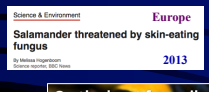
Skin Devouring Fungus:
The Next Threat to Salamanders and to Biodiversity

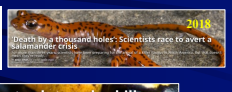
Matthew Gray, Davis Carter, Molly Bletz, Patrick Cusaac, Doug Woodhams, Laura Reinert, Louise Rollins-Smith, John Romansic, Jonah Piovia-Scott, Lori Williams, Pandy Upchurch, Priya Nanjappa, and Debra Miller

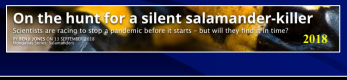
UTIA Center for Wildlife Health:
Department of Forestry, Wildlife, Fisheries

UTIA East Tennessee Research & Education Center
(Dr. Bobby Simpson, Alex Anderson)

Skin Devouring Fungus:
Batrachochytrium salamandrivorans (Bsal)







http://www.amphibians.org/news/watching-extinction-happen-origins-of-the-salamander-eater/

Emergence of Bsal

Salamandra salamandra

- 2010: 96% wild mortality in Netherlands
- 2013 & 2014: wild mortality in Belgium
- 2015: UK (trade) and Germany (captivity)
- 2016: Netherlands, Belgium, Germany (wild)
- Present in: (Vietnam, Thailand, Japan, China)
  - wild salamanders in Asia
  - museum records in Asia >150 yrs

Unknown to occur in North America

Martel et al. 2013, PNAS; Laking et al. 2017, Scientific Reports; Martel et al. 2014. Science; Cunningham et al. 2015, Veterinary Record; Sabino-Pinto et al. 2015, Amphibia-Reptilia

PATHOLOGY
Necrotic Skin Ulcerations

"Skin devouring"

14 of 55 sites: 3 species

Ichthyosaura alpestris
Lissotriton vulgaris

Spitzen-van der Sluijs et al. (2016); EID

How *Bsal* will enter?

Port of Entry (LEMIS: 120,000 sal/year)

440K/yr.
35K/yr

POLICY PERSPECTIVE 2018 WILEY Conservation Letters

Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species

Zhiyong Yuan[1,2] | An Martel[3] | Jun Wu[4] | Sarah Van Praet[3] | Stefano Canessa[5] | Frank Pasmans[3]

Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe

2017 (detected in German pet store; 3/36 = 8%)

Tao Thien Nguyen[1,2], Thinh Van Nguyen[1], Thomas Ziegler[3,4], Frank Pasmans[5], An Martel[5,6]

2.2M

3% Prevalence ⟹ 66,000 Bsal⁺ newts ('08)

Fitzpatrick et al. 2018

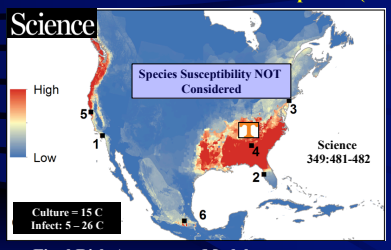
7/11 = 64%

Fomites on Recreational Gear

2-3 days

Stegen et al. (2017)

Bsal Invasion Risk Model: Yap et al. (2015)



Final Risk Assessment Model

- Relative Risk = SpRich * Log ClimSuit Bsal

Initial *Bsal* Research in the USA

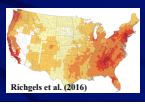
Test the susceptibility of various North American amphibian species to *Bsal*

- Tested 24 salamander and 6 anuran species
- Susceptibility: infection, mortality, & disease generally across 4 *Bsal* doses (n = 5-10 / dose)

Robustly estimate Invasion RISK

→ Direct Surveillance and Response

(Yap et al. 2015:NA, Richgels et al. 2016: USA, Feldmeier et al. 2016: Europe)



