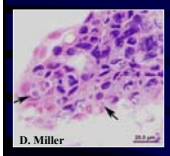
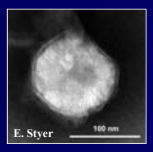
Emerging Infectious Diseases in Amphibians:
Chytrid Fungus and *Ranavirus*





Matthew J. Gray

Institute of Agriculture
Center for Wildlife Health

Outline

I. Amphibian Declines and EIDs

II. Chytridiomycosis

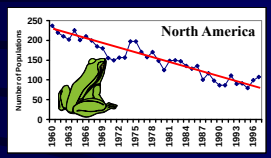
III. Ranaviral Disease

IV. Possible Conservation Strategies to Reduce *Ranavirus* Emergence

Amphibian Declines and Emerging Infectious Diseases

Science
306:1783-1786

EID 5:735-748

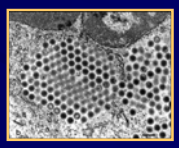


Nature
404:752-755

Biotropica
37:163-165

Chytrid Fungus

Adults: >95%
Larvae: 80-100% (Europe)



Ranaviruses

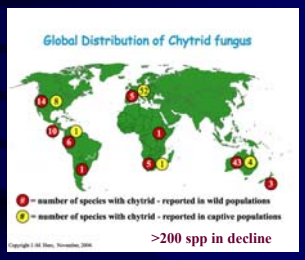
Chytrid Fungus

Batrachochytrium dendrobatidis

Western toad
Wyoming toad

Chiricahua leopard frog?

CA red-legged frog?



>200 spp in decline

- Mostly Tropical at High Elevations
- Western United States
- Some Species: Highly Pathogenic

Mountain yellow-legged frog
(*Rana muscosa*)

R. Brenes

Chytridiomycosis: An Emerging Infectious Disease of Amphibians

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The pathogen

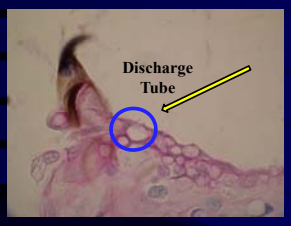
Phylum: Chytridiomycota
Class: Chytridiomycetes
Order: Chytridiales

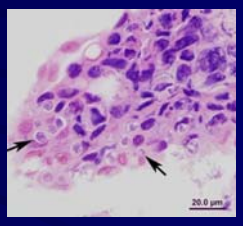
- *Batrachochytrium dendrobatidis* (*Bd*):
  - Non-hyphal parasitic fungus (only chytrid spp pathogenic to vertebrates)
- Infect keratinized tissue (stratum corneum and granulosum)
  - Larvae: Mouthparts
  - Adults: Pelvic Region
- Life stages
  - Zoospore – aquatic, flagellated (3-5µm)
  - Zoosporangium – zoospores discharged (300)

(4 days)

Histological Signs

Epidermis





Zoosporangia

Proliferation of Epidermal Cells

Stratum Corneum
Normal Thickness: 2 – 5 µm
Infected: 60 µm

Epidermal Hyperplasia
→ Sloughing

Cause of Mortality

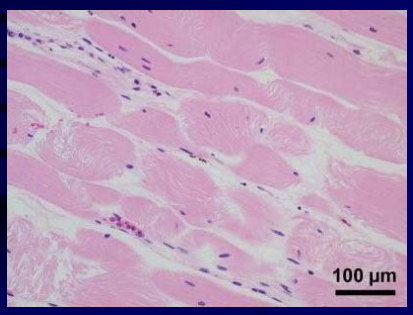
- Osmoregulatory Inhibition (suspected #1 cause)
  - Decreased water uptake & ion exchange; altered electrolyte/solute levels (decrease Ca → actin & myosin)
- Cutaneous Respiration
- Toxicosis

100 Zoospores

10 – 18 days

Histological Signs

Muscular Degeneration

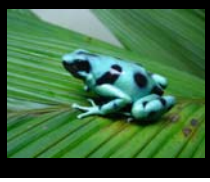


Field Signs of Bd Infection

- Infected individuals appear healthy
- Lethargic & paralysis
- Sloughing skin & lesions
- Loss of pigmentation in mouthparts of larvae

