastomyces
Chryosporium
Coccidioides
Epidermophyton
Geotrichium
Gliocladium
Histoplasma
Microsporum
Paecilomyces
Paracoccidiodes
Penicillium
Scopulariopsis
Sepedonium
Sporothrix
Trichoderma
Trichophyton

Family: Dematiaceae
Genera:

Alternaria
Aureobasidium
Bipolaris
Cladosporium
Curvularia
Drechslera
Exophiala
Fonsecaea
Helminthosporium
Nigrospora
Phialophora
Wangiella
Xylophyla

Family: Tuberculariaceae
Genera:

Epicoccum
Fusarium

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Review of Literature by Order of Reptiles

I. Chelonia

Hyalohyphomycosis

Hyalohyphomycosis is a term that includes mycotic infections involving any fungal agent with septate hyphae and non-pigmented (hyaline) walls in tissue. The term encompasses a large number of fungi, some very different from each other, with one common characteristic: septate hyaline hyphae. The term does not refer to a group of common clinically recognizable symptoms (Fisher and Cook, 1998).

Fusarium solani has been reported as the cause of cutaneous mycosis in a loggerhead sea turtle, *Caretta caretta* (Cabanes et al., 1997). The authors described the fungal lesions on the turtle's skin as "white-scaled." The skin lesions from which the fungus was isolated measured between 10mm and 35mm in diameter.

Paecilomyces lilicanus and *Candida albicans* were isolated from an Aldabra tortoise, *Geochelone gigantea* (Heard et al., 1986). *Paecilomyces lilicanus* was isolated from many macroscopic, firm yellow nodular lesions in the oral and gastric mucosas and throughout the liver.

A case of systemic mycosis caused by *Penicillium griseofulvum* has been reported in a Seychelles giant tortoise, *Geochelone gigantea* (Oros et al., 1996).

Aspergillosis

Aspergillosis is caused by members of the genus *Aspergillus*. Many *Aspergillus* species are widespread saprobes (soil dwellers), breaking down plant materials for nutrition (Fisher and Cook, 1998). A side-necked turtle, St. Hilaire's terrapin (*Hydraspis hilarii*), died from a generalized aspergillosis (Hamerton, 1934). Mycotic granulomas from the forefeet of a female musk turtle (*Sternotherus odoratus*) were found to contain yeast-like organisms presumed to be an *Aspergillus* sp. (Frye and Dutra, 1974).

Mucormycosis

Mucormycosis is caused most frequently by members of the family Mucoraceae, including *Rhizopus*, *Absidia*, *Rhizomucor*, *Mucor*, and *Apophysomyces* (Fisher and Cook, 1998). In chelonians, mucormycosis has been reported in juvenile Florida softshell turtles (*Apalone ferox*) that had necrotizing shell and skin lesions. Mucor was isolated from the lesions (Jacobson et al., 1980). A *Mucor* sp. was also isolated from infected skin of wood turtles, *Clemmys insculpta* (Lappin and Dunstan, 1992).

Mixed mycoses (Sporotrichosis, Phaeohyphomycosis, Hyalohyphomycosis)

Mariculture-reared green sea turtles (*Chelonia mydas*) with mycotic pneumonia were found upon necropsy to be infected with several different fungi including a *Sporothrix* sp., a *Cladosporium* sp., and a *Paecilomyces* sp. (Jacobson et al., 1979). The lesions were described as multifocal firm nodules that were more prominent in the right lung. Histopathology of the granulomas showed that each contained numerous fungal hyphae.

II. Crocodilia

Superficial and deep mycoses (Hyalohyphomycosis, Aspergillosis, Beauveriosis, Candidiasis)

One study isolated *Fusarium solani* from deep tissue mycoses in saltwater

crocodiles (*Crocodylus porosus*) and freshwater crocodiles (*Crocodylus johnstoni*) on

farms in Australia. In the same study, *Aspergillus niger*, *Penicllium oxalicum*, and

Curvularia lunata varaeria coexisted in superficial lesions on the skin and gingiva. The

authors did not conclude which organisms were the causative agents (Buenviaje et al.,

1994). In the cases exhibiting deep tissue mycoses, lesions were found in the liver, lungs,

intestines, and stomach of affected animals. In a subsequent study, several additional

species of fungi were noted as pathogens on crocodile farms. These included *Fusarium*

sp. and *Candida* sp. (Buenviaje and Ladds, 1998).

A report was published of a case of a fatal *Beauveria bassiana* infection

(hyalohyphomycosis) in a captive American alligator, *Alligator mississippiensis*

(Fromtling et al., 1979). On necropsy, it was determined that only the lungs were

infected. The lungs were reportedly thickly covered with "mats" of fungal hyphae.

A hyaline *Fusarium* sp. and a yeast-like *Trichosporon* sp. were isolated from skin

lesions on two different caiman, *Caiman crocodylus* (Kuttin et al., 1978).

Mixed fungal pneumonias

There have been several unique reports of fungal pneumonia in crocodilians. *Candida albicans* was identified as the causative agent of pneumonia in unspecified species of crocodile and caiman (Zwart, 1968). A fatal, diffuse, granulomatous pneumonia and accompanying necrotizing hepatitis was described in three six-month-old *Caiman sclerops* (Trevino, 1972). In this paper, hyphae with terminal chlamydospores morphologically consistent with *Cephalosporium* sp. were seen in tissue sections of the lesions. In another paper, three species of crocodilians, including a Morelet's crocodile (*Crocodylus moreleti*), an American crocodile (*C. acutus*), and a Nile crocodile (*C. niloticus)*, developed a fatal respiratory infection (Silberman et al., 1977). Lesions were primarily confined to the lungs, from which a *Mucor* species was identified. Several captive two to six-week-old American alligators (*Alligator mississippiensis*) were seen in a separate study with pneumonic lesions from which *Aspergillus fumigatus* and *A. ustus* were isolated (Jasmin et al., 1968).

Mixed necrotizing dermatitis (Aspergillosis, Mucormycosis)

Fungi from the genera *Aspergillus*, *Mucor*, and *Rhizopus* were isolated from cutaneous lesions in a 100-year-old American crocodile (*Crocodylus acutus*) that was infected with *Erysipelothrix indiosa* (Jasmin and Baucom, 1967). Interestingly, in the same study, members of the genera *Aspergillus*, *Rhizopus*, and *Penicillium* were isolated from seemingly normal skin and scales of an American alligator *(Alligator mississippiensis)*.

III. Squamata: Lacertilia

Cryptococcosis

     Cryptococcus neoformans, a yeast-like organism, has been isolated from a subcutaneous lesion of an eastern water skink, *Eulamprus quoyii* (Hough, 1998). The lesion was described as a "small, discrete swelling over the lower thoracic spine." Light microscopic examination revealed numerous vacuoles containing the yeast-like cells.

Hyalohyphomycosis

     A cutaneous fungal infection involving the *Chrysosporium* anamorph of *Nannizziopsis vriesii* has been reported in chameleons (Pare et al., 1997). *Chrysosporium keratinophilum* was seen in multifocal lung lesions and necrotic stomach lesions of two green iguanas, *Iguana iguana* (Zwart et al., 1968).

Aspergillosis

     Aspergillus terreus was isolated from two San Esteban chuckwallas, *Sauromalus varius* (Tappe et al. 1984). The lesions were described as edematous and necrotic. Biopsies revealed the presence of numerous fungal hyphae.

     A black-pointed teguexin (*Tupinambis nigropunctatus*) died following a generalized mycosis caused by an unidentified species of *Aspergillus* sp. (Hamerton, 1934).

Mucormycosis

Mucor sp. was isolated from cutaneous lesions in a bearded dragon, *Pogona barbata* (Frank, 1966).

Candidiasis

Candida albicans has been isolated from multiple necrotic areas of the liver of a two-banded chameleon, *Chameleo bitaeniatus* (Silberman et al., 1977), and from necrotic esophageal lesions in a crocodile tegu, *Crocodilurus lacertinus* (Zwart et al., 1968).

Unclassified mycoses

One of the first papers reporting a fungal infection in a reptile described hyphae that were seen in "tumours" of a green lizard, *Lacerta viridis* (Blanchard, 1890). The hyphae were consistent with either a *Fusarium* sp. or a *Selenosporium* sp.

IV. Squamata: Serpentes

Trichosporonosis

The only known causative agent of Trichosporonosis is *Trichosporon beigeleii* (Fisher and Cook, 1998). This fungus was isolated from the liver and kidneys of several captive banded rock rattlesnakes, *Crotalus lepidus klauberi*, but the authors were not certain that this was also the yeast-like organism observed in tissue (Reddacliffe et al., 1993).

Phaeohyphomycosis

Cladosporium sp. has been isolated from granulomatous lesions from the mandible of an adult anaconda, *Eunectes murinus* (Marcus, 1971).

 Chromoblastomycosis

Chromoblastomycosis is defined as a superficial or subcutaneous mycosis resulting from a fungus that produces round, non-budding forms called sclerotic bodies (Fisher and Cook, 1998). A case of chromoblastomycosis has been reported in a reticulated python, *Python reticulates* (Frank, 1970), with a severe ulcerative dermatitis on the ventral scales. A similar fungus has been associated with skin lesions in a boa constrictor, *Constrictor constrictor* (Frank, 1976).

 Geotrichosis

Geotrichosis is an infection caused by *Geotrichium candidum* (Fisher and Cook, 1998). Mycotic dermatitis (Geotrichosis) due to *G. candidum* was documented in a group of captive carpet pythons (*Morelia spilotes variegata*). Skin lesions were prominent on the ventral scales (McKenzie and Green, 1976). *Geotrichium candidum* was also seen in caseous subcutaneous nodules in a northern water snake, *Nerodia sipedon* (Karstad, 1961).

Aspergillosis

An *Aspergillus* sp. was isolated from a puff adder (*Bitis arietans*) with peritonitis (Hamerton, 1934).

Unclassified mycoses

Although fungal elements were seen on microscopic examination of histologic sections taken from a mangrove snake (*Boiga dendrophila*), no fungi were isolated (Jacobson, 1984). Similarly, fungal infections were diagnosed in a western Massasaugua rattlesnake, *Sistrurus catenatus* (Williams et al., 1979), a red milksnake, *Lampropeltis triangulum syspila* (Sindler et al., 1978), and an eastern indigo snake, *Drymarchon corais couperi* (Werner et al., 1978), but an infectious agent was not isolated in any of these cases. A zygomycete was observed in a captive gopher snake (*Pituophis melanoleucos*) and a captive copperhead (*Agkistrodon contortrix*). Systemic disease resulted in the deaths of these snakes (Jessup and Seely, 1981).

