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Cryptosporidium serpentis Surveillance in Free-ranging Snakes to Inform a Reintroduction Strategy for the Eastern Indigo Snake (*Drymarchon couperi*)

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ABSTRACT: Understanding risk factors associated with reintroductions is important for making informed decisions within an adaptive framework. Biosecurity measures minimizing the risk of the introduction or spread of transmissible diseases are a priority when considering the release of captive-reared wildlife. Eastern indigo snake (EIS; *Drymarchon couperi*) reintroductions have been occurring in Alabama since 2010 and in Florida since 2017. During this effort the pathogen *Cryptosporidium serpentis* was detected, affecting several of the captive breeding snakes. Infected snakes were quarantined and removed from breeding efforts, which reduced snakes available for the reintroduction projects. To make informed management decisions about future reintroduction strategies, 155 free-ranging snakes were sampled at the two release sites and a third site in Georgia to evaluate the natural occurrence of *C. serpentis*. Additionally, 72 free-ranging EIS and other species incidentally encountered throughout the EIS range were tested opportunistically. All snakes sampled at the three focal sites tested negative, but one opportunistically tested EIS from South Florida tested positive. These results indicate that *C. serpentis* is present in the environment in at least one location, but at low levels. Our results suggest that, pending additional surveillance, *C. serpentis*-positive snakes should not be included in reintroduction efforts, and that maintaining a high level of biosecurity is important in captive breeding programs.

Key words: *Cryptosporidium serpentis*, cryptosporidiosis, disease, *Drymarchon couperi*, serpentes.

Making informed and adaptive decisions that support species requires knowledge of species needs and risks associated with those decisions. The eastern indigo snake (EIS; *Drymarchon couperi*) is an endemic, federally (US) threatened species that historically occurred throughout southern Georgia, southern Alabama, southeastern Mississippi, and Florida (US Fish and Wildlife Service [USFWS] 2019a). Population strongholds remain in southeastern Georgia and peninsular Florida, but EIS are rare or presumed extirpated throughout the remainder of the range (Enge et al. 2013). These snakes are generalist apex predators that can consume nearly any appropriately sized prey animals, including other snakes (Stevenson et al. 2010; Steen et al. 2016).

Reintroduction of captive-reared juvenile EIS is a conservation strategy currently in use to assist with recovery of the species (USFWS 2019b). Long-term reintroduction efforts are underway in southern Alabama and the Florida Panhandle, where the species is believed to have been extirpated. Snakes released at these sites were raised from eggs of wild-caught EIS and reared at Auburn University (Auburn, Alabama) or were bred and reared at Central Florida Zoo & Botanical Gardens' Orianne Center for Indigo Conservation (OCIC; Eustis, Florida), the sole captive propagation facility for the EIS reintroduction program. In 2016, OCIC discovered the protozoan parasite *Cryptosporidium serpentis* in captive snakes at the facility and developed strict protocols to minimize the incidence of disease at OCIC. All animals are regularly screened; those animals that test positive for *C. serpentis* are housed in a separate quarantine room, regu-

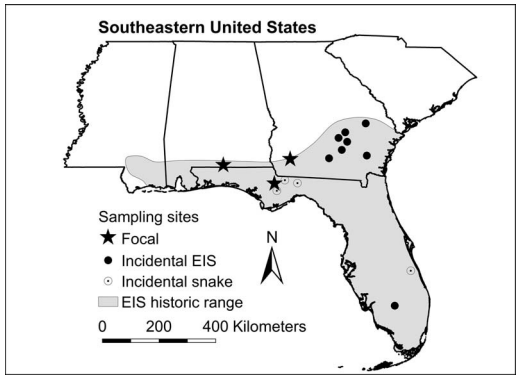

FIGURE 1. Historic eastern indigo snake (*Drymarchon couperi*) range in the southeastern US (Florida and the coastal plain region of Mississippi, Alabama, and Georgia, probably not reaching South Carolina in the east); three focal sites (from left to right: Conecuh National Forest [CNF], Apalachicola Bluffs and Ravines Preserve [ABRP], and The Jones Center at Ichauway [JC] in Alabama, Florida, and Georgia; and locations of incidental captures.

larly evaluated, and retested on a systematic schedule.