Origins

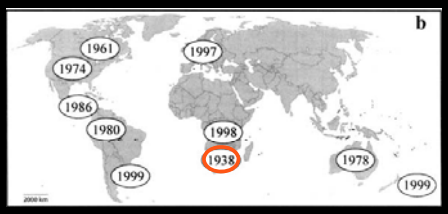
- Novel Pathogen Hypothesis
  - Out of Africa (Weldon 2004)
- Endemic Pathogen Hypothesis
  - Environmental changes (Pounds 2006)



Novel pathogen hypothesis

- Exotic, introduced pathogen
  - Low genetic variation globally in Bd
  - Recent global spread (Morehouse et al. 2003)
  - Broad range of host species
  - Few resistant individuals (tropics)
  - Lack of host immune response

Novel pathogen hypothesis

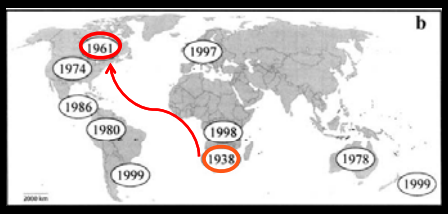


Rachowicz et al. (2005)

1. *Xenopus laevis ;* South Africa (1938)
2. *Xenopus gilli*; South Africa (1943)

(pregnancy test)

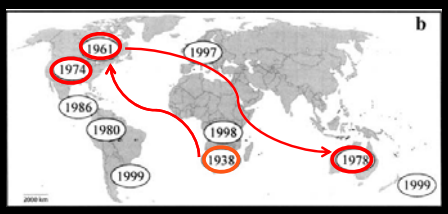
Novel pathogen hypothesis



Rachowicz et al. (2005)

1. *Xenopus laevis ;* South Africa (1938)
2. *Xenopus gilli*; South Africa (1943)
3. *Rana clamitans*, Canada (1961) 23 years later

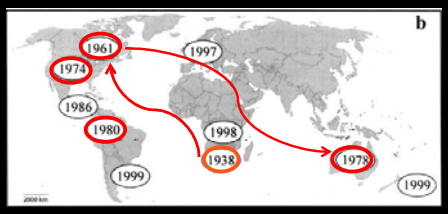
Novel pathogen hypothesis



Rachowicz et al. (2005)

1. *Xenopus laevis ;* South Africa (1938)
2. Xenopus gilli; South Africa (1943)
3. *Rana clamitans*, Canada (1961) 23 years later
4. 1970s North America and Australia

Novel pathogen hypothesis



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1. *Xenopus laevis ;* South Africa (1938)
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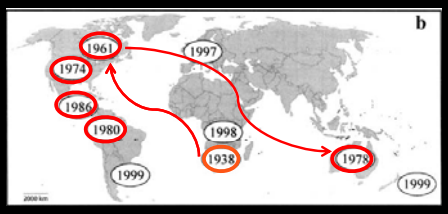
4. 1970s North America and Australia
5. Spread around the world

Novel pathogen hypothesis



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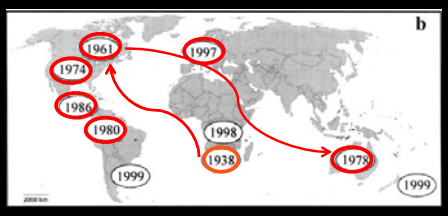

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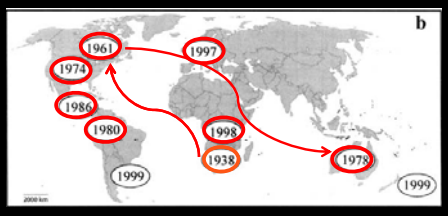

