Assessment and Evaluation of Prevalence of Fungal Dermatitis in New England Timber Rattlesnake (*Crotalus horridus*) Populations

Final Report

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Overview

This report provides an overall summary of the project as it was executed and the results of the sampling of 98 timber rattlesnakes (*Crotalus* horridus) performed during 2013 and 2014.

Procedures/Results

Orientation/Training

In the spring of 2013 we had a meeting at Roger Williams Park Zoo with biologists familiar with timber rattlesnakes in the New England states. During this meeting we discussed the goals of the health survey and identified 10 separate populations of rattlesnakes that the biologists felt were isolated from each other by distance or obstructions (either natural or man-made). At this meeting a biologist was identified to act as population coordinators for the purpose of this study. All information about each population was directed through the population coordinators and the population coordinators were responsible for organizing the survey and collection of rattlesnakes.

Based on conversations with the population coordinators it was decided that snake sampling would naturally divide itself into 4 "seasons" during the 2 year study. Population coordinators reported that snakes were easiest to find in the spring (as snakes moved out of known snake dens and dispersed) and the fall (as snakes returned to the same dens in preparation for winter). Biologists felt this would be the best time to try to collect snakes from their populations. Concerns were expressed by the population coordinators that attempts to find snakes outside of these times might result in a low capture rate.

Rather than set specific start and end times for each "season" it was decided to let the weather patterns determine the spring and fall seasons for each year. The start and end dates of each sampling season were determined by the weather and the ability of biologists to search for snakes randomly in their populations.

After confirming the population coordinators, two separate training sessions were instituted for all biologists who would be participating in snake sampling. These training sessions were held at Roger Williams Park Zoo and allowed the veterinary staff to review the protocols for sampling (included as Appendix A) and to give instruction on how to collect the biological samples to the biologists. Biologists were given the opportunity to collect blood and swabs from several captive timber rattlesnakes during these sessions. While all biologists were trained and given sampling materials the biologists were also given the option to bring any snakes they wanted to the zoo and the zoo staff would perform any sampling required for the study.

At the training sessions we provided each population with a field collection manual (Appendix A), a set of data collection forms (Appendix B) and the supplies necessary to collect the samples and ship them to the zoo.

Sampling

The following table shows the sampling seasons for 2013 and 2014:

Sampling Season	Dates of Sampling
Spring 2013	April 19 – May 28, 2013
Fall 2013	August 8 – October 14, 2013
Spring 2014	April 21 – July 29, 2014
Fall 2014	September 4 – October 17, 2014

We were able to sample snakes from nine of the ten populations. The one population which was not sampled was not sampled due to extremely small numbers of animals within that population and limited time that the biologists had to survey snakes in that population.

Biologists were sent out to collect random snakes from areas surrounding known den sites. Biologists were not to use radio-telemetry to find snakes. Instead, biologists were instructed to pick up each snake they encountered. If that snake had not been sampled yet, it was examined and sampled. It was hoped that this would provide a random sampling of data with minimal field labor.

No snake was sampled for this study more than once. Snakes which had been part of previous surveys or other studies on rattlesnakes were allowed to be used in this health survey as long as they were part of the "random" survey of rattlesnakes.

Once captured, the following procedures were performed:

- Each snake was given an ID which consisted of a three letter abbreviation for the population the snake was found in followed by the last four digits of the PIT tag.
- Biologists obtained GPS coordinates of where the snakes were captured.
- Gender was determined and an estimated age was recorded.
- Measurements were obtained (snout to vent length, vent to tail tip length, body weight).
- Snakes were checked for PIT tags. Snakes which did not have a PIT tag had one placed.
- Blood was drawn for hematology, serum biochemistry and heavy metal analysis.
- Two (2) cloacal swabs were obtained for paramyxovirus testing.
- The snake was examined for dermatitis lesions or other external abnormalities.
    - If the snake had dermatitis lesions it was transported to a veterinarian for biopsy of those lesions.

**It should be noted that biologists also obtained a small piece of scale for another study looking at the DNA of timber rattlesnakes to determine relatedness. While not specifically for our health survey biologists were encouraged to collect these samples and send them to us. We then provided these samples to the investigator.*

All snake data was recorded on standardized data sheets (Appendix B) except for GPS location information which was kept by the population coordinator.