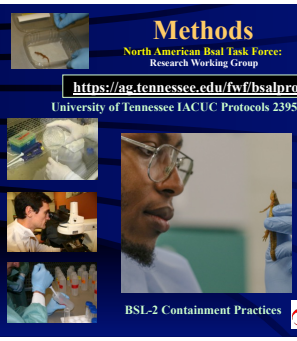
Richgels et al. (2016)

Methods

North American Bsal Task Force: Research Working Group

https://ag.tennessee.edu/fwf/bsalproject/

University of Tennessee IACUC Protocols 2395 and 2623



BSL-2 Containment Practices

Results

Of the 30 species tested (>1,000 animals), 75% became infected and 9 species (30%) developed *Bsal* chytridiomycosis

Spelerpinae (42 endemic spp)	Plethodontidae	Salamandridae
	1) *Eurycea wilderae*	1) *Notophthalmus perstriatus**
	2) *Pseudotriton ruber*	2) *N. meridionalis**
	3) *Ensatina e. klauberi*	3) *N. viridescens*
	4) *Aquiloeurycea cephalica*	4) *Taricha granulosa*
	5) *Chiropterotriton* spp.	

Bsal Infection Tolerance Rank

Low Tolerance — High Tolerance

High Risk — Low Risk

BAND

Epidemiological Role:

Amplification (Risk = 3; high)	Carrier (Risk = 2; moderate)	Carrier (Risk = 1; low)	Resistant (Risk = 0; no)
*Notophthalmus perstriatus**	*N. viridescens (adults)**	*Aneides aeneus**	*Hemidactylium scutatum**
*N. meridionalis**	*Taricha granulosa**	*Eurycea lucifuga**	*D. aeneus**
*N. viridescens (efts)**	*Taricha torosa**	*Desmognathus ocoee*	*D. monticola**
Ensatina e. klauberi	*Eurycea wilderae**	*Plethodon metcalfi*	*P. shermani x P. teyahalee*
Aquiloeurycea cephalica	*Pseudotriton ruber**	*Cryptobranchus alleganiensis**	*Necturus maculosus**
Chiropterotriton sp.		*Ambystoma mexicanum*	*Ambystoma opacum*
3=high mortality; high infection	2=low – mod mortality; high infection	*Anaxyrus americanus**	*A. laterale**
		*Hyla chrysoscelis**	*A. maculatum**
* = SWAP Species of Greatest Conservation Need		*L. chiricahuensis**	*Lithobates sylvaticus**
		*Scaphiopus holbrooki**	*L. catesbeianus*
14/21 SGCN (infected) 7/21 (chytridiomycosis)		1= No mortality; low infection	0=No mortality; No to low infection

Initial Evidence

- Most North American amphibian assemblages will be composed of suitable hosts (75%) with different tolerances to Bsal infection
  - USA 4.5x spp. > Europe → Ample susceptible hosts to facilitate Bsal emergence and persistence
- Four of six frog species tested were suitable hosts
  - Anurans: 95% of trade → Host range of Bsal is greater than expected, which increases the likelihood of entry through trade
- Significant conservation threat: 30% disease
  - → Representative sample: potentially 60 species

The combination of amplification and carrier species and *suitable environmental conditions* exist in the USA create the "perfect storm" for Bsal emergence.

Aneides aeneus susceptibility to *Bsal*

Bailee Augustino, Davis Carter, Markese Bohanon, Brittany Bajo, Pattarawan Watcharaanantapong, Daniel Malagon, Rajeev Kumar, Dr. Debra Miller, Dr. Matt Gray

Why Study Green Salamanders?