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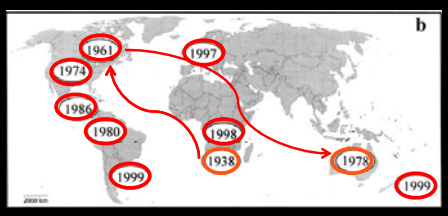

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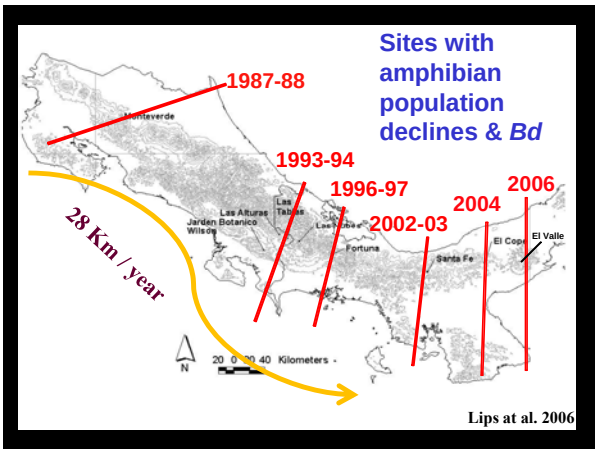


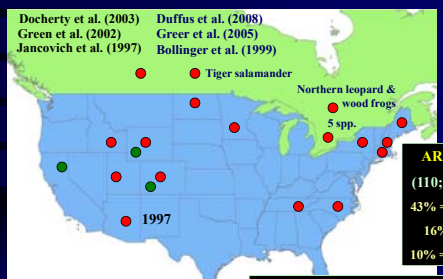
Lips at al. 2006

Endemic pathogen hypothesis

- Susceptibility of host may increase because of environmental changes
  - Immunosuppression (Carey 1993)
    - Climate (temperature & moisture, Pounds 2006)
    - UV-B radiation (Kiesecker and Blaustein 1995)
    - Downwind agriculture – Pesticide Deposition
- Species-specific Effects
  - Antimicrobial peptides (Rollins-Smith et al. 2002)
  - Life-history: habitat, basking behavior

Tropics and Subtropics: Novel Pathogen Hypothesis

Reported Amphibian Die-offs in North America: *Ranavirus*



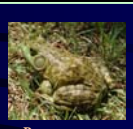
ARMI 2006
(110; 34 states)
43% = *Ranavirus*
16% = fungi
10% = protozoan

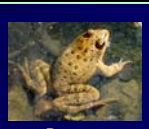
12 States & 20 Spp = *Ranavirus*
3 States & 3 Spp = Chytrid

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

Ranavirus Die-offs:
Species of Concern

Misnomer: *Ranavirus* only affects common species.
No evidence that *Ranavirus* discriminates based on USFWS ESA protection status!


Rana muscosa


R. aurora

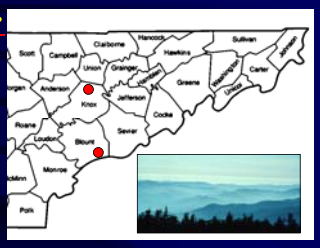

Bufo boreas


Ambystoma tigrinum stebbinsi

Ranavirus in Tennessee
Previous Documentation

What do we know?

- Widespread Geographic Distribution
- Across all Taxa
- Across all Elevations & Latitudes



2005: Cumberland Plateau
Green frog, American bullfrog
Gray et al. (2007)

2007: GSMNP
56 of 69 Plethodontids (10 species: adults)
(Gray & Miller, unpubl. data)

Blount County, Great Smoky Mountains National Park

5 Species (Larvae):
Green et al. (2002)
(Haislip et al. , unpubl. data)

- **1999:** Pickerel Frog, Spotted Salamander
- **2001:** Wood Frog, Eastern Newt, Marbled Salamander
- **2008:** Green Frog, American Bullfrog, Eastern Newt (Knox County!)