Over the course of this survey we sampled a total of 98 animals from the nine separate populations:

Number of animals sampled by sampling period and population

Population	Spring 2013	Fall 2013	Spring 2014	Fall 2014	Total
1	1	2	6	6	**15**
2	5	0	1	1	**7**
3	6	4	2	2	**14**
4	6	10	4	0	**20**
5	0	6	3	4	**13**
6	4	0	1	4	**9**
7	9	0	0	0	**9**
8	3	0	0	0	**3**
9	2	2	3	1	**8**
Total	**36**	**24**	**20**	**18**	**98**

Any snake which had abnormal scales that showed evidence of active disease (previous scars were ignored) were considered positive. Visual "maps" of the lesions were drawn on the data sheet. Using this information we were able to determine the prevalence rates for each population:

Prevalence of dermatitis lesions by population

Population	Number of snakes sampled (n)	Prevalence
1	15	53%
2	7	43%
3	14	17%
4	20	60%
5	13	15%
6	9	33%
7	9	0%
8	3	0%
9	8	25%
Total	**98**	**32.6%**

We can see from these numbers that there is a wide variation in prevalence of dermatitis between each population.

If we look at the prevalence rates between snakes sampled in the spring (combined 2013 and 2014) and the fall (combined 2013 and 2014) a striking difference in prevalence rates is seen:

Prevalence rates for each population by season of sampling

Population	Spring Prevalence	Fall Prevalence
1	71%	38%
2	50%	0%
3	13%	17%
4	100%	20%
5	33%	10%
6	60%	0%
7	*	*
8	*	*
9	40%	0%
Total	**53%**	**17%**

**Prevalence rates could not be calculated for comparison because snakes were only sampled during the spring 2013 for these populations.*

The overall prevalence rate went down in 86% of the populations and only increased slightly in the one remaining population. The belief of many of the biologists is that some animals are able to clear themselves of the infection during the summer months through shedding and increase basking. While individual snakes were not followed as part of the health survey this data suggests either snakes are able to clear themselves of visible lesions or snakes with lesions are less able to survive during the summer months and are removed from the population either through predation or scavenging.

We also looked at the prevalence seen between 2013 and 2014 (spring and fall combined for each year):

Prevalence rates for each population by calendar year

Population	2013 Prevalence	2014 Prevalence
BLH	100%	42%
BRE	40%	50%
BRW	10%	25%
CCT	50%	100%
CTW	17%	14%
EMT	50%	20%
GVT	*	*
NH	*	*
TEK	50%	0%
Total	**37%**	**34%**

**Prevalence rates could not be calculated for comparison because snakes were only sampled during the spring 2013 for these populations.*

No obvious trends are visible. Nearly as many populations (n=3) saw a drop in prevalence rates compared with those (n=4) who saw an increase in prevalence from 2013 and 2014.

Blood Work

Blood from 80 snakes (82%) were available for hematologic sampling. We collected data on 7 different hematological parameters to try to determine if any hematology tests could help predict which snakes would be positive for this disease. This could also have given us insight on how the animals' immune system was reacting to the dermatitis and/or to expand our understanding of how this disease is affecting the snakes (other than causing a dermatitis). The results are summarized below:

Hematology Parameter		n	Mean ± SD	Min	Max
White Blood Cell Count	Without Lesions	56	8,539 ± 4,470	2,500	20,500
	With Lesions	24	8,583 ± 4,189	2,400	18,900
HCT	Without Lesions	56	27% ± 7%	0%	42%
	With Lesions	24	27% ± 5%	19%	36%
Heterophils	Without Lesions	56	1,474 ± 1,765	0	8,195
	With Lesions	24	1,820 ± 1,681	83	7,750
Lymphocytes	Without Lesions	56	3,382 ± 2,827	320	13,260
	With Lesions	24	2,956 ± 3,140	318	13,419
Azurophils	Without Lesions	56	3,372 ± 2,173	318	11,890
	With Lesions	24	3,513 ± 1,753	1,032	8,580
Eosinophils	Without Lesions	56	194 ± 270	0	1,674
	With Lesions	24	218 ± 243	0	913
Basophils	Without Lesions	56	116 ± 105	0	497
	With Lesions	24	76 ± 62	0	189