CHAPTER 2
DESCRIPTION OF DISEASE AND PATHOGENESIS

Introduction

In order to understand demographic and ecological aspects of dusky pigmy

rattlesnakes, *Sistrurus miliarius barbouri*, a study site was established in 1992 at Lake

Woodruff National Wildlife Refuge, Volusia County, Florida. This research provided

new information on the life history of this snake (Rabatsky and Farrell, 1996; Bishop et

al., 1996; May et al., 1996; Jemison et al., 1995; Farrell et al., 1995; Roth et al., 1999).

All pigmy rattlesnakes with a mass greater than 25g encountered were manually

restrained using leather welder's gloves and a passive integrated transponder tag (PIT-

tags) was inserted into the coelom using a modified hypodermic syringe (Jemison et al.,

1995). PIT-tags (AVID Marketing Inc., Norco, California) are small glass-encapsulated

microchips that contain unique identification numbers.   These tags can be repeatedly

read with an external scanning device. This allows reliable, unique identification of

individual snakes.

In the fall and winter of 1997, field studies were conducted and nine pigmy

rattlesnakes at this site were observed with severe skin, eye, and mouth lesions. Several

ribbon snakes, *Thamnophis sauritis sauritis*, and a garter snake, *Thamnophis sirtalis

sirtalis*, with similar lesions were also seen at the site during further surveys. Of these, a

few were either found dead in the field or were moribund. During the same period, other

pigmy rattlesnakes were seen with less severe multifocal subcutaneous masses or crusted

scutes. Skin lesions in snakes can be caused by a variety of pathogens including bacteria, fungi, and parasites. Unfortunately, there is a paucity of information on wild reptiles However, there is a variety of reports of fungal infection in captive reptiles. In snakes, the integumentary system is commonly affected. The following fungi have been identified in skin lesions of snakes: *Geotrichium spp.* (Karstad, 1961), *Candida albican*s (Zwart, 1968), *Penicillium spp*. (Jacobson, 1980), and an unidentified Phycomycete (Werner *et al*, 1978). Fungal skin and granulomatous disease have been seen in a variety of species of captive snakes including an anaconda (*Eunectes murinus*) (Marcus, 1971), reticulated pythons (*Python reticulatus*) (Frank, 1970), a boa constrictor (*Constrictor constrictor*) (Frank, 1976), carpet pythons (*Morelia spilotes variegata*) (McKenzie and Green, 1976), a northern water snake (*Nerodia sipedon*) (Karstad, 1961). In this chapter, pathologic and microbiologic findings on snakes from the affected population are reported.

Materials and Methods

Between September 1997 and March 1998, a survey of pigmy rattlesnakes at Lake Woodruff National Wildlife Refuge (Deleon Springs, FL; 29° 07' N, 81° 22' 30"W) revealed three pigmy rattlesnakes with severe eye, head, mouth, and multifocal skin lesions (Figure 1A and Figure 1B). The snakes were transported to the University of Florida where they were euthanitized with a concentrated barbiturate solution and necropsied. The heads were removed and decalcified using a formic acid sodium citrate decalcification solution (Luna, 1968). Samples of all major organ systems were collected and placed in neutral buffered 10% formalin (NBF). For microbial isolation attempts,

samples of lesions were homogenized and streaked onto blood agar for bacterial isolation

and incubated at 36°C. Samples were pressed into Sabouraud dextrose agar (SAB) and

mycobiotic agar for fungal isolation and incubated at 23°C for 30 days.
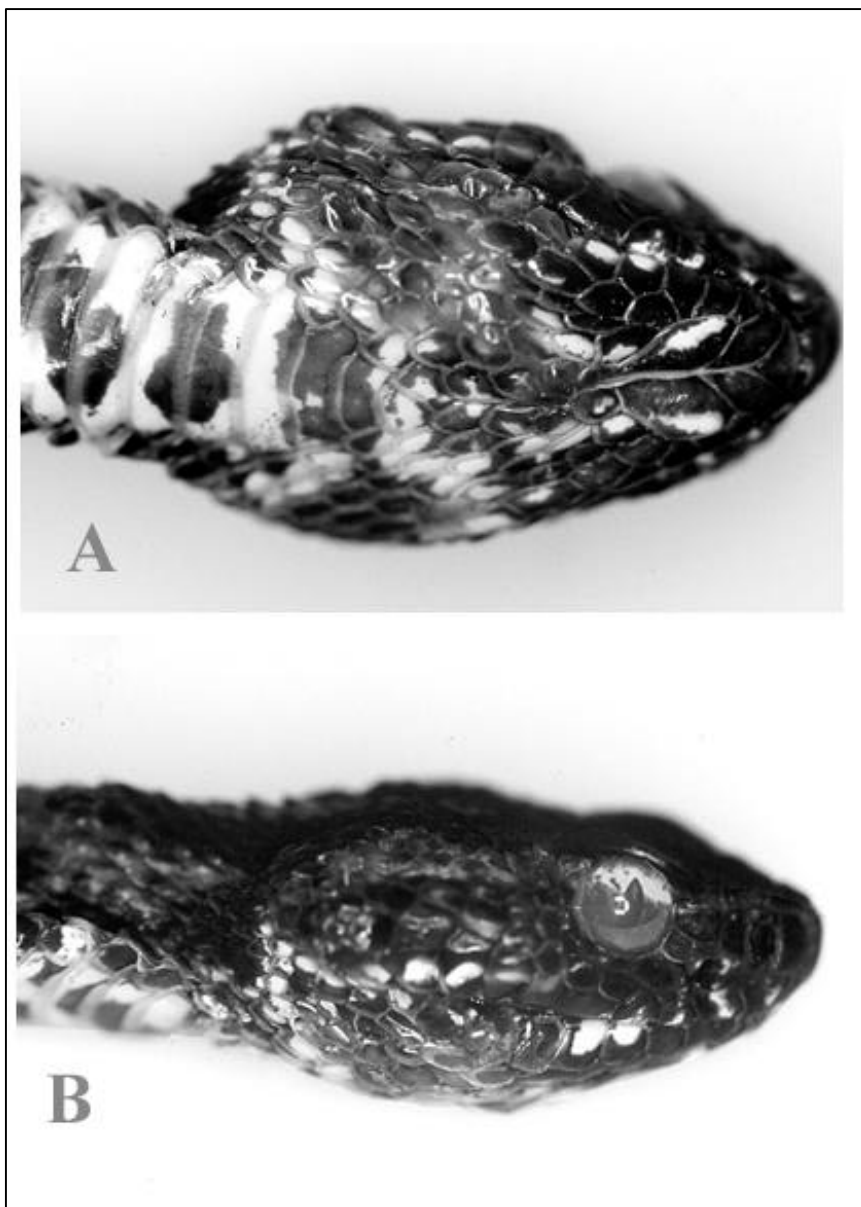
Figure 1 – Ventrodorsal (A) and lateral (B) views of a pigmy rattlesnake with severe facial skin disease. The spectacle is cloudy and bulging beyond normal limits. There are diffuse areas of epidermal necrosis with subcutaneous swelling that distorts the appearance of the head.

After the initial snakes were found, weekly inspections of the field site were conducted from September 1997 until November 1999. During these surveys, a ribbon snake, *Thamnophis sirtalis sirtalis*, and a garter snake, *Thamnophis sauritis sauritis*, with lesions similar to those seen in the pigmy rattlesnakes were found at the same study site. Both of these snakes were euthanitized and necropsied. Samples were taken for histology and fungal culture as described above.
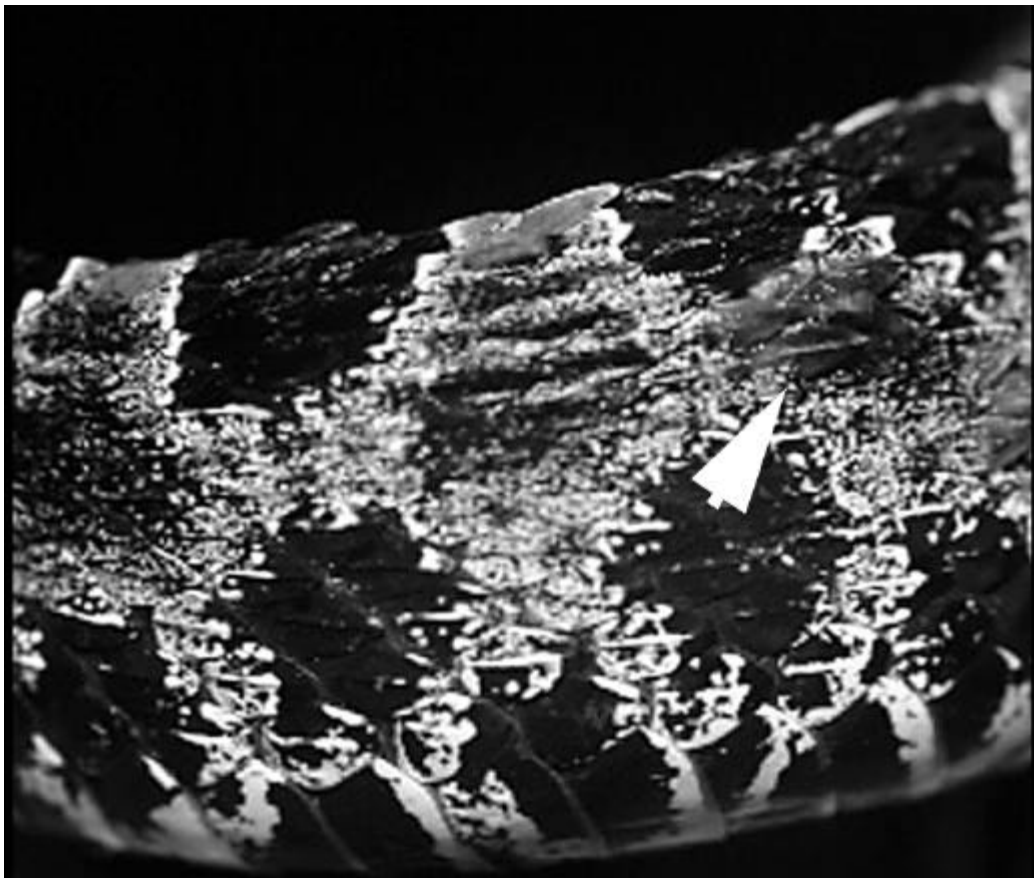
Figure 2 – Focal epidermal necrosis and subcutaneous masses in the skin of a pigmy rattlesnake, *Sistrurus miliarius barbouri*.