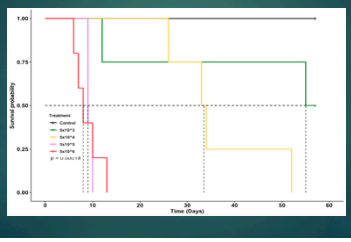
- Green salamanders are terrestrial, lungless, outcrop specialists listed as IUCN Near Threatened and state endangered in North Carolina
- Shown susceptibility to *B. dendrobitidis*
- Vulnerable to declining populations
  - Disease could play a role
- Understanding green salamander susceptibility to *Bsal* is crucial for anticipating future outbreaks - likely from international trade

Experimental Design

- 20 animals
  - 2 controls
  - 4 zoospore doses: $5x10^{3-6}$
- Housed terrestrially at 15 C with a PVC cover object
- Observed daily for signs of disease and behavior changes
- Skin swabs taken every 6 days
- qPCR

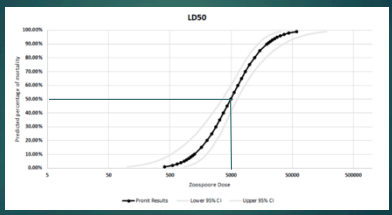
Survival Was Dose-Dependent

Days of survival post-exposure

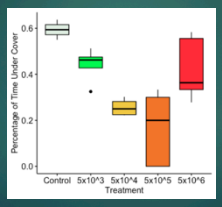


Estimated LD-50

Lethal Dose - 50 is the dose required to kill 50% of the po



Infected Individuals Expressed Behavioral Changes

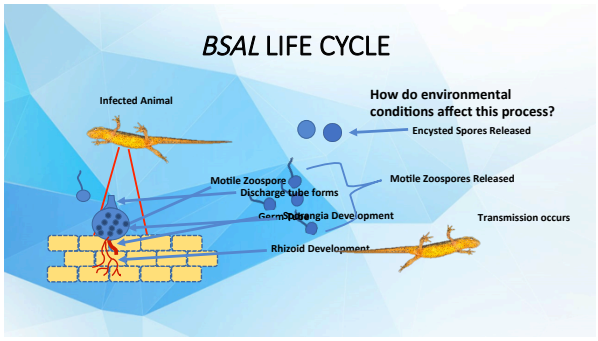


Green salamanders are highly susceptible to *Bsal*

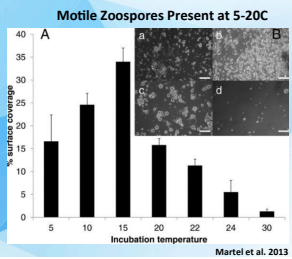
- Habitat loss has restricted their current range
- Bsal may contribute to further population declines
  - Dose-Dependent mortality
  - Low LD-50
  - Behavioral changes influencing their availability to predators
- Overall, *Bsal* poses an impending risk to green salamander survival and could devastate populations

Winter is coming: Temperature dependent virulence of *Batrachochytrium salamandrivorans*

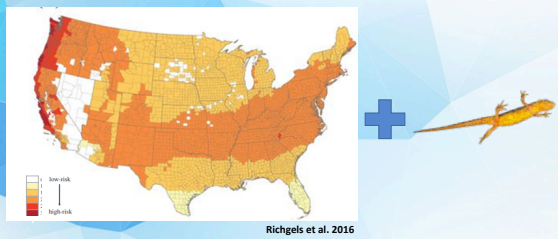
BSAL LIFE CYCLE



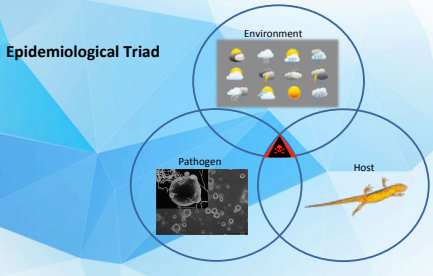
OPTIMAL GROWTH TEMPERATURE FOR BSAL



BSAL RISK MODELS FOR THE US



WHEN DOES DISEASE OCCUR?



