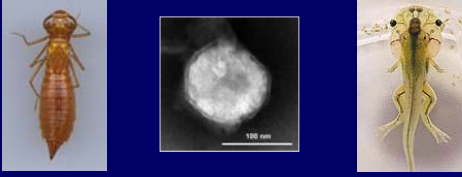
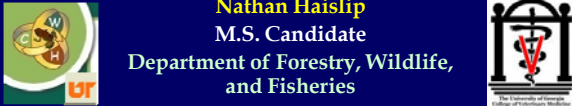


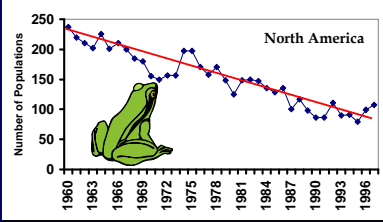
Possible Impacts of Predation Risk and Development on Susceptibility of North American Anurans to Ranaviruses



Nathan Haislip
M.S. Candidate
Department of Forestry, Wildlife,
and Fisheries



Amphibian Declines



32% of Anurans (frogs and toads) and 46% of Urodeles (salamanders) **Risk of Extinction**

Causes of Declines

- Habitat Loss and Degradation
- Pollution
- Global Climate Change
- Infectious Diseases



Emerging Pathogens

(1) *Batrachochytrium dendrobatidis* (chytrid fungus)

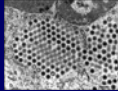


Post-metamorphic Stages



Occur Mostly in the Tropics at High Elevations

(2) *Ranaviruses*

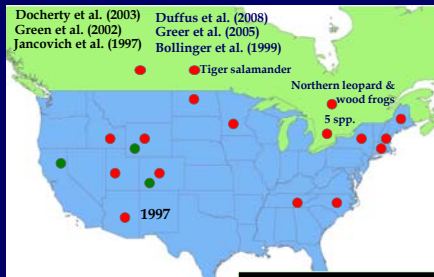


Affects Larvae



Occur at All Latitudes and Elevations!

Reported Amphibian Die-offs in North America



>20 States & 20 Spp = *Ranavirus*
3 States & 3 Spp = Chytrid

Ranaviruses Represent The Greatest Pathogen Threat to Loss of Amphibian Biodiversity in North America.

Why is *Ranavirus* Emerging?

Anthropogenic Stressors
Pesticides
Global Climate Change



Baseline Information

Ecological Stressors
Predation
Development



Predators as a Stressor


Prey alter - behavior, morphology, and development
Lima and Dill (1990)

Persistent physiological stress may negatively impact immune function
Griffin (1989)


Stressed tadpoles - **3X** greater infection by pathogenic trematodes
Belden and Kiesecker (2005)


No studies Conducted on Other Amphibian Pathogens!

Predator



Control





Predation


(1) *Anax* spp.


(2) *Belostoma flumineum*


Anax = Greater Susceptibility


Tadpoles tend to exhibit greater stress responses to *Anax* than *Belostoma*

Relyea (2001a), (2001b), (2003)









Objectives: Predation Study

1. Effect of **predation risk** on susceptibility of tadpoles to *Ranavirus* infection and disease
2. Determine **species-specific trends** in susceptibility to *Ranavirus* (4 species)
3. Effect of predator-cue **exposure duration** on susceptibility to *Ranavirus* (21 days)

Response Variables:

1. Growth
2. Activity Level
3. Percent with Gross Signs of Disease
4. Percent Infected
5. Mean Viral Load
6. Percent Survival

Anuran Development

Gosner 1960

Ranaviruses?

Development as a Stressor

Pre-metamorphic Immune Function

Egg
 Hatchling
 Larval
 Meta

Immune function
 Developmental stage
 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46

Chan and Asashima (2006)
 Hansen and Zapata (1998)
 Rollins-Smith (1998)
 Du Pasquier et al. (1989)
 Du Pasquier and Weiss (1973)
 Manning and Horton (1969)
 Fox (1963)

Susceptibility to Pathogens = Lowest at Larval Stage

Limited information on Ranaviruses

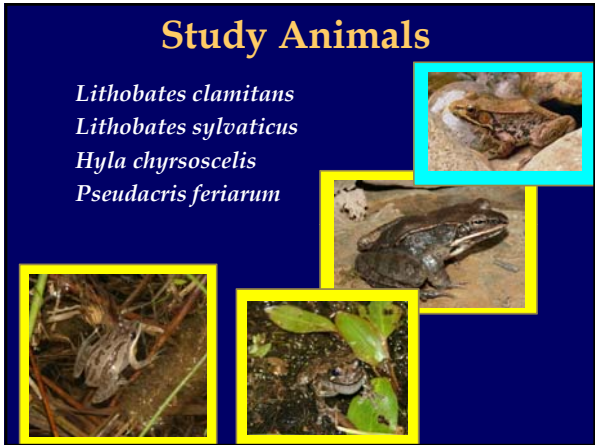
Objectives: Development Study

1. Effects of **developmental stage** on susceptibility to *Ranavirus* Stage 11, 21, 30, and 41
2. **Species-specific differences** in susceptibility to *Ranavirus*

Response Variables:

1. Growth
2. Percent Infected
3. Mean Viral Load
4. Percent Survival







JARTU Facility: Rearing

Outdoors in 300-L wading pools

Filled with aged tap water



Initial Sample




$n = 10$ individuals

- Mass
- Length
 - Snout-to-vent length (SVL)
 - Total length (TL)
- Developmental stage
 - Gosner (1960)