Blood was available on 89 of the snakes (91%) for biochemical analysis. We looked at 13 different parameters in the biochemical profile of each snake. These parameters could provide insight into how this disease might be affecting organs other than the skin. The results are summarized below:

Biochemical Parameter		n	Mean ± SD	Min	Max
Albumin	Without Lesions	61	1.7 ± 0.2	1.1	2.1
	With Lesions	28	1.6 ± 0.2	1.2	2.0
ALP	Without Lesions	61	80 ± 41	24	260
	With Lesions	28	72 ± 35	28	197
ALT	Without Lesions	61	19 ± 7	0	51
	With Lesions	28	18 ± 7	10	42
Amylase	Without Lesions	61	901 ± 345	0	1,786
	With Lesions	28	749 ± 194	429	1,179
AST	Without Lesions	61	28 ± 22	1	126
	With Lesions	28	30 ± 22	0	83
Calcium	Without Lesions	61	14 ± 9	9	78
	With Lesions	28	18 ± 20	9	100
CPK	Without Lesions	61	559 ± 827	11	5,993
	With Lesions	28	549 ± 641	10	2,900
Globulins	Without Lesions	61	3.1 ± 0.4	2.4	3.9
	With Lesions	28	3.1 ± 0.5	2.3	4.5
Glucose	Without Lesions	61	69 ± 31	25	145
	With Lesions	28	72 ± 43	25	181
LDH	Without Lesions	61	739 ± 1,080	50	6,489
	With Lesions	28	532 ± 553	63	2,485
Phosphorus	Without Lesions	61	3.8 ± 1.7	1.3	10.5
	With Lesions	28	4.6 ± 3.8	1.7	21.4
Total Protein	Without Lesions	61	4.7 ± 0.6	3.7	6.0
	With Lesions	28	4.6 ± 0.7	3.5	6.3
Uric Acid	Without Lesions	61	3.2 ± 1.9	0.1	10.2
	With Lesions	28	3.6 ± 1.7	1.2	8.7
Calcium/ Phosphorus Ratio	Without Lesions	61	3.72 ± 1.32	1.43	8.23
	With Lesions	28	3.71 ± 0.98	2.48	7.02
A/G Ratio	Without Lesions	61	0.55 ± 0.06	0.38	0.67
	With Lesions	28	0.52 ± 0.05	0.40	0.67

Paramyxovirus

Cloacal swabs were collected from 84 of the 98 snakes (86%). All swabs were tested at the University of Florida using PCR primers specifically for ophidian paramyxovirus. All swabs were negative for paramyxovirus DNA.

Heavy Metals

Blood was available on 58 snakes (59%) that was suitable for testing for heavy metals. All samples were sent for testing to the Michigan State University Diagnostic Center for Population and Animal Health.

Assays for the following heavy metals were at or below detectable limits of the equipment used for analysis:

- Antimony
- Arsenic
- Beryllium
- Chromium
- Mercury
- Thallium
- Vanadium

The remaining results are summarized below:

Heavy Metal	Lowest Value	Highest Value
Cadmium	< 5 ppb	11 ppb
Lead	5 ppb	87 ppb
Nickel	27 ppb	83 ppb
Selenium	95 ng/ml	347 ng/ml

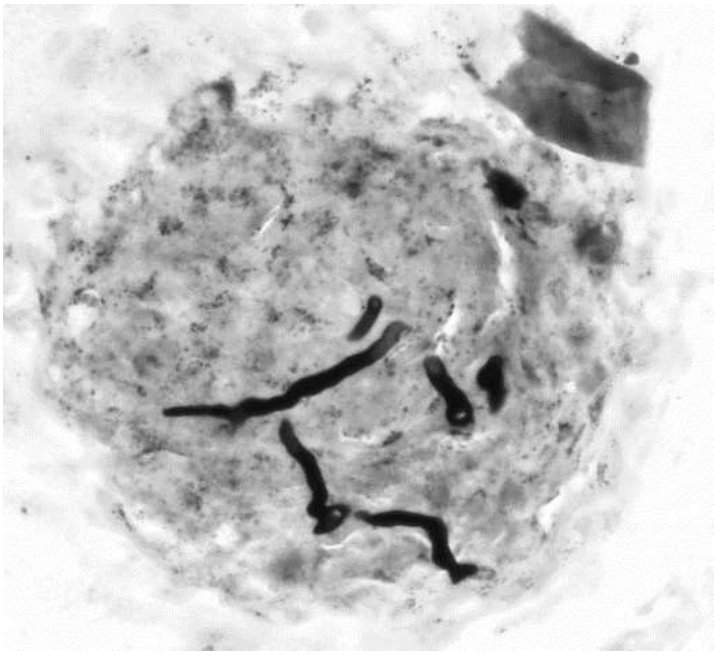
Based on these results heavy metal exposure does not seem to be a likely variable in dermatitis in timber rattlesnakes.