OBJECTIVES

1. Determine how *Bsal* virulence is affected by temperature at three environmentally relevant temperatures (6,14 and 22C)
2. Evaluate whether host susceptibility is influenced by temperature

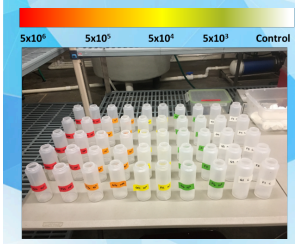
- Eastern Newt
- (*Notophthalmus viridescens*)

n=45 for each Temperature

METHODS: HOUSING

https://ag.tennessee.edu/fwf/bsalproject

IACUC# 2623

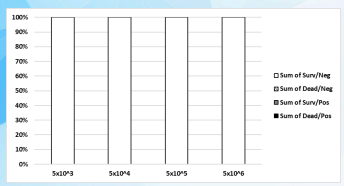


SURVIVAL & PREVALENCE 22C

No Mortality

Treatment	Percent Mortality
Control	0%
5x10^3	0%
5x10^4	0%
5x10^5	0%
5x10^6	0%

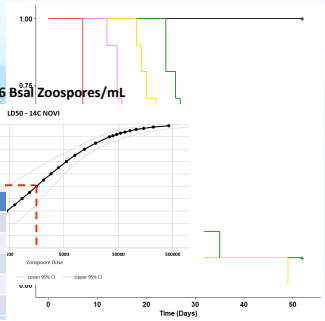
No Positive Animals Throughout Experiment



SURVIVAL 14C

Treatment	Percent Mortality
Control	0%
5x10^3	
5x10^4	
5x10^5	
5x10^6	

LD50= 1,576 Bsal Zoospores/mL

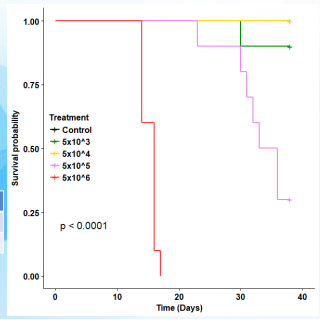


Treatment	Median Days Survival
Control	NA
5x10^3	27.5
5x10^4	23
5x10^5	16
5x10^6	8

SURVIVAL 6C
(Experiment Ongoing)

Treatment	Percent Mortality
Control	0%
5x10^3	60%
5x10^4	10%
5x10^5	70%
5x10^6	100%

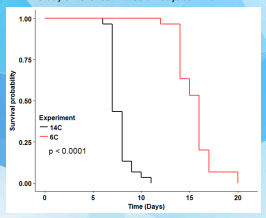
Treatment	Median Days Survival
5x10^5	34.5
5x10^6	16



DIFFERENCES IN SURVIVAL TIME AT EACH TEMPERATURE

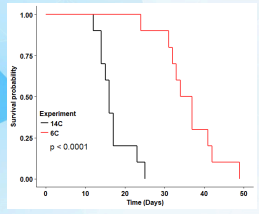
5x10^6

8 day difference in median days survival



5x10^5

20 day difference in median days survival



TEMPERATURE INFLUENCES HOST'S BSAL LOAD THRESHOLD

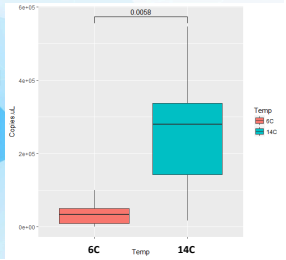
1. Lower *Bsal* replication rate at lower temperatures
2. Host immune response is limited by cold temperatures

Temp

Host Threshold

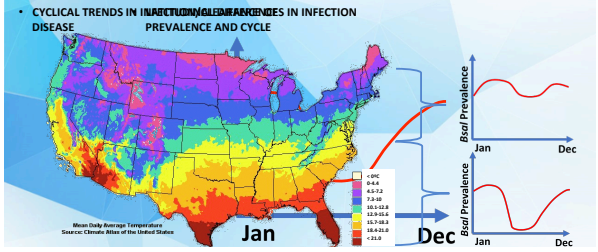
Bsal zoospore copies/uL

Temperature	N=	Mean	Max	SD	SE
6C	10	37,453	100,381	34,122	10,790
14C	10	252,254	546,151	165,789	52,427