Calculate Average Growth

Frozen at -80°C

Confirm Control -- Negative



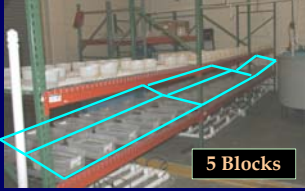
Methods: Predation Study



Experimental Design


Virus Treatments	Predator Treatments		
	Control (no predator)	Belostoma	Anax
Control (no virus)	5	5	5
10 ³ PFUs mL ⁻¹	5	5	5
	Total		30

Bollinger et al. (1999), Brunner et al. (2005), Pearman (2005)



5 Blocks

Randomized Complete Block





11.7-L tub


n = 11 tadpoles/tub

Predator Treatment

- Predators housed individually
- Predators contained in cages
 - 250 mL cups
 - Inverted with pocket of air
 - Opening closed with window screen








- 200-300 mg of tadpoles

Cue added at a standardized concentration (0.28 mg cue per L) every day

Virus Exposure

- Isolate: Frog virus 3 (GA Ranaculture Facility)
- 8 days after Predator-cue Exposure
 - Sufficient Time → Predator Stress Relyea 2003
- Exposure = 4 days
 - Shown to initiate infection Hoverman et al. unpublished data



- After 4 days, water will be changed and virus not be added again

Tadpole Euthanasia

- **3 Days** after Viral Exposure
1 random tadpole per tub
Baseline Viral Load Prior to Mortality
- Severe **Gross Signs** of Disease
- **21 Days** after Viral Exposure
Sufficient time for Mortality Hoverman et al. unpublished data
2 random tadpoles per tub tested
End-of-Experiment Viral Load

Benzocaine
Hydrochloride
(250 mg/L)

IACUC Protocol #1816

Methods: Development Study



Experimental Design

Virus	Developmental Stage			
	Egg	Hatchling	Larval	Pro-metamorphosis
Control	20	20	20	20
10 ³ PFUs mL ⁻¹	20	20	20	20



1-L tub
n = 1 tadpole/tub

2 Blocks




Randomized Complete Block

Virus Exposure & Euthanasia

- Exposure = 3 days
Shown to initiate infection
Hoverman et al. unpublished data
- Euthanized
Severe gross signs of disease
14 days post-exposure Hoverman et al. unpublished data
All individuals tested for *Ranavirus*



IACUC Protocol #1755

Combined Methods



Tadpole Feeding

- Tetramin - Daily ration of 8% of body weight
Relyea 2002
- $n = 10$ non-experimental tadpoles
Weighed - Average Weight
- Water Changes: 3 - 4 days
Maintain water quality



Observations

Tadpoles monitored 3 times daily for signs of disease (e.g., edema, hemorrhages)



Predation Study:

Tadpole Activity
Activity measured 10x per tub to estimate average activity



Post-Mortem Processing

Following Death or Euthanasia:

Measured: Mass and Length → Growth



Necropsied
Internal Signs
Liver & Kidney



Frozen at -80°C



Ranavirus Testing

gDNA Extraction

QIAamp DNA Mini Kit

DNA Quantification

Qubit™ fluorometer and the Quant-iT™ dsDNA BR Assay Kit

Viral Infection & Load Quantification

TaqMan qPCR assay



Predator Study Data Analysis

<p>Repeated Variables</p> <hr/> <p>Repeated-measures ANOVA</p> <p>Variables:</p> <ul style="list-style-type: none"> Percent Active Percent with Gross Signs Percent Infection Mean Viral Load <p>Effects:</p> <ul style="list-style-type: none"> Predator Treatment Virus Treatment Weeks of Exposure Species 	<p>End-of-Experiment Variables</p> <hr/> <p>3-way ANOVA</p> <p>Variables:</p> <ul style="list-style-type: none"> Mean Growth Percent Infected Mean Viral Load Percent Survival <p>Effects:</p> <ul style="list-style-type: none"> Predator Treatment Virus Treatment Species
--	--

Development Study Data Analysis

<p>Continuous Variables</p> <hr/> <p>3-way ANOVA</p> <p>Variables:</p> <ul style="list-style-type: none"> Mean Growth Mean Viral Load <p>Effects:</p> <ul style="list-style-type: none"> Developmental Stage Virus Treatment Species 	<p>Binary Variables</p> <hr/> <p>Logistic Analysis</p> <p>Variables:</p> <ul style="list-style-type: none"> Percent Infected Percent Survival <p>Effects:</p> <ul style="list-style-type: none"> Developmental Stage Virus Treatment Species
--	--

SAS® System at $\alpha = 0.05$

Both Studies Survival Analyses

Survival Curves

Differences tested among treatments ($\alpha = 0.05$) using Wilcoxon's test in PROC LIFETEST in SAS®.

Day of experiment	Control (%)	FV3 (%)	Georgia isolate (%)
0	100	100	100
1	100	100	100
2	100	100	100
3	100	100	100
4	100	100	100
5	100	100	100
6	100	100	100
7	100	100	100
8	100	100	100
9	100	100	100
10	100	100	100
11	100	100	100
12	100	100	100
13	100	100	100

Funding

Tennessee Wildlife Resources Agency
UGA Veterinary Diagnostic & Investigational
Laboratory (Tifton)
UT East Tennessee Research
and Education Center
UT Department of Forestry,
Wildlife & Fisheries



Acknowledgments

Committee Members

Dr. Matt Gray
Dr. Jason Hoverman
Dr. Debra Miller
Dr. Arnold Saxton

UT Staff & Students

Drs. John Hodges & Mark Campbell
Roger Long
Dr. Michael Keene
Wetlands Lab
Multiple Student Volunteers
Erica Young