Histopathology

Thirty-two biopsies were sent to a single pathologist experienced in zoo and wildlife pathology. They were stained using routine hematoxylin and eosin stains. Additional cuts were also stained with Gomori–Grocott methenamine silver stain to highlight fungal hyphae. Reports were categorized on whether there was evidence of fungal disease without significant bacterial infection, fungal disease with evidence of bacterial infection or non-fungal disease causes.

Category	Result
Fungal disease, no bacterial disease	78%
Fungal and bacterial disease	19%
Non-fungal disease	3%

Fungal hyphae were easily detected in 31 (97%) of the biopsies of lesions. An example is shown below:



High magnification (400x) photograph of dermal granuloma stained with Gomori–Grocott methenamine silver (GMS) stain revealing fungal hyphae

One of the 32 samples (3%) was not typical of the histologic examination of all the other samples. This sample was primarily lymphohistiocytic inflammation. The original sections did not show any fungal hyphae thought additional sections were examined and some fungal hyphae were seen. This sample tested negative for fungal PCR. The significance of this lesion is currently unknown.

Each snake which was biopsied also had a single biopsy of skin without evidence of dermatitis submitted to the same pathologist. They were processed the same as the abnormal samples. All 32 of the skin samples without lesions were normal on histologic exam without evidence of fungal hyphae.

Fungal Identification

One biopsy was sent from each snake with lesions to the University of Florida. DNA was extracted and amplified from each biopsy. This DNA was sequenced and compared with known fungal gene sequences. The results are as follows:

Organism	Prevalence
Ophidiomyces ophiodiicola	N=24 (75%)
Candida palmiolephila	N=2 (6%)
Cladosporium spp	N=1 (3%)
Curvularia lunata	N=1 (3%)
No fungal PCR isolated (“Negative”)	N=4 (12.5%)

Ophidiomyces ophiodiicola has been implicated by other researchers as a possible cause of dermatitis in snakes. This data presents strong evidence that *O. ophiodiicola* is associated with dermatitis in timber rattlesnakes.

Conclusion

We believe we accomplished the objectives of this study. This data:

- Provides an initial prevalence rate for each of the nine populations. This can be used by biologists going forward to determine if the incidence of dermatitis is going up or down in the respective populations.
- Shows that the overall prevalence rate of infection with dermatitis in the nine populations of timber rattlesnakes is approximately 33%.
- Shows no evidence that this is an opportunistic infection by snakes that are immune suppressed.
- Show no evidence that paramyxovirus is currently a significant problem for wild timber rattlesnakes in the nine sampled populations
- Shows that in spite of a high incidence of dermatitis that the timber rattlesnakes sampled appear to be in overall good health. Overall sampled snakes were in good body condition with minimal pathological changes.
- Provides strong evidence that O. ophiodiicola has a strong association with dermatitis in timber rattlesnakes in the northeastern populations.
- Shows that the prevalence of dermatitis in these populations of snakes is much higher in the spring than in the summer.

Additional statistical analysis is in progress at this time. Once completed this data will be prepared for publication in the Journal of Zoo and Wildlife Medicine.

Appendix A

Timber Rattlesnake Health Assessment
Field sample collection manual 2013

Supplies:

- Cooler with ice packs
- Alcohol swabs
- Syringes – 1 cc and 3 cc
- Needles – 25 and 23 gauge 1”
- Sterile culturettes
- Whirl-pack bags
- Lithium heparin (green top) microtainers
- EDTA (purple top) microtainers
- Scissors
- Ziplock plastic bags
- Fine-point slide marker for labeling
- White tape for labeling
- Gauze
- Slides
- Slide holder
- Exam gloves
- Sharps container

In order to allow processing of blood at RWPZ in-house laboratory, blood can only be collected from Monday - Wednesday**, and any Wednesday samples must be shipped by the end of the day. If possible **ALL** samples should be shipped the same day as they are collected so that the blood can be analyzed the following day. Some FedEx locations are open until early evening hours. See the last section in the document for further shipping instructions.