WHAT DOES THIS MEAN FOR BSAL DISEASE DYNAMICS IN THE UNITED STATES

- CYCLICAL TRENDS IN INFECTION DISEASE
- PREVALENCE AND CYCLE



HOW CAN WE STOP BSAL SPREAD IN THE UNITED STATES

Build a wall

DEPARTMENT OF THE INTERIOR

Fish and Wildlife Service

50 CFR Part 16

RIN 1018–BA77

[Docket No. FWS–HQ–FAC–2015–0005; FXFR13360900000–156–FF09F14000]

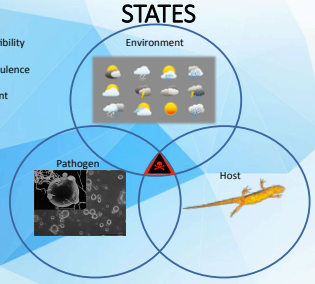
Injurious Wildlife Species; Listing Salamanders Due to Risk of Salamander Chytrid Fungus

AGENCY: Fish and Wildlife Service, Interior.

ACTION: Interim rule; request for comments; notice of availability of economic analysis.

HOW CAN WE STOP BSAL SPREAD IN THE UNITED STATES

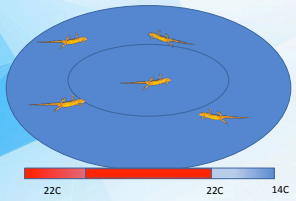
1. Decrease Host Susceptibility
2. Decrease Pathogen Virulence
3. Modify the Environment



POTENTIAL MANAGEMENT IMPLICATIONS

MANAGEMENT STRATEGIES

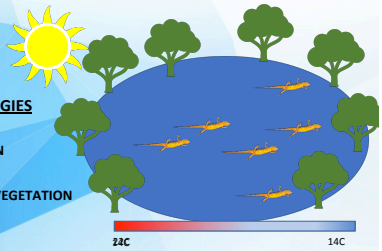
- **WATER LEVEL REDUCTION**



POTENTIAL MANAGEMENT IMPLICATIONS

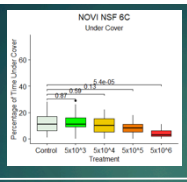
MANAGEMENT STRATEGIES

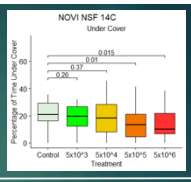
- **WATER LEVEL REDUCTION**
- **REMOVAL OF RIPARIAN VEGETATION**



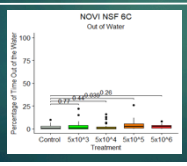
Behavioral Shifts in Other Species

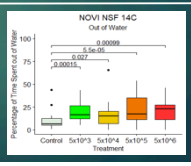
Under Cover



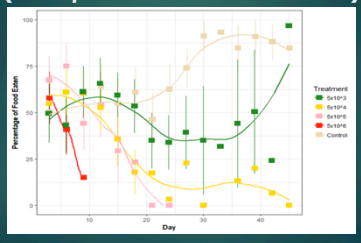


Out of Water





Feeding Trends at 14 C (*Notophthalmus viridescens*)

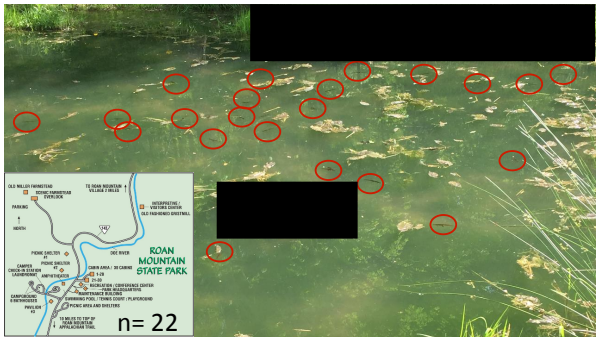


QUESTIONS

Thank You!