Ranavirus Characteristics

Docherty et al. (2003)

Granoff et al. (1965); Rafferty (1965)

- dsDNA, 150-280K bp
- 120-300 nm in diameter (3x smaller than bacteria)
- Icosahedral Shape (20)

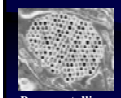
Chinchar et al. (2006)

Jancovich et al. (1997)

Family: Iridoviridae

Genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Megalocytivirus*, and *Lymphocystivirus*

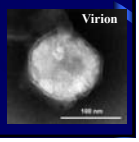
Invertebrates — Ectothermic Vertebrates


Paracrystalline Array

Major Capsid Protein (MCP)

Species (6)

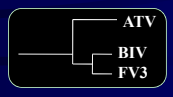
Amphibian Declines:
- *Ambystoma tigrinum* virus (ATV)
- Bohle iridovirus (BIV)
- Frog virus 3 (FV3)



Candidate Species: *R. catesbeiana* virus Z (RCV-Z)

Ranavirus Phylogeny

New Species in the Smokies?



9 = ATV MCP

13 = FV3 MCP

% Identity:

Our Species → | ← Previous Isolates →

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
1		99.5	100.0	97.5	19.7	19.9	21.5	23.3	20.9	24.6	21.6	21.8	22.8	21.8	21.4	98.4	21.6	21.1	21.1	24.1	21.4	1
2	0.0		100.0	97.5	19.7	19.9	21.5	23.3	20.7	24.6	21.6	21.8	22.8	21.8	21.4	97.9	21.6	21.1	21.1	24.1	21.4	2
3	0.0	0.0		97.5	19.7	19.9	21.5	23.3	21.7	21.7	21.7	21.7	22.9	21.9	21.5	98.4	21.7	21.2	21.2	21.5	21.5	3
4	2.4	2.4	2.4		19.7	20.3	20.6	24.1	21.0	24.6	20.3	21.2	21.9	21.9	20.8	99.1	21.7	20.8	20.8	22.2	20.8	4
5	37.5	37.9	38.3	39.8		98.4	95.1	93.7	21.1	92.1	96.3	96.8	94.4	96.1	96.8	19.5	96.3	96.5	96.5	93.3	96.8	5
6	36.6	37.0	37.4	38.9	0.0		96.5	95.2	21.5	93.3	97.9	98.4	95.6	97.7	98.4	20.3	97.9	98.2	98.2	94.5	98.4	6
7	38.5	38.5	38.5	38.5	2.4	2.4		78.5	23.3	97.4	98.3	98.3	98.8	97.1	98.8	21.5	97.7	98.8	98.8	97.4	98.8	7
8	27.7	27.7	27.7	27.7	2.4	2.4	0.0		28.1	96.7	97.8	97.8	97.8	95.9	98.5	25.2	97.0	98.1	98.1	95.9	98.5	8

D. quadramaculatus

D. monticola

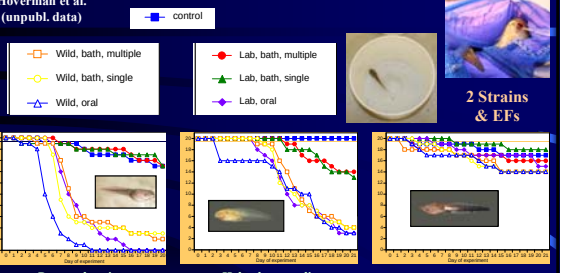
G. porphyriticus

D. imitator

Species/Strain Virulence

Are all isolates equally virulent?

Hoverman et al. (unpubl. data)



2 Strains & EFs

Rana palustris *Hyla chrysoscelis* *Gastrophryne carolinensis*

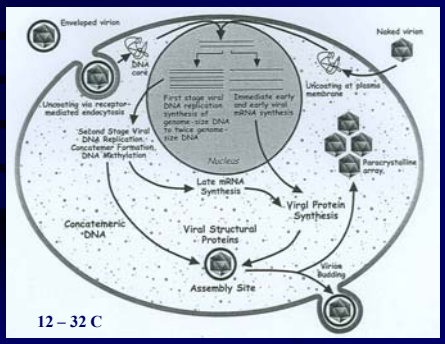
Schock et al. (2008): ATV and FV3 most virulent in salamanders and anurans, respectively