Blood collection

1. With snake restrained in snake tube, draw blood from ventral tail vein in front of rattle and caudal to hemipenes and scent glands
    a. Wipe area with alcohol swab prior to blood collection
    b. A maximum of 0.8% of body weight can safely be obtained. Please refer to the chart below for volumes:

Snake Weight (grams)	Volume of blood (mls)
Less than 200	Do not draw blood sample
200 – 300	1.5
301 – 400	2.25
Greater than 400	3.0

2. Place blood immediately on slides and into tubes (**Tube order: One green top, then one purple top, then rest of blood into green tops**)
    a. Remove needle from syringe and deposit needle in sharps container
        i. Do not push blood back through the needle as this may rupture cells
    b. Place one drop of blood on two glass slides
        i. Smear to create thin blood layer
        ii. Air dry slides horizontally. Slides must be dry before being placed in the slide holder
        iii. At least 2 good quality slides are needed for the complete blood count
    c. Fill one green top tube to the top line of the tube where the diameter widens (above the 0.5 ml mark)

d. Fill one purple top tube to the 250 µL line (ONLY ONE PURPLE TUBE PER SNAKE) – Do not fill over the line marked below.

e. Fill green top tubes with the remainder of the blood to the top line of the tube where the diameter widens (above the 0.5 ml mark)

   i. Fill as many green top tubes as blood volume allows

f. Close the lids and gently invert tubes 4-5 times after filling to prevent clotting

g. Label all tubes and slides with the date, sampling location, your initials, and the ID # of the snake

   i. Label slides on the frosted white portion

   ii. The marker may smudge on the plastic tubes so label a tab of white tape and affix near bottom of tube

   iii. Place tubes into plastic bag.

   iv. Place slides into plastic slide holder. Place the slide holder in the bag and seal the bag

   v. Place the bag into cooler with an icepack

Cloacal swab

1. Open sterile culturette and gently swab cloaca
   a. Do not touch tip of culturette with fingers or other objects
2. Place swab into whirl-pack bag
   a. Cut swab handle with scissors to fit into bag
   b. Fold top of bag over to seal
   c. Label bag with the date, sampling location, your initials, and the ID # of the snake
3. Repeat above procedure with second culturette – please place each swab in a separate whirl-pak bag
   a. Store bagged swabs in cooler with blood

Transponder placement

1. Insert transponder chip subcutaneously along left lateral body just cranial to the vent
    a. Clean skin with alcohol swab before insertion
    b. Close skin defect with drop of skin glue
2. It is acceptable to use another location/protocol for insertion if preferred by biologist

Record keeping

1. Please complete all fields of the provided data sheet and make sure that this information matches what is labeled on the samples

Sample transport in field

1. Blood and swabs must be kept in cooler with ice pack
2. Samples should stay chilled but not frozen
3. After returning from field sampling place samples into a refrigerator if not being immediately processed for shipping

Sample storage and transfer to RWPZ

1. Store blood and swabs in refrigerator – **Samples can only be collected from Monday-Wednesday**
2. Ship to Roger Williams Park Zoo within 24 hours of collection. If possible try to ship **ALL** samples the same day as they are collected. A delay in sample submission will alter blood results. Some FedEx locations accept packages until the early evening hours.
3. Any samples collected on Wednesdays **must** be shipped the same day to allow time for processing at the RWPZ laboratory
4. Samples must be sent via FedEx overnight shipping
    a. Package with ice pack to keep cool
    b. Include a copy of the datasheet – Keep original for your records
    c. Clearly label box "Refrigerate on Arrival"
    d. Use provided shipping label with RWPZ account and address
    e. Call or email Bonnie Soule to let RWPZ laboratory staff know that sample is being shipped. Email is preferred.
        i. bsoule@rwpzoo.org
        ii. 401-785-3510 x 307