Influence of Host Density and Habitat Structure on Eastern Newt Contact Rates and Pathogen Transmission

Daniel A. Malagon, Luis Melara, Olivia F. Prosper, Suzanne Lenhardt,
E. Davis Carter, Markese Bohanon, Debra L. Miller, Matthew J. Gray



Influence of Host Density and Habitat Structure on Eastern Newt Contact Rates and Pathogen Transmission – Objectives

01

Estimate contact rates among different densities and and habitat structures.

02

Estimate probability of transmission under different contact scenarios.

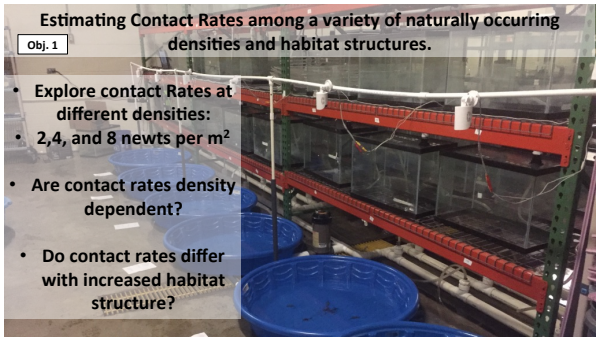
03

$$\dot{S} = -\beta\left(\frac{c}{K+N}\right)$$

Model transmission given contact using a system of ordinary differential equations.

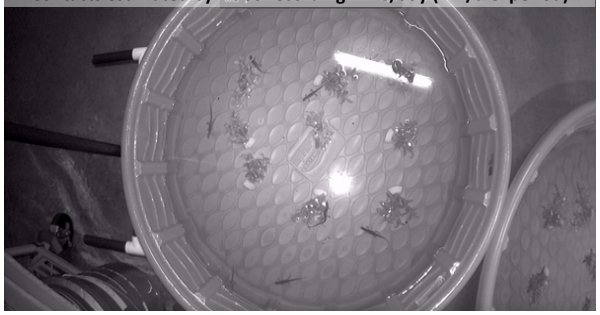
Estimating Contact Rates among a variety of naturally occurring densities and habitat structures.

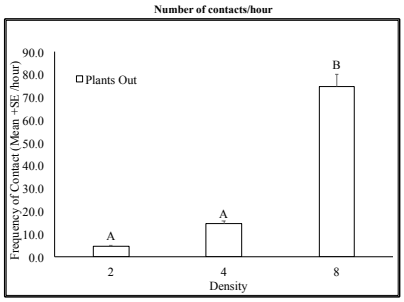
Obj. 1



- Explore contact Rates at different densities:
- 2,4, and 8 newts per m^2
- Are contact rates density dependent?
- Do contact rates differ with increased habitat structure?

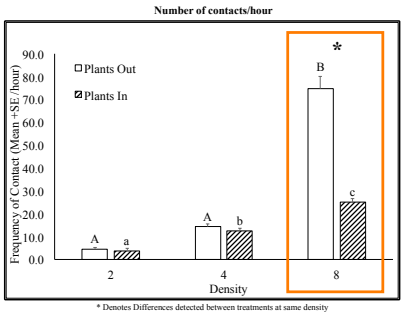
Contacts estimated by video recording 4 hrs/day (1hr/diel period)





White bars with unlike upper case letters are different.