Cryptosporidium serpentis parasitizes the stomachs of its hosts; infected snakes may develop clinical signs or the infection may remain subclinical (Bogan 2019). Severe clinical infections are progressively debilitating and may lead to fatal gastric cryptosporidiosis (Bogan 2019). Clinically affected snakes may display lethargy, regurgitation, or anorexia (Cranfield and Graczyk 2006; Fayer et al. 2018). Spread of the parasite occurs through feco-oral transmission (Cranfield and Graczyk 2006) and it may affect both ophiophagous and nonophiophagous snake species (Cranfield et al. 1999). Subclinically infected snakes may nevertheless shed oocysts into the environment; gravid snakes may contaminate eggshells and offspring through cloacal contact (Cranfield et al. 1999). Infection has been best documented in captive snake populations but also occurs in wild populations (Matsubara Karasawa et al. 2002; Kuroki et al. 2008). Few studies have addressed the prevalence of *C. serpentis* in free-ranging snakes, although prevalence of up to 26% has been documented in Brazil and Japan (Matsubara Karasawa et al. 2002; Kuroki et al. 2008). A *Cryptosporidium* sp. has been detected in the gopher tortoise (*Gopherus polyphemus*; McGuire et al. 2013), whose burrows provide winter refuge to EIS; however, the particular species of *Cryptosporidium* was not determined.

Although *C. serpentis* is not known to be vertically transmitted (Bogan 2019), OCIC initiated strict disease surveillance testing at the facility to ensure that the juvenile EIS scheduled for release as part of the repatriation efforts repeatedly tested negative for *C. serpentis*. This testing is expensive and creates colony management challenges. It is difficult to determine what causes a subclinically infected snake to develop clinical signs, and challenging to determine prevalence among subclinically infected animals. It is possible that some wild snakes may be subclinically infected with *C. serpentis* and never develop signs of disease. We tested free-ranging snakes across the range of the EIS with a focus on current reintroduction sites for *C. serpentis*.

Snake species sampled for the presence of *C. serpentis* included EIS, other ophiophagous snakes, and prey species. Free-ranging snakes were collected and sampled from the two EIS reintroduction sites: in 2018 from the Conecuh National Forest (CNF; 33,994 ha) in Covington and Escambia counties, Alabama and in 2019 from The Nature Conservancy's Apalachicola Bluffs and Ravines Preserve (ABRP; 2,547 ha) in Liberty County, Florida. Sampled snakes were also collected at The Jones Center at Ichauway (JC; 11,700 ha) in Baker County, Georgia from 2008 through 2017 (Fig. 1). Although EIS were released at JC in 1994, they have not been detected on the site since 1998 (D. Speake, pers. comm.). Incidentally encountered EIS and conspecific snakes across Alabama, Georgia, and Florida were also sampled (Fig. 1).

At CNF and ABRP, snakes were captured opportunistically by hand or systematically with box traps (Burgdorf et al. 2005). Snakes from JC were collected dead on roads and frozen; they were later thawed, necropsied, and stomach tissue samples from necropsies were submitted for analysis. Snakes from

CNF were euthanized with an intracoelomic injection of phenytoin plus pentobarbitol (Beuthanasia_D Special, Merck Animal Health, Madison, New Jersey, USA) and sampled by cloacal swab and stomach sample. Stomach samples were obtained after incising the coelom of the dead snake, exteriorizing the stomach, incising the stomach, and swabbing the entire gastric mucosa with a nylon-fiber swab (FLOQSwab, COPAN Diagnostics, Murrieta, California, USA). Snakes captured from ABRP were sampled by cloacal swab and released. Incidentally encountered EIS and conspecific snakes from Georgia and Florida were sampled by a combination of cloacal swab, stomach swab, and stomach biopsy. All collected samples were submitted for *C. serpentis*-specific probe hybridization quantitative PCR analysis to the University of Florida's Zoological Medicine and Wildlife Disease Laboratory. Analyses were performed following Bogan et al. (2021).

This research was performed under Alabama Department of Conservation and Natural Resources permit 2018116543268680, Auburn University Institutional Animal Care and Use Committee 2015-2658 & 2017-3102, authorizations from Florida Administrative Code Chapter 68A-9.002(2), Florida Fish and Wildlife Conservation Commission permit LSSC-19-00027A, Georgia Department of Natural Resources Scientific Collection Permit 1000545407, and USFWS permits TE26554C-1 & TE32397A-2.

At the three focal sites, 155 snakes representing 18 species were sampled for *C. serpentis*, including 56 snakes from ABRP, 51 from CNF, and 48 from JC (Table 1); all these wild-caught snakes tested negative for *C. serpentis*. Additionally, 64 EIS were captured opportunistically, including one from Hendry County, Florida that tested positive for *C. serpentis*. With a single snake testing positive of 227 snakes sampled, our results suggest that *C. serpentis* is potentially locally present in the Southeast, but occurrence rates are low.