During weekly surveys from January 1998 through December 1999, 22 total pigmy

rattlesnakes were seen at the study site with multifocal minimal to moderate necrotizing

skin lesions overlying subcutaneous masses (Figure 2). Some of these snakes were

captured multiple times over the course of their infection (and after their recovery) while

others were observed only once. Snakes were manually restrained using heavy leather

welding gloves and a ring block of 2% lidocaine (Butler Company, Columbus, Ohio,

USA) was used for local anesthesia. Field biopsies were obtained from six pigmy

rattlesnakes with subcutaneous masses only. Biopsies were taken aseptically by cleansing

the scales with Nolvasan (Fort Dodge, Fort Dodge, Iowa, USA) and immediately

removing the affected scales and subdermal masses with a sterile number-15 scalpel

blade. The incision site was then cleansed with an organic iodine solution (Betadine, Fort

Dodge, Fort Dodge, Iowa, USA) and the snakes were released. The tissues excised from

the snakes were divided in half and placed in either NBF or sterile water. Samples placed

in NBF were processed for histology. For isolation of fungi, biopsies in sterile water were

placed on SAB agar or mycobiotic agar and incubated at 23°C.

All tissues in NBF were routinely processed, embedded in paraffin, and sectioned

at 5µm. Sections of each lesion were stained with hematoxylin and eosin, Periodic Acid

Schiff (PAS) stain, or Gomori's methenamine silver (GMS) stain and were evaluated by

light microscopy.

Cultures for fungal isolation were observed for fungal growth over a 30-day

period. Fungi forming colonies on the plates were separated into pure culture on

additional SAB plates. Samples of pure fungal cultures were placed on malt extract agar

to encourage the production of reproductive structures. All fungi growing on plates were

identified by morphological characteristics and colony presentation using several

previously published keys (Fisher and Cook, 1998; Ellis, 1972; Ellis, 1976). Samples of

the mature cultures were placed on a slide in a drop of lactophenol cotton blue to help

delineate the morphological features of the fungi (Rippon, 1988). Measurements of fungi

in tissue were made with an optical micrometer.

Since a database was available that contained records of all captures since the

beginning of the ecological study, it was possible to conduct a retrospective evaluation to

determine the presence of lesions in previously captured snakes. Fields contained in the

database included mass, capture location, length, gravidity, gender, presence or absence

of prey, and any special comments necessary to describe the individual. In this database,

cutaneous masses were referred to as "lumps," "bumps," or "tumors" and snakes with

severe oro-facial lesions were described as having "mouth rot." Mild to moderate

multifocal skin lesions were often not recorded in the database. Querying the database

for these terms using the database software (Microsoft Access 97 for Microsoft Windows

platform) revealed how many snakes with each type of lesion had been seen during the

years of the study.

Results

Between February 1992 and November 1999, a total of 10,727 dusky pigmy

rattlesnake captures and recaptures were made at the field site. This number represents

approximately 600 individual snakes (May et al., 1996). Since the initiation of the

ecological study, sixteen snakes have been seen with severe head and oral cavity lesions

(Table 2). Of the sixteen snakes with severe head and oral cavity lesions, six had been previously captured and PIT-tagged during the course of the study.

During the same period, 48 individual snakes were found with small (3-5mm), raised, firm, mild to moderate multifocal skin lesions scattered over the body surface. Twenty-three (23) males and thirty-one (31) females were affected and ages of the snakes ranged from less than one year old to greater than six years of age. Of the 45 snakes with multifocal granulomatous lesions, 19 were seen with lesions for the first time during weekly surveys for this study between January 1998 and November 1999.

Table 2 - Incidence of disease by year (% affected)

1992	1993	1994	1995	1996	1997	1998	1999
6/912	6/800	10/1047	20/670	3/533	10/829	30/474	1/285
(0.66)	(0.75)	(0.96)	(2.99)	(0.56)	(1.21)	(6.32)	(0.35)

By light microscopy, the severe facial and orbital lesions seen in the pigmy rattlesnakes and several garter and ribbon snakes also found at the study site had similar features. Affected skin, spectacles, and mucosa lining the oral cavity were diffusely necrotic with either diffuse infiltrates of mixed inflammatory cells including heterophils, small mononuclear cells, and macrophages (i.e. immature granulomas) or more organized, mature granulomas. The mature granulomas observed in both the severe and multifocal infections had a necrotic, deeply eosinophilic center with H&E staining (Figure 3A). Using PAS and GMS stains, branching septate hyphae were seen (Figure 3B). Hyphae of several different widths (1.1µm to 5.5µm) and morphologies were observed in tissues, suggesting that there were multiple fungal agents associated with the granulomatous response in this population of snakes. Some hyphae in tissue branched

often (every 3-6µm) and others had much longer regions between branches (20-30µm).

Fungal hyphae were seen in all granulomas, both immature and mature. Evaluation of all

internal tissues did not reveal any fungal granulomas. The only lesion seen was a sperm

granuloma in the kidney of one pigmy rattlesnake.
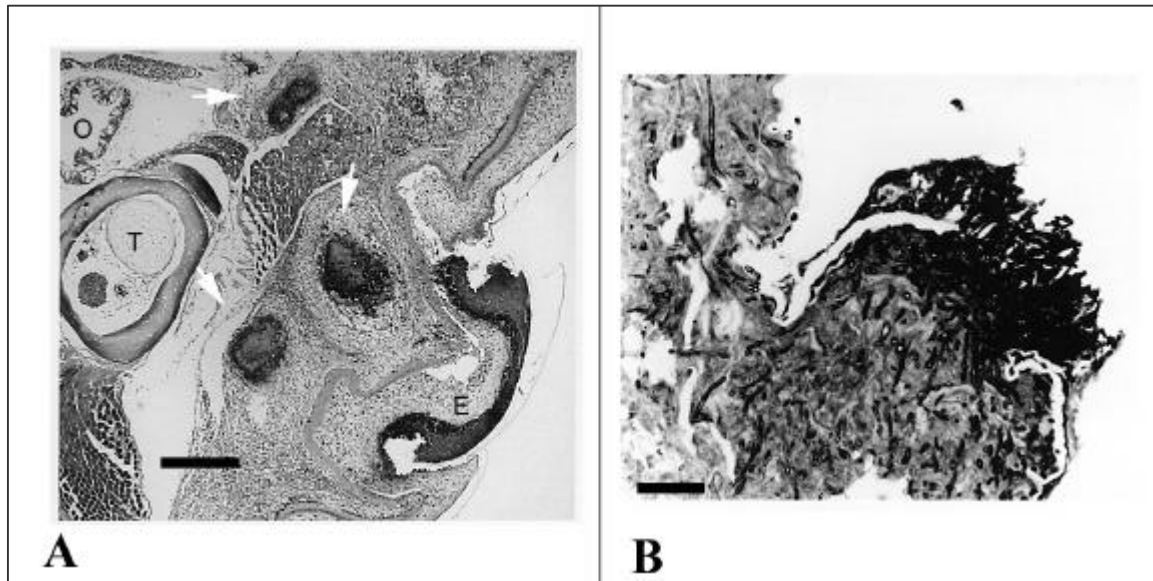
Figure 3 –
 A. Photomicrograph of the head of a pigmy rattlesnake showing necrotic epidermis
    (E), subcutaneous granulomas (arrows), tooth (T), and oral cavity (O). H&E stain.
    40x. Bar=.2mm.
 B. At a higher magnification, numerous hyphae can be seen in an area of epidermal
    necrosis. PAS stain. 400x. Bar=10µm.

Biopsies of mild to moderate skin lesions showed epidermal hyperplasia, often

with ulceration, subtended by an edematous dermis and subdermis. In skin samples from

one affected pigmy rattlesnake, there was a severe epidermitis with focal to diffuse

coagulation necrosis of the epidermis and dermis. In the dermis there were multiple

mature granulomas with deeply eosinophilic centers clustered together (Figure 4).
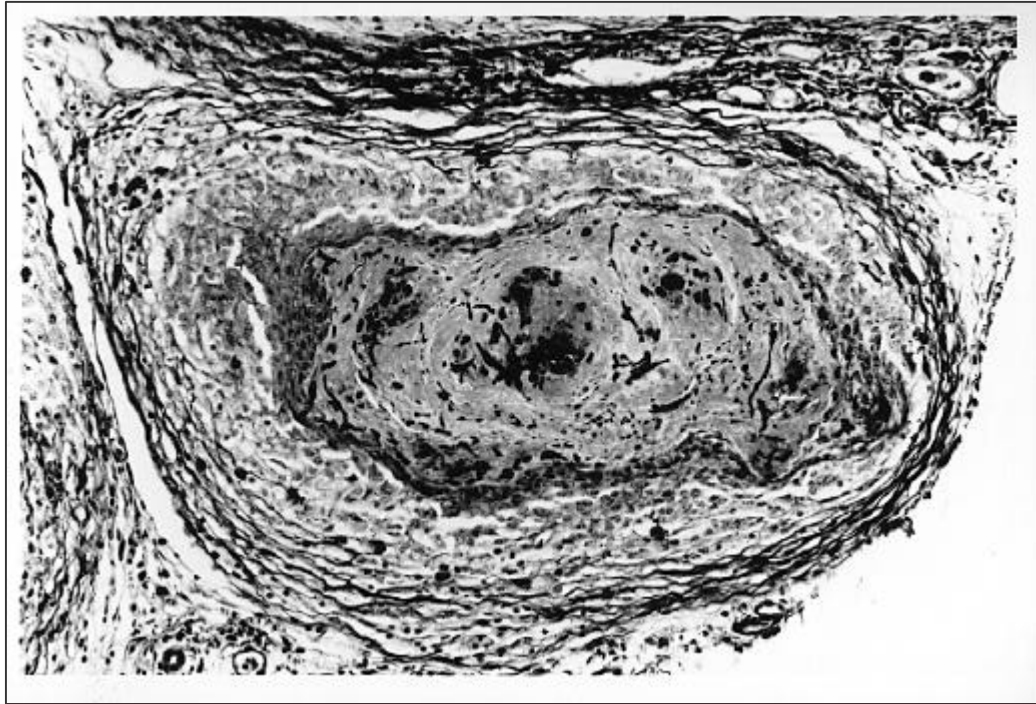
Figure 4 – Photomicrograph of a subcutaneous granuloma in a pigmy rattlesnake with multifocal skin lesions. Hyphae can be seen within the center of the granuloma. GMS stain. 400x.