Storfer et al. (2007): ATV isolate from bait shop more virulent than wild ATV

Majji et al. (2006): RCV-Z more virulent than FV3 (exposure order)

Ranavirus Replication Cycle

Chinchar (2002), Chinchar et al. (2006)



12 – 32 C

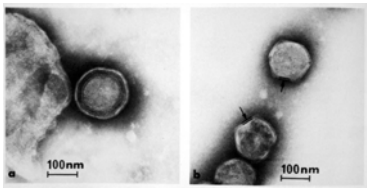
Protein synthesis within hours of infection

Cell death occurs within 6 – 9 PI

Doubling rate 0.7 – 1.8 days

Ranavirus Replication Cycle

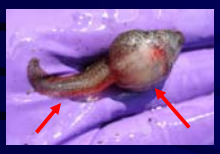
Chinchar (2002), Chinchar et al. (2006)



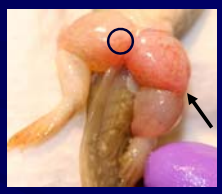
Enveloped Virion Non-enveloped

Ranavirus: Gross Signs

Edema



Erythema and Dermal Ulcerations



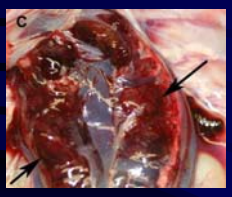
Signs can be Associated with Other Pathogens