Because *C. serpentis* may be difficult to detect in wild snakes, a negative test does not mean that *C. serpentis* is absent from the wild population. Additionally, since *C. serpentis* is intermittently shed (Bogan 2019), multiple serial cloacal swabs would be needed to thoroughly assess the prevalence of this disease in a wild snake population. Because of the cryptic nature of most snake species, this would be difficult to reliably obtain. The gastric sampling of snake cadavers from CNF and JC helped to alleviate this shortfall, but only 43.6% (99/227) of the samples were collected in this manner.

The single positive EIS found in South Florida indicates that *C. serpentis* does occur in wild snake populations. The initial indication is that *C. serpentis* is either absent or not present at levels that infrequent or intermittent sampling would likely detect among free-ranging snakes at the EIS repatriation release sites. Therefore, we do not recommend release of any *C. serpentis*-positive animals at repatriation sites and we support continued testing of snakes at OCIC and other facilities that may breed or rear snakes for repatriation purposes, to ensure that released animals are not disease carriers.

It is difficult to predict the actual prevalence of a disease with no positive detections, as observed at the focal sites. On the basis of a search of published and gray literature (e.g., PubMed, Web of Science, ScienceDirect, JSTOR), our survey of 227 snakes is the largest *C. serpentis* sampling effort in the US to date. To better understand the prevalence of *C. serpentis* among wild snakes, future surveillance efforts should target areas where positive results have been recorded, where robust snake surveys are performed, and at planned reintroduction sites. Salvaged carcasses of species known to carry *C. serpentis* should be tested whenever possible. Ophiophagous snakes, such as EIS, may be indicator species for *C. serpentis* in the wild. As clinically affected snakes show lethargy, they may be easier for ophiophagous species to capture and consume. Subclinically infected snakes may be difficult to detect through standard herpetological survey methods as oocysts may only be intermittently shed. If unknown *Cryptosporidium* spp. are encountered, for example in gopher tortoises, speci-

TABLE 1. Snakes sampled for *Cryptosporidium serpentis* at two eastern indigo snake (*Drymarchon couperi*) reintroduction sites (Apalachicola Bluffs and Ravines Preserve [ABRP] and Conecuh National Forest [CNF]) and The Jones Center at Ichauway (JC) in Florida, Alabama, and Georgia from 2008 to 2019, along with incidental captures throughout the range. Samples were collected by cloacal swab at ABRP, cloacal swab and stomach tissue at CNF, and by stomach tissue at JC.

Snake species	ABRP	CNF	JC	Incidental Alabama, Florida, and Georgia
Agkistrodon contortrix[ab]	8	8	2	0
Agkistrodon piscivorus[ab]	0	1	0	0
Coluber constrictor[ab]	7	18	6	0
Coluber flagellum[ab]	15	2	12	1
Crotalus adamanteus[b]	3	2	2	2
Crotalus horridus[b]	0	0	0	1
Drymarchon couperi[a]	0	0	0	64
Farancia abacura[b]	0	0	0	1
Heterodon platirhinos[ab]	2	0	0	0
Lampropeltis getula[ab]	0	0	5	0
Lampropeltis elapsoides[a]	1	0	2	0
Nerodia fasciata[b]	0	13	0	0
Pantherophis alleghaniensis[b]	5	0	0	0
Pantherophis guttatus[ab]	4	1	0	0
Pantherophis spiloides[b]	0	4	0	1
Pituophis melanoleucus	4	1	19	2
Sistrurus miliarius[ab]	6	0	0	0
Thamnophis sirtalis[b]	0	1	0	0
Virginia valeriae	1	0	0	0
Total	56	51	48	72

[a] Ophiophagous species (Gibbons 2017).

[b] Eastern indigo snake prey species (Stevenson et al. 2010; Steen et al. 2016).

ation through molecular diagnostics should be pursued to further understand their role in ophidian cryptosporidiosis.

Biosecurity measures in captive reptile and amphibian repatriation programs, such as intake quarantine and serial screening for infectious diseases, help prevent spread of disease within captive colonies and when releasing animals into the wild. Globally, reptiles and amphibians are affected by many diseases that may cause local to widespread mortality events. As reintroduction and translocation strategies increase in frequency for endangered species management, an adaptive framework to mitigate the spread of diseases is essential for the long-term well-being of reintroduction site populations.

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