Over the course of the study, biopsy samples were collected from nine snakes with mild to moderate multifocal skin lesions. Fungi were isolated from five of the samples. Based upon the morphology of conidia, spores, and other sexual structures, the following fungi were identified on malt extract agar plates: from severe orofacial lesions, *Sporothrix schenkii* (two snakes), a *Paecilomyces* sp. (one snake), *Pestalotia pezizoides* (one snake) and *Geotrichum candidum* (=*Galactomyces geotrichum*) (one snake) were isolated. The first three fungi were isolated from the initial cultures from the severely affected snakes. *Galactomyces geotrichum* was also isolated from cultures of two biopsies of granulomatous lesions from different snakes.

The samples taken from the gloves used to handle snakes while measurements were made resulted in the isolation of an unidentified actinomycete and two species of fungi: *Pestalotia pezizoides* and *Cladosporium sphaerospermum.*

In addition to the fungi, the following bacteria were isolated from the initial severe oro-facial lesions of two snakes: a *Xanthomonas* sp., a *Klebsiella-Enterobacter* sp., a *Corynebacterium* sp., and *Bacillus* spp.

Discussion

The study site, Lake Woodruff National Wildlife Refuge, is within the floodplain of the Saint Johns River in Volusia County, Florida. Habitats in the refuge include sandy uplands, seasonally damp oak hammocks, pine flatlands, and areas of tall marsh grass. A diverse reptile population is present in the refuge. During this study, we observed seven species of turtles, one species of crocodilian, five species of lizards, and seventeen species of snakes. Of these, pigmy rattlesnakes were the most plentiful reptile encountered at the site. The most recent estimates of the size and density of the population, published in 1996, indicated that there were approximately 600 individuals in the research site, providing a density of greater than fifty pigmy rattlesnakes per hectare (May et al., 1996). Density and population size fluctuate yearly, partially because the number of juveniles born each year varies. One study published concerning the reproduction strategy in the study population found that over a two-year period, approximately 68% of the adult females were fertilized, with some snakes reproducing in both years (Farrell et al., 1995). Each gravid female produces an average of approximately six offspring. Neonate pigmy rattlesnakes typically have an average mass

of 4.79 grams (Farrell et al., 1995). The mean weight of adult pigmy rattlesnakes at the site is approximately 47.5g, with a maximum weight in the population of 182.0 grams.

In the spring of 1998, surveys at the study site revealed pigmy rattlesnakes with severe oral and integumentary lesions. Twenty-two of the eighty-five snakes (23%) captured in the first three months of 1998 represented new cases of either mild to moderate granulomatous lesions, crusted scutes, or a severe necrotizing fungal infection involving the head. In the previous quarter, the winter months of 1997, five new cases were observed in 92 snakes (5.4%). In a review of field records, similar appearing lesions were recognized in pigmy rattlesnakes in this population sampled during previous years. However, the number and frequency of the lesions was highest during the 1997-1998 epidemic. Prior to the winter of 1997, 50 total cases had been documented. During the sixth month period between October 1997 and March 1998, 27 new cases were documented.   Before this report, no previous histopathologic evaluation was undertaken to determine the nature of these lesions.

In field studies on this population after the severe oral and integumentary lesions were recognized, snakes with focal to multifocal less severe integumentary lesions also were observed. In a review of field records, previous reports of similar lesions were found. Calculated incidences based on the number of new cases and the numbers of individual snakes captured per year (Table 2) indicate a high degree of variability in the incidence of the disease in the population. The number of snakes captured each year varied with a maximum of 1047 in 1994 and a minimum of 285 in 1999.

Lesions were not limited to pigmy rattlesnakes. Similar severe gross lesions were observed in a ribbon snake and a garter snake at the site in the fall of 1997. Prior to 1997,

records indicated that only pigmy rattlesnakes were observed with these lesions. Similar

lesions were not recognized in any other reptiles at Lake Woodruff.

The severe facial and orbital lesions distorted the appearance of affected snakes.

Spectacles of severely affected snakes were edematous, white or clouded, and abnormally

bulged from the margins of the orbit. The oral cavity and surrounding tissues were

similarly edematous, thickened, and necrotic. The mild to moderate focal to multifocal

integumentary lesions were easily overlooked and only slightly elevated the overlying

epidermis, often resulting in a superficial necrosis. Histologic evaluation indicated that

the more severe lesions consisted of granulomatous inflammation intermixed with areas

of cellulitis and necrosis. The overlying mucosa and epidermis were often necrotic. The

mild to moderate integumentary lesions consisted of mature granulomas containing

eosinophilic centers when stained with H&E.

In reptiles, granulomatous inflammation can be caused by a wide variety of

pathogens including bacteria, fungi, and parasites. While several bacteria were isolated

from lesions in snakes in this study and were identified in tissue section using special

stains, in GMS and PAS stained tissue sections, fungal hyphae were consistently seen

within the center of organized granulomas, in areas of less organized granulomatous

inflammation, and also within necrotic tissue on the body surface. Similar appearing

integumentary lesions have been seen in captive snakes with fungal epidermitis and

dermatitis (Williams et al., 1979; Jacobson, 1984). One veterinarian has seen similar

fungal associated integumentary in other wild snakes in the southeast United States

including a corn snake (*Elaphe guttata guttata*), water snakes (*Nerodia* spp.), garter

snakes (*Thamnophis* spp.), and eastern indigo snakes (*Drymarchon corais*) (Jacobson,

pers comm.). Most of these lesions probably go unreported or unrecognized by investigators working on snakes in the field.

Hyphae with several different morphologies were observed in the lesions. Fungi seen in the tissues were irregularly branching, septate, hyaline hyphae ranging from 1μm to 5μm in width. It is often difficult to determine the identity of fungi in tissue without special techniques such as immunoflourescent antibody assays, immunohistochemistry, or molecular (i.e. polymerase chain reaction) assays (Fisher and Cook, 1998). In some cases, these can be used to identify fungi to genus and species (Fisher and Cook, 1998; Sandhu et al., 1995; Makimura et al., 1994). Identification via morphological characteristics of cultures grown out on media is still considered the gold standard. Thus, in this report, direct examination of paraffin embedded tissue sections by light microscopy was not useful in identifying fungi to a generic and specific level.

Based on hyphae and spore characteristics (Kwon–Chung and Bennett, 1992; Fisher and Cook, 1998), the following four species of fungi were isolated from snakes with severe orofacial lesions: *Sporothrix schenkii, Pestalotia pezizoides, Geotrichum candidum* (=*Galactomyces geotrichum*), and a *Paecilomyces* sp. *Sporothrix schenkii* is a well-known pathogen. It has been reported to cause subcutaneous lesions in primates (Costa et al., 1994; Vieira-Dias et al., 1997; Vismer and Hull, 1997; Conias and Wilson, 1998; Tomimori-Yamashita et al., 1998; Hajjeh et al., 1997; Kauffman, 1999; Werner and Werner, 1994), ungulates (Irizarry-Rovira et al., 2000; Greydanus-van der Putten et al., 1994), felids (Costa et al., 1994; Davies and Troy, 1996; Nakamura et al., 1996; Marques et al., 1993; Reed et al., 1993), and armadillos (Wenker et al., 1998) that are similar to those that we have observed in pigmy rattlesnakes. Previous isolation of *S.*

schenkii from reptiles has been limited to a group of mariculture-reared green sea turtles (*Chelonia mydas*) with mixed mycotic pneumonia (Jacobson et al., 1979). A *Paecilomyces* sp. was also isolated from the lungs of the same sea turtles. Only two additional reports could be found indicating *Paecilomyces* as a pathogenic fungus in reptiles. Infections in a captive Aldabra tortoise (*Geochelone gigantea*) that died with macroscopic, firm yellow nodular lesions distributed across the oral surface, gastric mucosa, and throughout the liver (Heard et al., 1986) and a systemic infection of a captive crocodile (*Crocodylus porosus*) (Maslen et al., 1988) showed granulomatous lesions of fungal origin in the liver, left lung, and spleen. *Pestalotia pezizoides* has never been implicated as a pathogenic fungus, except in some plant species, and was probably a contaminant in the tissue. However, it is possible that the organism was acting as a falcultative pathogen because of the advanced necrotic condition of the lesions from which it was isolated. *Geotrichum candidum* (*Galactomyces geotrichum*) has been previously reported as a pathogen in a group of captive carpet pythons (*Morelia spilotes variegata*), (McKenzie and Green, 1976) a northern water snake (*Nerodia sipedon*) (Karstad, 1961) and most recently an unspecified garter snake (*Thamnophis* sp.) (Vissiennon et al., 1999).

Based upon our findings, more than one fungus was probably involved. It is possible, since we do not know the identities of all of the fungi seen in paraffin sections, that other pathogenic or opportunistic fungi may have been present in the lesions but not isolated in culture. It is also possible that some of the fungi that were isolated were surface contaminants and not actually in the tissues. Transmission studies needed to demonstrate a causal relationship were beyond the scope of this report.

The pathogenesis of these lesions, both the severe orofacial lesions and mild to moderate integumentary lesions, is unclear. Penetrating wounds in the integument may have resulted in infection and granuloma formation in the dermis in those snakes with mild to moderate integumentary lesions. Though no ticks or other external parasites of any kind were observed on pigmy rattlesnakes at the time of capture, this is also a potential route of infection. This type of infection could explain the multifocal distribution of the subcutaneous granulomas. Ticks do exist in the habitat, but their role, if any, as a parasite of pigmy rattlesnakes is unknown. Another explanation could be the association with subcutaneous parasites. Fungal granulomas associated with subcutaneous pentastomid parasites in indigo snakes in Florida have been seen by a specialist on diseases of reptiles, Dr. Elliott Jacobson (pers comm.). No pentastomes were seen in any of the snakes in this study, but this does not exclude the possibility that they may be in the population. Full necropsies on several snakes with severe lesions did not indicate systemic disease. While this suggests that lesions commenced locally and spread to surrounding tissues, it is still possible that the pathogen(s) spread via the circulatory system, causing inflammation at the affected sites. Fungal hyphae may have spread locally from granulomas to surrounding areas via the circulatory system. In humans, the spread of *S. schenkii* and other systemic fungi has been shown to occur through the circulatory system and lymphatics (Rippon, 1988).