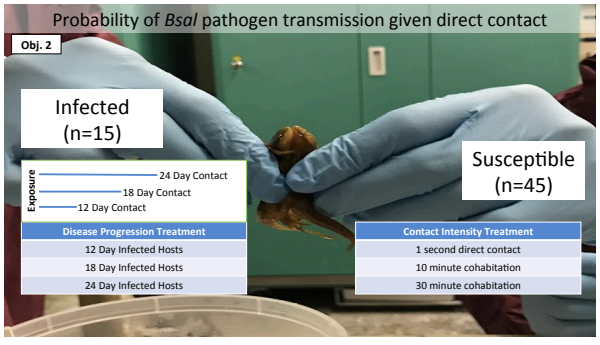
- Contact Rates varied by density



* Denotes Differences detected between treatments at same density

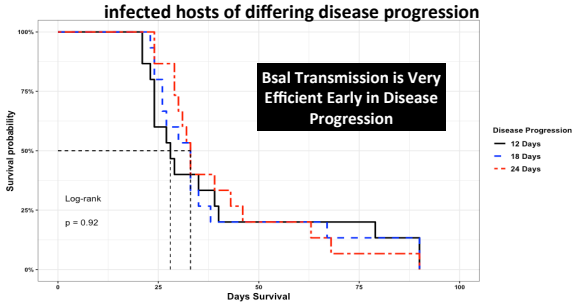
White bars with unlike upper case letters are different; cross hatched bars with unlike lower case letters are different

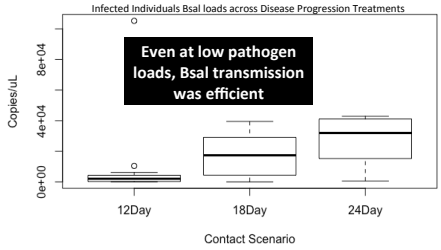
- Contact Rates varied by density
- Adding Emergent Vegetation Decreased Number of Contacts Per Hour at highest density



Cohabitation contact scenarios were recorded and contacts estimated

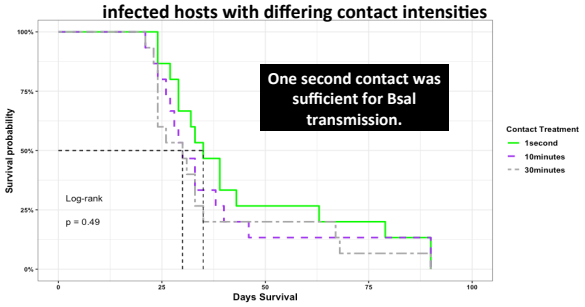
Survival does not differ between between susceptible hosts paired to infected hosts of differing disease progression





Kruskal-Wallis chi-squared = 6.2625, df = 2, p-value = 0.04366

Survival does not differ between between susceptible hosts paired to infected hosts with differing contact intensities

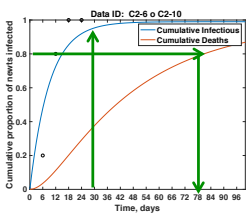


Obj. 3

SEI Modeling:

$$\dot{S} = -\beta\left(\frac{c}{K+N}\right)\chi_{[0,10]}(t)SI$$
$$\dot{E} = \beta\left(\frac{c}{K+N}\right)\chi_{[0,10]}(t)SI - \gamma E$$
$$\dot{I} = \gamma E - dI$$

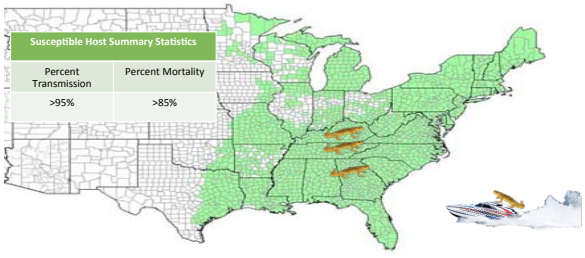
β (transmission) = 0.98
ϒ (latency) = 7.5 days
d (mortality) = 0.89