Aeromonas hydrophila

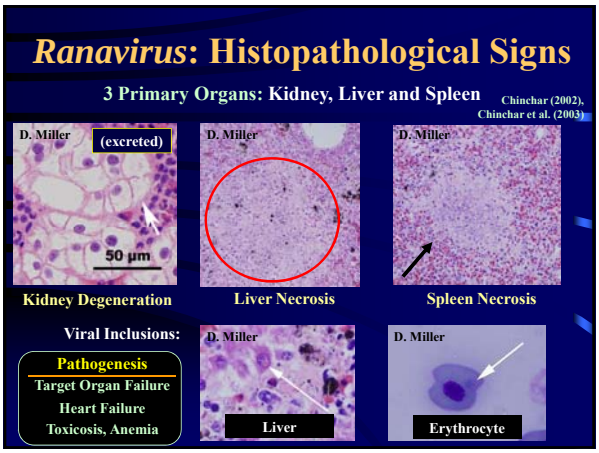
Inanition, incoordination, emaciation

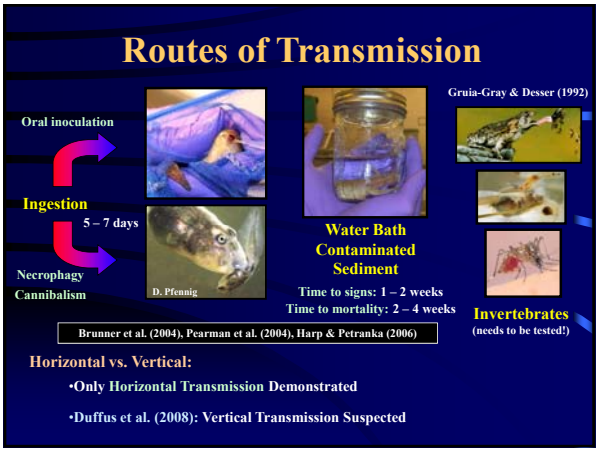
Ranavirus: Gross Signs

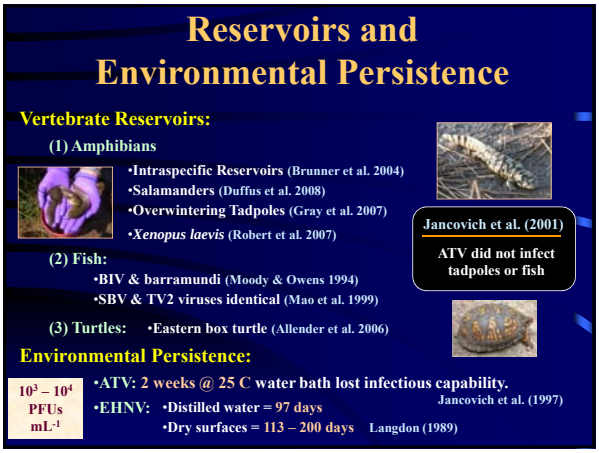
Kidney Hemorrhages



Pale and Swollen Liver


Ranavirus: Histopathological Signs
3 Primary Organs: Kidney, Liver and Spleen
Chinchar (2002), Chinchar et al. (2003)
D. Miller
(excreted)
50 µm
Kidney Degeneration
D. Miller
Liver Necrosis
D. Miller
Spleen Necrosis
Viral Inclusions:
Pathogenesis
Target Organ Failure
Heart Failure
Toxicosis, Anemia
D. Miller
Liver
D. Miller
Erythrocyte


Routes of Transmission
Oral inoculation
Ingestion
5 – 7 days
Necrophagy
Cannibalism
D. Pfennig
Water Bath
Contaminated
Sediment
Time to signs: 1 – 2 weeks
Time to mortality: 2 – 4 weeks
Gruia-Gray & Desser (1992)
Invertebrates
(needs to be tested!)
Brunner et al. (2004), Pearman et al. (2004), Harp & Petranka (2006)
Horizontal vs. Vertical:
•Only Horizontal Transmission Demonstrated
•Duffus et al. (2008): Vertical Transmission Suspected


Reservoirs and
Environmental Persistence
Vertebrate Reservoirs:
(1) Amphibians
•Intraspecific Reservoirs (Brunner et al. 2004)
•Salamanders (Duffus et al. 2008)
•Overwintering Tadpoles (Gray et al. 2007)
•Xenopus laevis (Robert et al. 2007)
(2) Fish:
•BIV & barramundi (Moody & Owens 1994)
•SBV & TV2 viruses identical (Mao et al. 1999)
(3) Turtles: •Eastern box turtle (Allender et al. 2006)
Jancovich et al. (2001)
ATV did not infect tadpoles or fish
Environmental Persistence:
10³ – 10⁴ PFUs mL⁻¹
•ATV: 2 weeks @ 25 C water bath lost infectious capability.
Jancovich et al. (1997)
•EHNV: •Distilled water = 97 days
•Dry surfaces = 113 – 200 days Langdon (1989)

Potential Natural Stressors

1) Water Temperature
- Negative Relationship: Immune Function & Ambient ºC

2) Development
- Immune Function: Egg, Hatchling, Larval, Metamorph, Adult

3) Population Density
- Contact Probability
- Competition

No relationship detected
Gray et al. (2007)
Harp & Petranka (2006)

4) Genetic Diversity Pearman and Garner (2005)
- Genetically Isolated Populations More Susceptible

5) Predation
- Exposure to Predators: Corticosterone Synthesis
- Elevated Corticosterone: Increased Parasite Infection

Belden and Kiesecker (2005)

6) Other Pathogens
- Secondary Infection: Ranavirues, Bd, *Aeromonas hydrophila*, *Saprolegnia*

7) Adult Breeding: Transmission & Shedding

Factors Contributing to Emergence

Anthropogenic Stressors: Forson & Storfer (2006); Gray et al. (2007)

A. tigrinum
1) Herbicide (Atrazine)
Fertilizer (sodium nitrate)
→ Leukocytes
- ATV Susceptibility ↑
- Inconclusive

2) Cattle Land Use: FV3 Prevalence → 150 head per ha per month

Other Possible Stressors: Pesticides, Fertilizers, Heavy Metals, pH, UV-B, Thermal Pollution

Pathogen Pollution: (Cunningham et al. 2003)

Anthropogenic introduction of novel strains to naïve populations

Picco et al. (2007)