Another possibility is showering of hyphae or spores from visceral structures. Fungi have been previously cultured from kidney of apparently healthy snakes without specific lesions seen in the kidney (Jacobson, pers comm.). It appears that reptiles may harbor organisms in visceral structures that can cause disease when conditions allow the

organism to proliferate, stimulating an inflammatory response. A similar situation has been described in amphibians where culture of the kidney of free ranging anurans in Brazil indicated presence of multiple species of fungi (Mok et al., 1982).

An anthropogenic basis for the epidemic also was considered. One of the initial concerns in this study was that fungi were transferred between snakes and entered tissues due to handling and sampling techniques being used in the field. Due to their small size, all pigmy rattlesnakes were manually restrained using a pair of leather welder's gloves. The pair of gloves being used at the time of the 1997/1998 outbreak had been in use for a period of approximately two years. Two fungi were identified in pure culture from samples taken from the gloves: *Cladosporium sphaerospermum* and *Pestalotia pezizoides*. No reports were found in the literature to indicate that *C. sphaerospermum* is a primary pathogen of vertebrates. However, it was isolated from lesions in a lesser octopus, *Eledone cirrhosa*, and transmission studies were conducted that confirmed the pathogenic nature of the fungus in a marine environment (Polglase et al., 1984). It was not isolated from any lesions in this study and probably did not play a role in the 1997/1998 outbreak. Though *P. pezizoides*, was isolated from both the severe necrotizing lesions on one of the pigmy rattlesnakes and the gloves used for manual restraint, it is unlikely that the fungus was a primary pathogen, since *P. pezizoides* has never been reported as a pathogen of vertebrates. Thus, there is no evidence to suggest that handling techniques were involved in the outbreak, other than the possible effects of stress and abrasion of the skin, which could make infection more likely. Field records indicated that twelve of the sixteen snakes with the severe oral and facial lesions observed during the 1997-1998 epidemic had never been captured previously or handled

by the study team. Still, field equipment can represent important mechanisms for transfer of pathogens between animals and should be disinfected as needed. This may necessitate having more than one set of equipment available when handling snakes, especially if snakes with lesions are encountered. A protocol for handling snakes in the field to reduce the spread of transmission of pathogens between animals is needed and is currently being developed by us.

CHAPTER 3
OUTBREAK SEASONALITY AND CYCLICITY

Introduction

This study was conducted to search for abiotic factors that could be related to outbreaks of fungal dermatitis and stomatitis in a wild population of pigmy rattlesnakes in Florida. Tests for seasonal and cyclical patterns in the incidence of the disease were also conducted to better explain the occurrence and cyclicity of the outbreaks.

Temporal fluctuations have been described in a wide variety of biological systems. Yearly changes in the sizes of mammal populations are the best studied, and many have been shown to vary in a predictable way based on population and environmental conditions (Oli and Dobson, 1999; Kendall et al., 1998; Seldal et al., 1994). Within populations of wild and captive animals, the prevalence of diseases and parasites has also been demonstrated to fluctuate temporally. The incidence of salmonellosis in horses presented to a veterinary hospital in California, for example, was shown to be strongly seasonal, occurring most frequently in June through September (Carter et al., 1986). A study of rabies in Chile concluded that outbreaks follow a seasonal trend, with cases increasing during November and December, and also exhibit a cyclic behavior, repeating regularly every five years (Ernst and Fabrega, 1989). Occurrence of human hemorrhagic septicemia has been shown to have a strong seasonality as well, with outbreaks occurring regularly during the rainy season in several

areas of India (Dutta et al., 1990). A similar study on foot-and-mouth disease in India also found a strong pattern of seasonality, again correlating occurrence of disease with the rainy season (Sharma et al., 1991). Retrospective studies of data from historic outbreaks of diseases in human populations have shown cyclic patterns. Analysis of the widespread smallpox epidemics in Britain in the seventeenth and eighteenth centuries, for example, show two distinctly different repeating patterns of occurrence based on the number of people in an outbreak area (Duncan et al., 1994). Studies of epidemics of whooping cough in London from 1701-1812 (Duncan et al., 1996a) and smallpox in London from 1647-1893 (Duncan et al., 1996b) have determined the existence of similar cyclical patterns of disease.

Outbreaks that occur seasonally during periods of increased rainfall (Sharma et al., 1991; Dutta et al., 1990) provide support for the idea that disease incidence fluctuations are sometimes due to changes in environmental conditions. Rainfall, temperature, food availability, or any other stress factor that makes a population of animals more susceptible to pathogens could all play roles of varying importance. Studying these factors could conceivably help in the development of a model to predict future outbreaks. Since these factors may be correlated to or dependent upon one another (e.g. rainfall and availability of frogs as prey items for pigmy rattlesnakes), establishing a linear relationship of one or more environmental variables and the incidence of a disease can be difficult.

Materials and Methods

Case Data

Cases were obtained by searching a database from an eight-year-long

mark/recapture study involving a population of pigmy rattlesnakes, *Sistrurus miliarius*

barbouri, in Florida. The database, obtained from researchers at Stetson University in

Deland, Florida, contains weekly entries that describe the number of snakes captured

during each sample date. These entries also contain notes on the condition of snakes.

During the study period, a subset of the study population was found with what have been

determined to be subdermal granulomas formed as a response to fungi in the tissue.

Snakes that were found with these lesions during the eight-year study were noted in the

database as having "bumps" or "lumps" on their skin. Snakes with a more severe fungal

infection involving the head were similarly designated in the database as having "head

rot" or "mouth rot." A search of the database for snakes described using these terms was

used to calculate the number of affected animals in the population during any given

period. Results returned by the queries of the database were carefully screened to be

certain that they were actual accounts of cases and not records describing snakes without

lesions of interest.

Monthly and quarterly incidences of disease were calculated for the study period

by querying the database for new cases and total snakes captured in a given month. The

number of new cases was subsequently divided by the total number of snakes captured in

a month, and the resulting figure was multiplied by 100 to yield an incidence per 100

snakes captured. The same process was repeated to determine quarterly incidences of

disease for the study period.

Environmental Data

During weekly surveys at the study site, a water level reading was recorded for the habitat. The water level value represents the depth of water covering the ground in the habitat at a given point. A negative value reflects the depth at which wet soil can be found. Readings are taken with the help of a permanently placed stake marked at 1cm intervals. Using this method, the lowest measurable water depth is –10cm. Weekly measurements were collected and averaged appropriately to yield mean monthly and quarterly water level measurements for the habitat.

For the purpose of this study, this water level reading is more representative of the conditions in the habitat than rainfall. The habitat for the study is part of a floodplain for the Saint Johns River, which runs along the East coast of Florida. Because it is attached to the river, the amount of water present in the habitat is dependent upon the water level in the river. Since the river flows from South to North, emptying into the Atlantic Ocean in Jacksonville, Florida, increased rain in South Florida or increased wind in Jacksonville could theoretically have a large impact on water level in the habitat. This necessitates a "bigger picture" measure of water present in the habitat than considering rainfall alone. Thus, water level was chosen.

Daily minimum and maximum temperature measurements were obtained from a local weather station for the duration of the study. Minimum and maximum temperatures for each day were averaged to yield an average daily temperature.

Data Analysis

The null hypotheses of this study were as follows:

1) There is no difference between annual incidence of disease in the population

2) There is no linear relationship between abiotic factors and disease

3) There is no increased "seasonal index" for any given month

4) There is no cyclical component of the disease outbreak

Descriptive Statistics

Incidence rates were calculated for each year by dividing the number of new cases identified during the year by the total number of snakes captured. The X^2 test (Rao, 1998) was used to compare annual incidence rates from 1992 to 1999.

Regression Analyses

Simple linear regressions and multiple linear regressions were performed with Microsoft Excel and verified by SAS. In the simple linear regressions, incidence in a given quarter was compared to either water level in the same quarter, mean temperature for the quarter, or a combined factor representing the multiplicative effects of water level and temperature. Multiple linear regressions were conducted using all three factors in the model.

Time series

Incidence rates were calculated for each month by dividing the number of new cases identified during the month by the total number of snakes captured. The resulting number was multiplied by 100 and expressed as a percentage. The time series model was

used to break the data into four components: trend, seasonal variation, cyclical variation, and random variation as previously described by Carter et al (1986).

Previously calculated incidence rates were examined for seasonal patterns. This was done by calculating a "seasonal index" for each calendar month. These indexes were derived by dividing the previously calculated monthly incidence by the 12-month moving average (12-month period centered around the month in question). The resulting value is then added for each calendar month (e.g., all December values are added to get a cumulative total for December). Dividing the cumulative index by the average index value (obtained by adding all indexes together and dividing by 12) will allow the expression of a monthly index value as a percentage of the mean. Months having index values higher than 100 are months in which there is a seasonal increase in the incidence of disease.

In order to determine the long-term trend in incidence rates, a regression analysis was performed using time in months (numbered consecutively starting at 1) as the independent variable (x) and deseasonalized monthly incidence as the independent variable. Deseasonalized data were calculated by dividing the monthly incidence rates by the cumulative seasonal index for the corresponding month.

Data used to look for cyclical patterns in incidence were calculated by dividing the deseasonalized incidence for each month by the corresponding trend value for the month. Trend values are continuous, beginning with the y-intercept (m) calculated in the above regression analysis and increasing in steps by the slope value (b) for the same number of points as the deseasonalized data.