Appendix B

Timber Rattlesnake Health Survey Data Record

Location Data

Date Located: _____ /_____ /_____ Population Name: _____

Date Animal Collected: _____ /_____ /_____ Time: _____ Notes: _____

Snake Data

Snake ID #: _____ Gender: _____ PIT Tag: _____

Length (Snout to Vent): _____ cm Length (Vent to Tail Tip): _____ cm Weight: _____ grams

Estimated Age: _____ Other Identifiers: _____

Sample Collection Data

Date of Blood Draw: _____ /_____ /_____ Time: _____ Location: ☐ Tail ☐ Heart Volume _____ ml

Samples Collected By (Initials): _____ Number of Slides: _____ Number of Blood tubes: _____

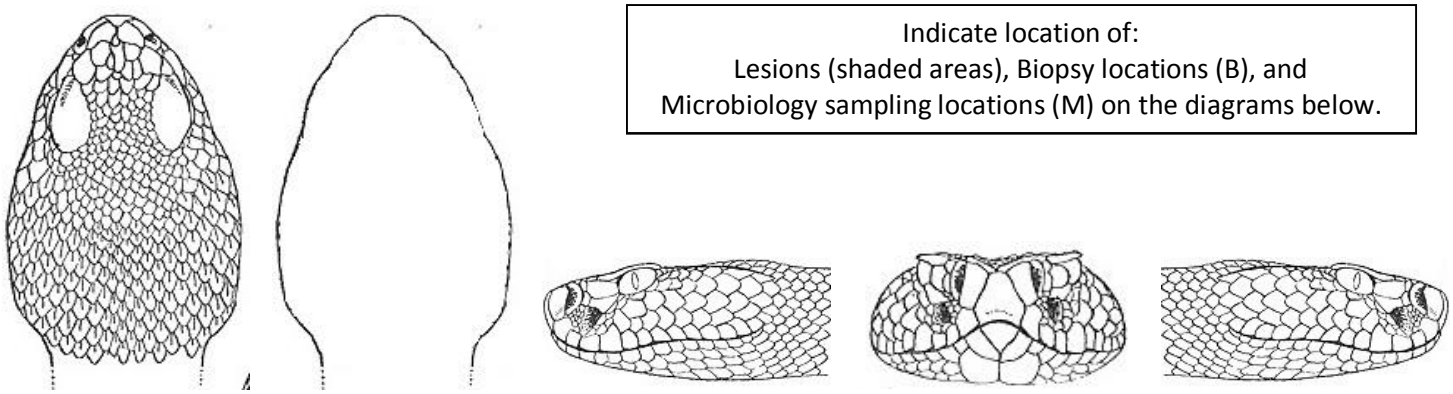
Cloacal Swabs Obtained: ☐ Yes ☐ No PIT Tag Placed: ☐ Yes ☐ No Scale Clip Obtained: ☐ Yes ☐ No

Dermatitis

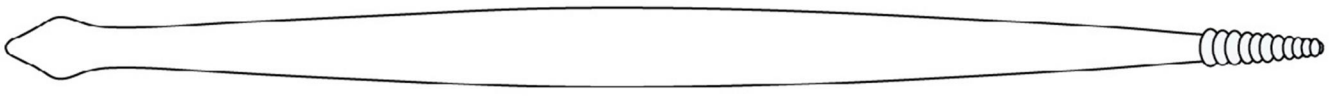
Does this animal have visible dermatitis lesions? □ Yes □ No

Distribution

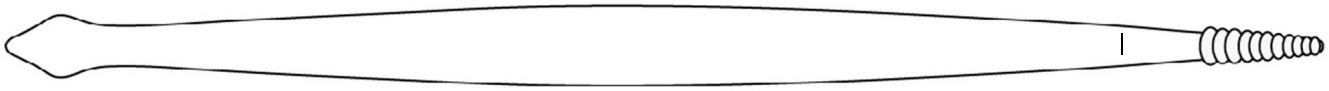
Indicate location of:
Lesions (shaded areas), Biopsy locations (B), and
Microbiology sampling locations (M) on the diagrams below.



Dorsal Ventral Left Front Right



Dorsal



Ventral

Sampling

Date of Biopsy: _____ /_____ /_____ Were microbiological samples collected: ☐ Yes ☐ No

Biopsy Collected By (Initials): _____

Please fax this page to (401) 680-8669 when completed and keep this copy in your records