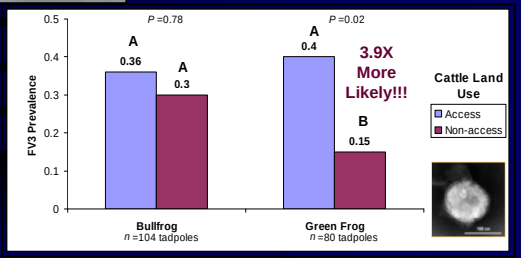
- Fishing Bait
- Ranaculture Facilities
- Biological Supply Companies
- Contaminated Fomites
- International Trade

Daszak et al. (2006)

Results

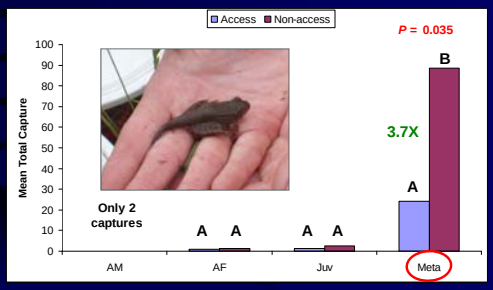
Cattle Land Use

Disease of Aquatic Organisms 77:97-103



Statistical Tests: Logistic Regression and Maximum Likelihood Estimation

Does Higher FV3 Prevalence Imply Negative Consequences to Population?



Postmetamorphic Green Frogs

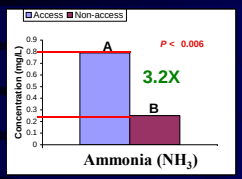
Water Quality Differences

Cattle Access | **No Access**




Possible Stressor Driving Trends

Water Quality

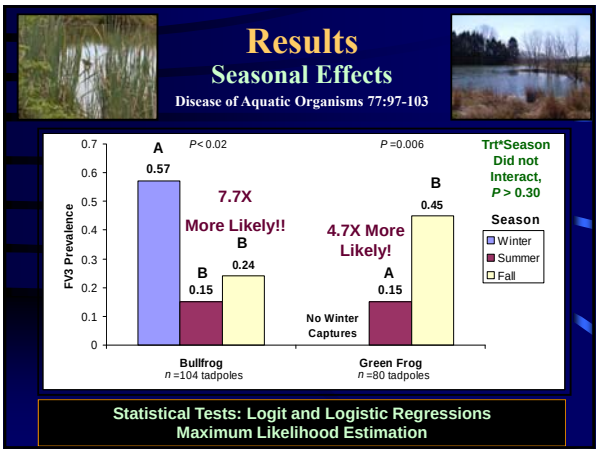


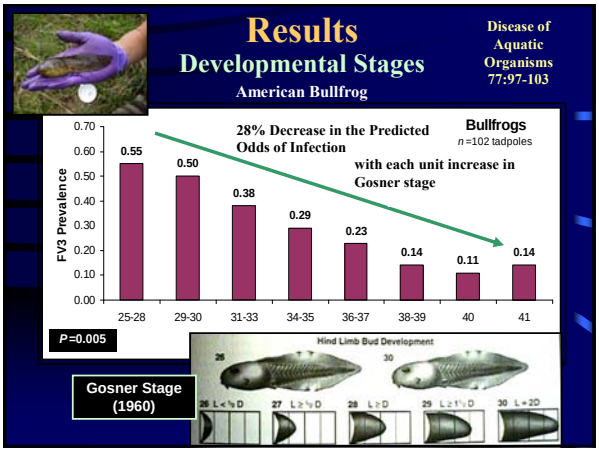

Ammonia (NH_3): Jofre and Karasov (1999)