Results

Descriptive Statistics

Figure 5 shows yearly incidence rates for all years in the study (1992-1999). The year containing the majority of the outbreak addressed in Chapter 2 (1998) has a much higher incidence than any other year in the study period. Groups developed based on results of the X^2 tests are shown in Table 3. The groups consist of years whose incidences are not significantly different from one another ($\alpha>0.05$). Some years are in more than one group. The only year whose incidence is statistically different from all others is 1998, the year of the outbreak that inspired this study.
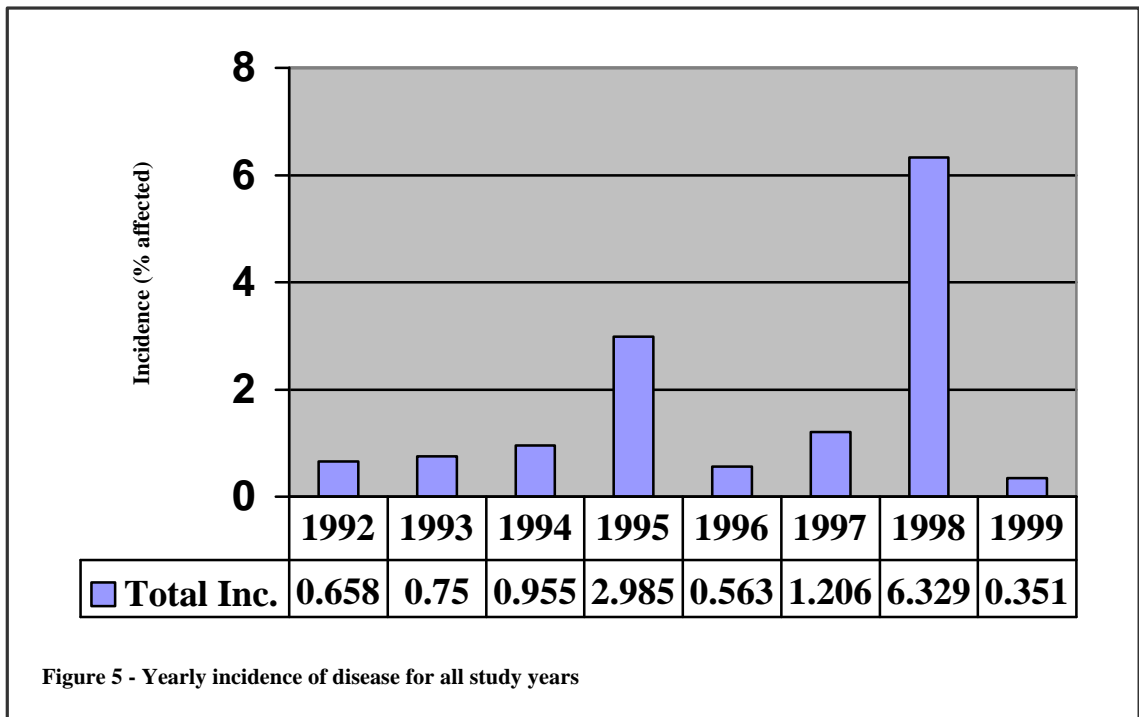

	1992	1993	1994	1995	1996	1997	1998	1999
Total Inc.	0.658	0.75	0.955	2.985	0.563	1.206	6.329	0.351

Figure 5 - Yearly incidence of disease for all study years

Table 3 - Groups by yearly incidence

Group A	Group B	Group C	Group D
1992	1993	1995	1998
1993	1994	1997	
1994	1996		
1996	1997		
1999	1999		

Regression Analyses

The results of regression analyses conducted to test for a linear relationship between environmental conditions (habitat water level and temperature) and the incidence of disease in the population are shown in Table 4.

Table 4 - Results of regression analyses

Factor	Intercept	Coefficient	P-value	R^2
Water level (W)	2.53	0.19	$\alpha=0.09$	0.10
Temperature (T)	15.19	0.19	$\alpha=0.09$	0.10

Time Series

The raw monthly incidence rates, 12-month centered moving average, and the trend are depicted in Figure 6.

Seasonal indices, calculated as previously described, are shown graphically in Figure 7, below. Values greater than 100 indicate a month in which the incidence of

disease is consistently elevated above the mean incidence across the study period.

Indexes for December, January, March, and April are all greater than 100. All other

months are below 100.

The cyclical portion of this time series is found in Figure 8. Cycles seen

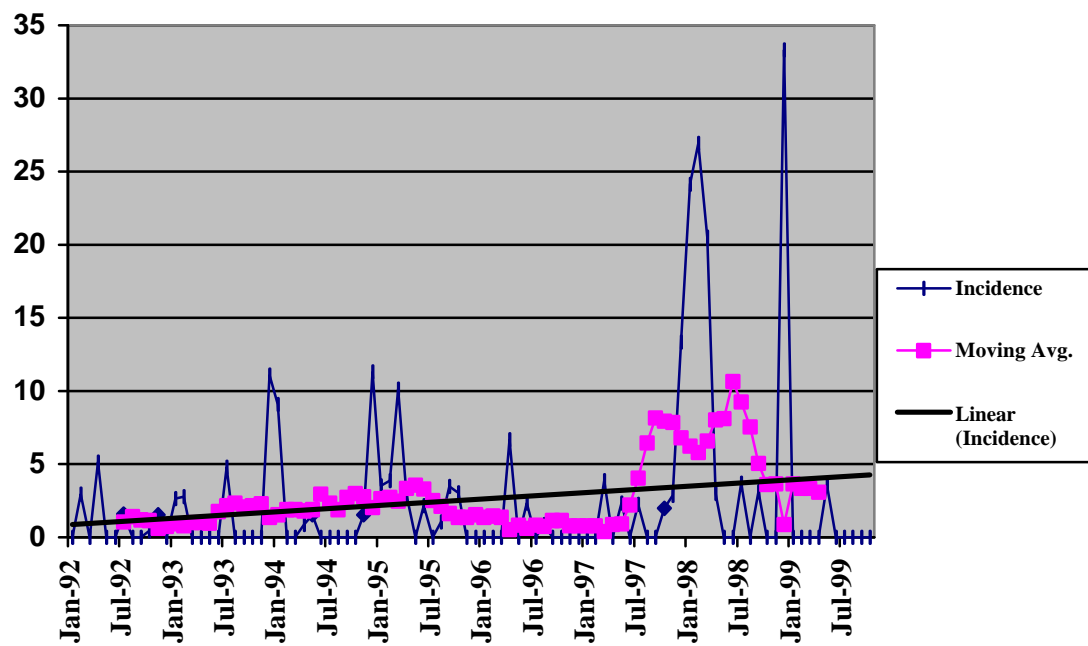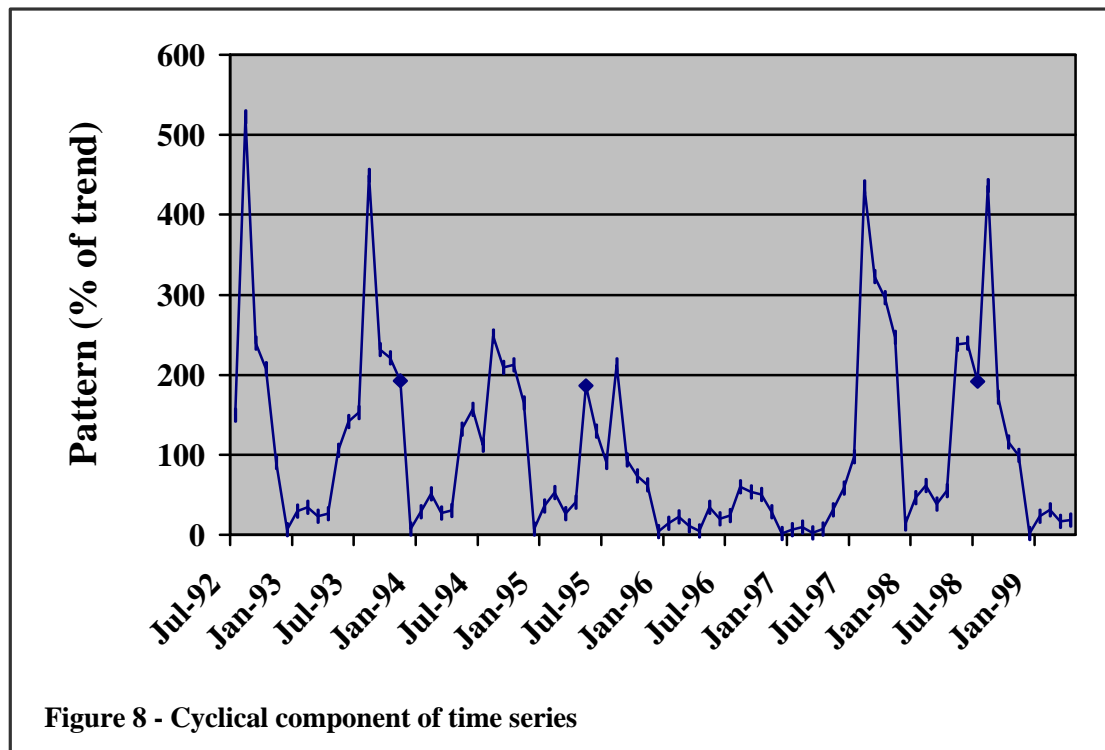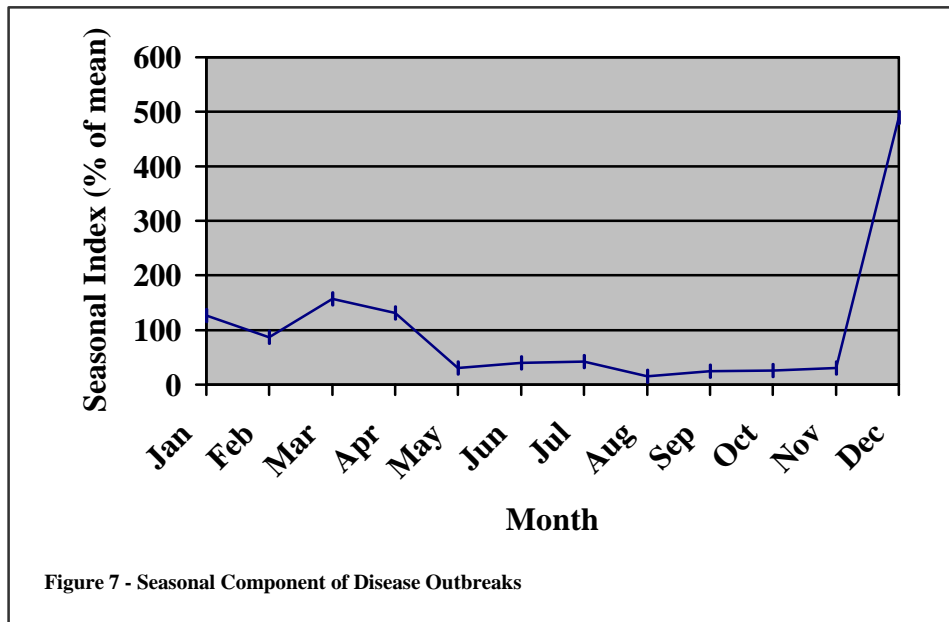here represent patterns in the data not accounted for by the trend or seasonal variation.

Figure 6 -Monthly incidence, 12-month centered moving average, and trend of disease

Figure 7 - Seasonal Component of Disease Outbreaks

Figure 8 - Cyclical component of time series

Discussion

The regression analyses in Table 3 indicate that there is no statistically significant linear relationship between the environmental factors of interest and the incidence of disease. It is possible, however, that the relationships are not statistically significant because the incidence of disease is profoundly affected by variables that were not considered in this study. Some possible variables that could contribute to the susceptibility of snakes to fungal disease are chemical pollutants, immune system disease, prey abundance, or an unknown periodic physiological change in the snakes.

Table 4 demonstrates that incidence rates are different between the years examined by the study. This indicates that the factors contributing to the outbreak, whatever they may be, are not the same from year to year.

The seasonal effect noted in Figure 7 may be the result of changes in environmental conditions in the habitat (i.e. water level, temperature, etc.) that occur in the winter months, which encourage the growth of fungi. Likewise, it is also possible that a repressive element exists in the summer months, helping to keep the occurrence of disease low.

Figure 8 indicates a clear cycle of disease after seasonal effects are removed. The cycle repeats yearly, but does not reach the "outbreak" level in 1996 (i.e. does not pass the "100%" mark). The peaks indicate that outbreaks occurred in six out of the seven high cycles in the study. Even in years that never exceeded the outbreak classification value of 100, the highest points in the cycles were seen in the same months as in years that clearly exhibited outbreaks.

CHAPTER 4

HANDLING AND SAMPLING PROTOCOL FOR HERPETOLOGICAL RESEARCH

Introduction and Background

Infectious diseases affecting reptiles are caused by many different pathogens. Bacterial, viral, fungal, and parasitic infections have been documented in both wild and captive reptiles (McLaughlin et al., 2000; Homer et al., 1998; Lackovich et al., 1999; Lamirande et al., 1999; Graczyk and Cranfield, 2000; Jacobson et al., 2000; Mader, 1996). All genera of reptiles are susceptible to infection by pathogenic organisms. Recently, several diseases have surfaced as causes of illness and mortality in wild reptiles. Examples include fibropapillomatosis of marine turtles (Jacobson et al., 1989), mycoplasma of tortoises (Brown et al., 1999), and, as reported in this thesis, fungal infections of pigmy rattlesnakes. Outbreaks of disease have been studied in many populations of captive reptiles, but few studies have been conducted with wild populations (Jacobson et al., 2000). This is not to say that wild populations are less susceptible to disease than captive animals. One reason for a relative lack of information on diseases of wild populations of reptiles (compared to captive reptiles) is the simple fact that they are monitored for disease much more infrequently than captive reptiles. Most free-ranging populations of wild reptiles are not regularly studied by herpetologists. In populations where studies are conducted, it is possible not to encounter affected animals, not to recognize disease, or simply not to document cases. In captive

populations, however, caretakers and pet owners have regular and repeated opportunities to inspect individual reptiles for signs of illness or disease and seek veterinary treatment. This difference may account for the higher case report rate for captive reptiles than wild reptiles. Also, when a reptile dies in the wild it is seldom found. The reason that disease is reported more often for wild chelonians than other groups of reptiles is because the shell of a dead chelonian will not decompose and will persist for a long period after mortality.

In captive reptiles, fungal and bacterial infections of the skin and integument often result from predisposing factors like unsanitary living space or improper regulation of environmental conditions (i.e. too wet, too cold, etc.). One recent study of the causes of death of captive mammals, birds, and reptiles found that improper husbandry, such as a poor diet, improper environmental conditions, etc., was responsible for a higher percentage of animal deaths than infectious disease alone (Ferreira et al., 1999). In addition, the condition of the reptile's immune system, like any other animal, is important in the development of disease. A strong response can help keep the infection subclinical. Conversely, a repressed immune system, whatever the cause, can predispose a reptile (or any other animal) to infections that are less likely to develop in the presence of a normal immune response. In the relatively close quarters of captivity, healthy reptiles may have a higher rate of exposure to diseased individuals due to increased animal density. This high rate of contact increases a reptile's chances of encountering and contracting whatever pathogens may be in the collection. This type of horizontal transmission has been documented in populations of captive animals, such as farm-reared broiler chicks (Shanker et al., 1990) and sheep (Li et al., 2000).

Wild reptiles are made more or less vulnerable to infection by the same factors that affect captive reptiles. Free-ranging reptiles, however, have a greater ability than captives do to change their surroundings. Their chances of developing disease are increased by the same variables, but wild reptiles can relocate to conditions that are more satisfactory with greater ease. In addition, healthy free-ranging reptiles may have less exposure to diseased individuals, reducing the spread of disease. This is also highly density dependent and populations that are steadily increasing in density may be increasingly at risk. This could help explain the fact that large-scale outbreaks of disease are rarely documented in free-ranging populations. The more obvious side affect of disease in a population is a reduction of population size.

When herpetologists study populations of free-ranging reptiles, the possibility exists of that whatever pathogens may be in the population can be transferred from animal to animal via contaminated equipment. Thus, there is a chance of unwittingly facilitating the spread of an infectious agent. Pathogens can be spread through a population simply by touching an uninfected reptile after handling a diseased animal with hands or capture and restraint tools. Unfortunately, it is often difficult to determine the status of a free-ranging reptile before it is captured. This fact necessitates the development of a standardized set of common, safe, and widely accepted handling and sampling protocols so that researchers can avoid spreading potentially pathogenic organisms from infected to healthy reptiles.

Preventing the spread of diseases between animals is the goal of preventative medicine programs in both veterinary medicine and the human medical profession. Practices designed to reduce the chances of spreading infections between patients have

become compulsory in hospitals around the world. Sets of guidelines designed to encourage practitioners to thoroughly cleanse equipment and avoid spreading diseases have been widely distributed. The recommended practices include hand washing, wearing latex gloves, avoiding mixing of healthy and ill patients, proper methods of disinfection, and sterilization of appropriate equipment (Anonymous, 2000).

Proper maintenance of instruments used to examine or manipulate reptile patients is also important. Much of the equipment used in modern human and veterinary hospitals is disposable. This equipment, such as syringes, are used once and then disposed of. When instruments designed for multiple uses are used for invasive procedures, they are routinely disinfected or sterilized to prevent the spread of infectious agents. Chemicals used frequently to disinfect equipment between uses include glutaraldehyde, hydrogen peroxide, peracetic acid, sodium hypochlorite, alcohol, iodophors, phenolics, and quaternary ammonium compounds (Rutala and Weber, 1999). However, gas or heat sterilization are the methods of choice.

In the spring and summer of 1999, a survey was conducted through an internet site at the University of Florida College of Veterinary Medicine to determine the handling and sampling protocols of herpetologists from around the United States and the world (Appendix A). Over 40 invitations to complete the survey were distributed via Email and 14 herpetologists from the United States, Europe, and Australia responded. All 14 responders (100%) reported conducting invasive procedures on free-ranging animals as part of their research, either in the field or in the lab. Of these 14, only six (42%) reported having received some kind of formal training on surgical and biomedical sampling procedures. Seven responders (50%) stated that they clean and sterilize their

non-surgical equipment (tongs, restraint tubes, etc.) at least periodically, if not between

animals. Complete survey results are presented in Table 5.

Table 5 - Summary of survey responses

Question	Responses
Invasive procedures used	14/14 (100%)
Assess gender with a probe	13/14 (93%)
Implant radio transmitters	9/14 (64%)
Insert ID microchips (PIT-tags)	9/14 (64%)
Sterile/aseptic techniques training	6/14 (43%)
Use anesthesia for surgery	10/14 (71%)
Sterilize non-surgical tools periodically	9/14 (64%)
Sterilize non-surg. tools after each use	0/14 (0%)
Sterilize surgical tools periodically	14/14 (100%)
Sterilize surgical tools after each use	6/14 (43%)
Observed health problems in study pop.	8/14 (57%)

Protocol for Safe Handling and Sampling of Reptiles

Basic Protocol

For mark-recapture studies and surveys, it is important to try to avoid contributing

the spread of infectious agents through the study population. Following a basic protocol

for safe handling and sampling can help reduce the chances of spreading pathogens

between study animals. First, it is important to regularly clean and disinfect capture and

restraint equipment to the highest possible level. Tongs can be washed with a sanitizing

solution like Nolvasan or a diluted bleach solution. Since snake bags and containers are

frequently soiled by specimens, they should be washed in a bleach solution after each

time they are used to hold an animal.

Small instruments, like gender (sexing) probes, can be autoclaved between uses or

trips to the study site. Gender probes are an area of special concern since they are tools

used for an invasive procedure. Carrying several autoclaved probes can allow the gender

of several snakes to be identified, should the need arise, so that no instruments are reused.

Minimally, tools should be cleansed with a glutaraldehyde, bleach, or a Nolvasan

solution and rinsed with sterile water between animals. Pathogen transmission via body

fluids has been documented for several pathogens including the transmission of bacteria

and viruses through human urine, though no references to vertical transmission in reptiles

via cloacal contact could be found in searches of the primary literature (Knutsson and

Kidd-Ljunggren, 2000). There are reports in the literature of horizontal cloaca-to-cloaca

transfer of sexually transmitted diseases in avians (Westneat and Rambo, 2000). Since

this is also a possible risk for reptiles, gender-probing bears further investigation to determine the risk of transmitting pathogens between animals.

Second, it is important to handle reptiles as gently as possible during capture and data recording to avoid abrading the skin or scales. It has been shown conclusively that skin abrasion can predispose reptiles to fungal or bacterial infections (Lillywhite, 1996). Because of this, every effort should be made to maintain the integrity of the skin. Complete instructions on handling many types of reptiles in a way that is safe for the animal and the herpetologist can be found in several books (Jenkins, 1996; Cunningham and Gili, 1994; Barnard, 1996).

Thirdly, try to handle individuals that have obvious signs of disease with different tools than healthy animals. These animals frequently exhibit brown or crusted scales or scutes, open sores, infections of the structures of the head, or subdermal masses. The best way to accomplish this is to carry two sets of capture and restraint tools, especially those integral to restraining the infected animal (i.e. tongs). Alternately, a disinfectant solution may be used in the field to cleanse tools that must be reused for the capture and restraint of healthy reptiles. If hand capture is used, carrying several sets of disposable latex-type gloves for use with obviously infected individuals is recommended. It has long been accepted that washing hands with soap and water helps reduce the likelihood of spreading pathogens between animals. Hand washing has also been shown to be protective against the transmission of *Salmonella enteritidis*, a common cause of salmonellosis, from reptiles to humans (Friedman et al., 1998). Minimally, three minutes of hand washing is necessary for proper cleansing.