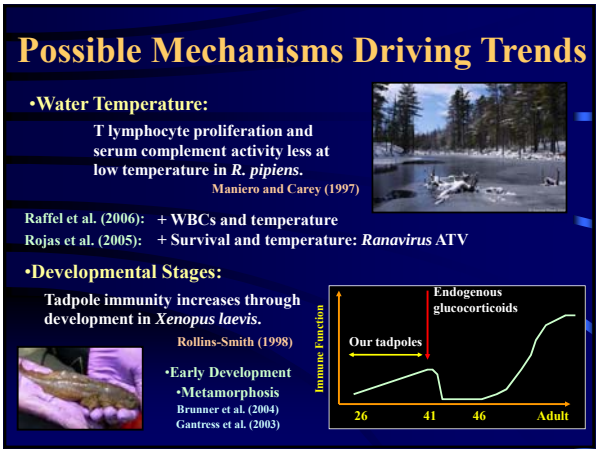
>0.5 mg/L

•Decrease in egg & green frog tadpole survival

Sublethal Effects?
Stressor: Immune Function
Increased Susceptibility

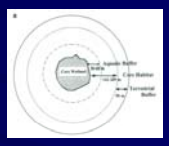

Results
Seasonal Effects
Disease of Aquatic Organisms 77:97-103
FV3 Prevalence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
0.57
P< 0.02
7.7X
More Likely!!
B
0.15
B
0.24
P =0.006
B
0.45
4.7X More
Likely!
A
0.15
No Winter
Captures
Trt*Season
Did not
Interact,
P > 0.30
Season
Winter
Summer
Fall
Bullfrog
n =104 tadpoles
Green Frog
n =80 tadpoles
Statistical Tests: Logit and Logistic Regressions
Maximum Likelihood Estimation


Results
Developmental Stages
American Bullfrog
Disease of
Aquatic
Organisms
77:97-103
FV3 Prevalence
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
28% Decrease in the Predicted
Odds of Infection
with each unit increase in
Gosner stage
Bullfrogs
n =102 tadpoles
0.55
0.50
0.38
0.29
0.23
0.14
0.11
0.14
25-28
29-30
31-33
34-35
36-37
38-39
40
41
P =0.005
Hind Limb Bud Development
Gosner Stage
(1960)


Possible Mechanisms Driving Trends
•Water Temperature:
T lymphocyte proliferation and
serum complement activity less at
low temperature in R. pipiens.
Maniero and Carey (1997)
Raffel et al. (2006): + WBCs and temperature
Rojas et al. (2005): + Survival and temperature: Ranavirus ATV
•Developmental Stages:
Tadpole immunity increases through
development in Xenopus laevis.
Rollins-Smith (1998)
•Early Development
•Metamorphosis
Brunner et al. (2004)
Gantress et al. (2003)
Endogenous
glucocorticoids
Immune Function
Our tadpoles
26
41
46
Adult

Conservation Strategies

Minimize Stress on the Aquatic Environment



Semlitsch & Bodie (2003)

1) Establish Buffers

- EPA: >10 m
- TN BMPs: >15 m
- Terrestrial Environment
  - •Rittenhouse & Semlitsch (2007): 200 m

2) Minimize Aerial Drift of Pesticides

- A) Eliminate Flyovers
- B) Aerial Application on Calm Days

3) For Cattle,

- A) Reduce Density
- B) Rotational Grazing

Conservation Strategies

Minimize Overland Transmission

4) Decontaminate Surfaces: Fomites

➡ "Pathogen Pollution" (Cunningham et al. 2003)

A)

Boot Bath

B)

Spray Nets, Waders

Chlorhexidine diacetate

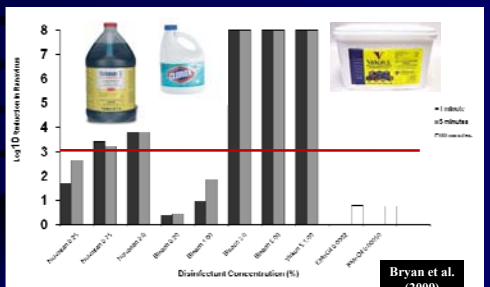
5) Regulate Movement among Watersheds:

- •Salamander Larvae as Bait
- •Introduction of Novel Strains

Jancovich et al. (2005), Picco et al. (2007)

Inactivating *Ranavirus*

Disinfectant Efficacy



Bryan et al. (2009)

Ineffective: •Nolvasan <0.75% •Potassium Permanganate •Bleach <3%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Questions??
Vector?