When reptiles with signs of disease are encountered, a report sheet specially designed for that species should be completed. This report sheet, often referred to as a health sheet, can be used to document the location, size, color, shape, and other physical characteristics of any lesions that may be found on the specimen. The sheet may also be used to record information such as location, behavior, breeding status, gender, mass, length, and any distinguishing marks that the subject may have. An example health sheet for use in documenting diseases in reptile populations can be found in Appendix B.

Surgical Protocol

Many herpetologists who are now performing surgical procedures are doing so under the direct supervision of a veterinarian. This is advisable, whenever possible, to ensure the health and safety of the animals. The guidelines provided here are a basic outline of the principles needed to reduce the risk of disease transmission between reptiles and the risk of postoperative infection.

Surgeries that are conducted in the field setting should be performed with the utmost attention to aseptic techniques in order to avoid spreading pathogens or encouraging post-operative infection. First, cleansing the skin and scales with an antimicrobial solution prior to starting surgery will help reduce the number of organisms on the surface of the skin and can help prevent postoperative infections. Commonly used solutions for surgery on reptile patients include povidone-iodine and chlorhexidine (Bennett and Mader, 1996).

Second, it is important to use an anesthetic when any type of surgery is conducted on a reptile. The anesthetic will reduce the amount of pain that the subject feels and, therefore, help keep the subject from writhing around during the procedure. In the field,

for minimally invasive surgeries, a local anesthetic is usually preferred because of the ease with which they can be administered and the reduced necessity for post-operative recovery time and observation. This method is especially useful when biopsying skin or subcutaneous masses. A lidocaine ring block can be performed by injecting an appropriate quantity a 2% solution in a circle (ring-block) around the biopsy or incision site. When surgeries are conducted in a laboratory or operating room, general anesthetics may be used, per a veterinarian's advice. Common injectable anesthetics used for reptiles include sodium pentobarbital, methohexital, ketamine, and telazol. Isoflurane is the preferred gas anesthetic for reptiles (Bennett, 1996). Consultation of a contemporary text on reptile anesthesia is recommended to help determine which protocols should be employed.

Thirdly, it is imperative that sterile surgical tools be used for each subject as previously discussed. For transmitter implants or biological sampling, sterile scalpels should be used for only one surgery and then replaced. For PIT-tag implants, a sterile insertion needle should be used for each individual. This level of sterility can be achieved by carrying several sterile surgical packs into the field when transmitter implants or biological sampling are anticipated.

All surgical wounds, especially when large, should be closed using either surgical glue or sterile suture material and the animal treated with an appropriate antimicrobial agent, usually an iodine solution (e.g. Betadine). Commonly used suture materials include nylon or polypropylene sutures. Skin staples designed for human surgery can also be used to close wounds in reptiles (Bennett and Mader, 1996). This is a concern for animals that will be released immediately after surgery, however, since most suture

materials require removal. For field surgeries, therefore, a skin glue product specifically

designed for postoperative wound closure may be more appropriate.

If animals are kept for observation after surgery or are part of a collection, closely

monitor the wound for signs of infection after the surgery is complete. If the subjects are

released after surgery, pay close attention to the surgical wound at the time of any

subsequent recaptures and beware of possible infections. If any infections are noticed,

contact a veterinarian immediately for instructions on treatment.

Conclusion

By applying the techniques described in this paper, researchers can reduce the

chances of causing disease outbreaks by spreading pathogens between individuals and

may lower postoperative infection rates. These simple steps can help insure the health of

reptile populations. The guidelines stated here are basic approaches to handling and

sampling reptiles in the field and in the lab. These simple rules can be applied to many

research protocols to help protect the health of wild and captive reptile populations.

Using the safest techniques possible will help reduce the risk of transmitting potentially

pathogenic organisms between animals, thereby helping keep wild and captive study

populations healthy.

APPENDIX A
HANDLING AND SAMPLING PROTOCOL SURVEY

Thank you very much for taking time out of your busy schedule to help us. This questionnaire was developed to document the handling and sampling methods of snake researchers worldwide. Within the last year, individuals in a Florida population of pigmy rattlesnakes, *Sistrurus miliarius barbouri*, developed <u>severe lesions</u> of the orbital, facial, and oral regions of the head. Several species of fungi have been isolated from these lesions and may be the causative agent(s).

The investigation into the cause of these lesions leads us to the question: "Is there some way that human interaction with this population could have induced the appearance of the lesions?" To answer this question, we must compare what is known of the handling and sampling protocol used in the study of the Florida population to that of studies in populations across the world. Please take the time now to complete the questionnaire below. Your input is **invaluable** to us. If you have any questions or comments about the nature, structure, or intent of this questionnaire, please feel free to send those concerns via email to <u>cheatwoo@ufl.edu</u>. Thank you.

Please enter your name:

Please enter your email address:

1) Which snakes (genus and species) do you study?

2) How long have you been studying each of the above mentioned snakes?

3) Have you published one or more papers about the handling and sampling methods you use?

Yes◯ **No**◯

(If "yes", please include pertinent references below.)

4) What type(s) of data do you collect when studying your snakes?

Dimensions ☐	Mass ☐	Gender ☐	Geographic location ☐
Parasites ☐	Prey ☐	Fecundity ☐	Disease ☐
Air temperature ☐	Ground temperature ☐	Body temperature ☐	Feeding status ☐
Shed status ☐	Skin abnormalities ☐	Other ☐	

(If "other", please elaborate below.)

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5) Do any of your methods require perforation of the skin or other invasive procedures?

Yes○ **No**○

(If "yes", please specify which data you are collecting invasively.)

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6) Do you use radio telemetry or inject a microchip into any of your research animals?

Yes transmittor○ **No transmittor** ○

Yes microchip○ **No microchip** ○

7) Have you had any formal training in aseptic surgical techniques and biomedical sampling?

Yes○ **No**○

8) Do you use anesthesia in any of your procedures?

Yes○ No○

(If "yes", please specify which anesthetics you use.)

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9) Are your snakes considered venomous?

Yes○ No○

10) What techniques do you use for capturing and restraining snakes if samples and data are to be collected?

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│                                 │
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11) How often do you sterilize your data collection tools and precautionary tools?

A) After each use ○
B) After several uses ○
C) Weekly (without regard to number of uses) ○
D) Monthly (without regard to number of uses) ○
E) Irregularly ◎
F) Rarely ○
G) Never ○

12) How often do you sterilize any surgical tools you are using?
(Including materials used for injections of any kind)
A) After each use ○
B) After several uses ○
C) Weekly (without regard to number of uses) ○
D) Monthly (without regard to number of uses) ○
E) Irregularly ○
F) Rarely ○
G) Never ○

13) Have you noticed any health problems in any of your research snakes?

Yes ○ No ○
(If "yes", please specify which health problems you have noticed.)

14) Have you conducted any studies specifically designed to test the safety of your data collection methods or surgical procedures?

Yes ○ No ○

15) Do you have any questions, comments, or additional information about your research that you would like to share with us?

Please click on the button below when you have completed answering all of the questions.

[Submit Answers]

APPENDIX B
SAMPLE DATA SHEET

Snake Observation Data Sheet

Field Worker:_____

Snake Species:_____

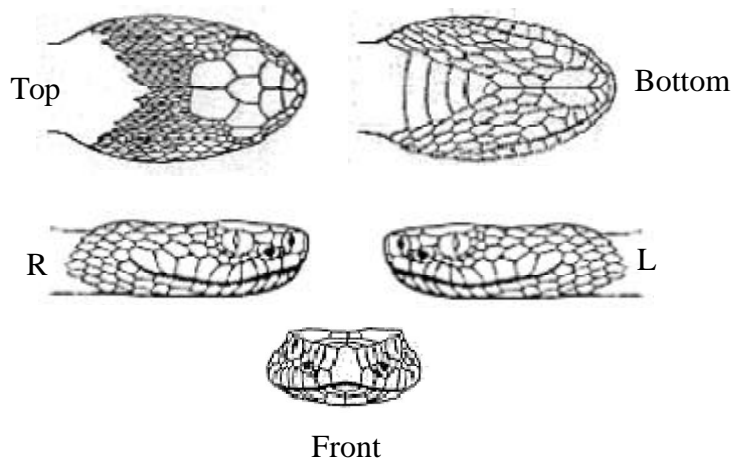Snake ID:_____

Sex: (circle one)   M   F

Location:_____

Date:_____

Mass (g):_____

Length (cm):_____

Posture:_____

Ambient temp. (C):_____

Photographs Taken?:   Y   N

HEAD/NARES

Nasal exudate: Y N

Nares crusted: Y N

Lesion/lumps: Y N

Eyes cloudy: Y N

Eyes swollen: Y N

Description of lesions:

_____

_____

_____

Other Comments:_____

_____

_____

Draw any lesions on diagrams below:

Top

Bottom

R

L

Front

ORAL CAVITY

Observed: Y N

Discharge: Y N

Membranes pink: Y N

Membranes swollen: Y N

Open lesions: Y N

Parasites observed: Y N

BEHAVIOR

Alert & responsive: Y N

Normal tracking: Y N

Defensive behavior obs.: Y N

Coiled when found: Y N

Moving when found: Y N

Tongue flicking: Y N

INTEGUMENT

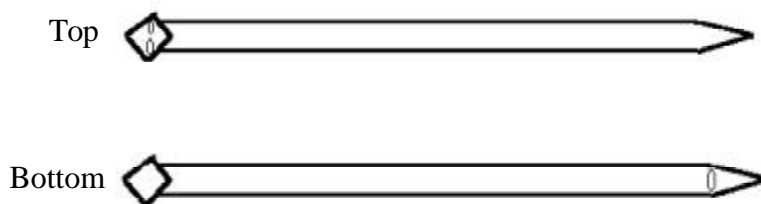Normal elasticity: Y N

Near/in ecdysis: Y N

Abnormal shed: Y N

BODY

Lesions/lumps: Y N

Normal muscle tone: Y N

Normal vertebral column: Y N

Description of lumps/lesions:

_____

_____

_____

Comments:_____

_____

Draw any lesions on diagrams below:

Top

Bottom

SAMPLES COLLECTED

Blood: Y N   Swabs: Y N

Tissue: Y N   Feces: Y N

OTHER COMMENTS

_____

_____

_____

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