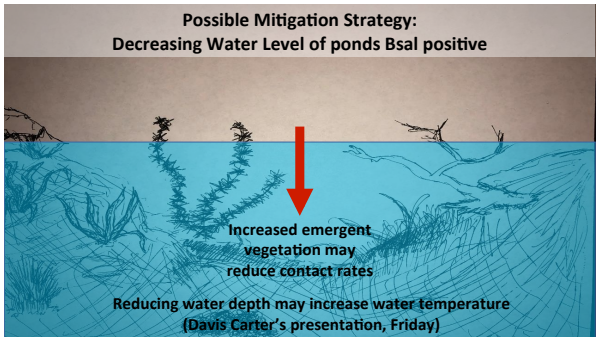


>90% infected in 1 month
80% mortality in 2.5 months

Stegen et al. (2017) – 100% infected in 1 month, all dead in 3 months

***Bsal* has a high invasion probability – spread rapidly through North America**



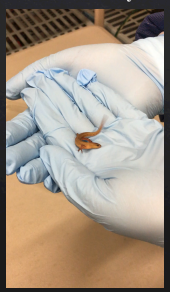


Bsal Pathology and Treatment

Pathogenesis of Bsal Chytridiomycosis

Convulsions, lethargy, loss of righting reflex, paralysis

D. Miller, A. Grzelak



Hypothesis

Epidermal Destruction resulting in….

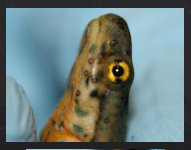
Impaired osmoregulation…

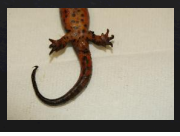
Electrolyte imbalance?

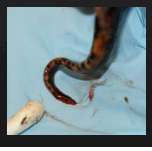
Actin-myosin cross-bridge cycle:

Muscle contraction – lead to paralysis

Gross Lesions of Bsal

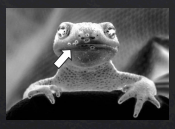




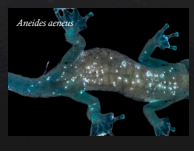


Gross Lesions of Bsal

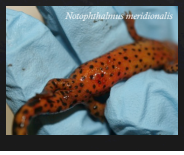
Taricha granulosa

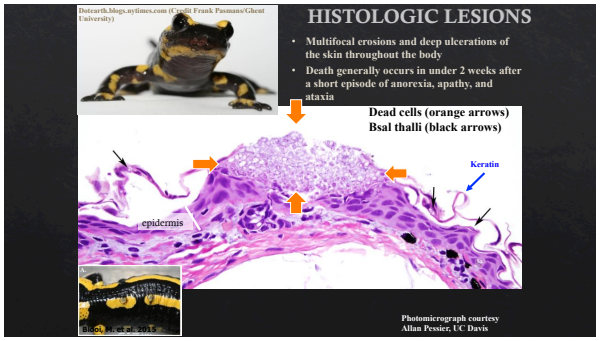


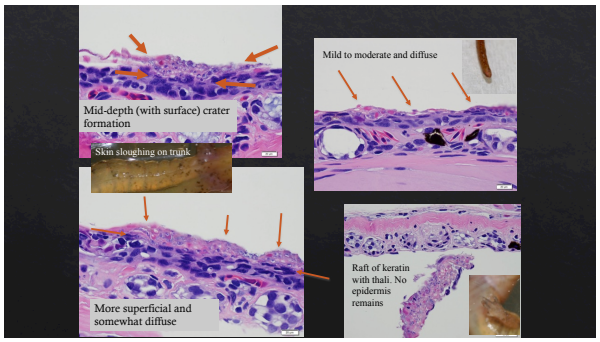
Aneides aeneus



Notophthalmus meridionalis



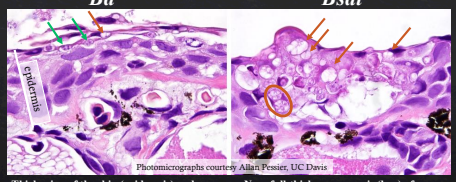

HISTOLOGIC LESIONS
• Multifocal erosions and deep ulcerations of the skin throughout the body
• Death generally occurs in under 2 weeks after a short episode of anorexia, apathy, and ataxia
Dead cells (orange arrows)
Bsal thalli (black arrows)
Keratin
epidermis
Photomicrograph courtesy
Allan Pessier, UC Davis


Mid-depth (with surface) crater formation
Skin sloughing on trunk
Mild to moderate and diffuse
More superficial and somewhat diffuse
Raft of keratin with thali. No epidermis remains


Bsal replacing the epidermis and extending into the dermis (green arrow) and a gland (red arrows; gland is designated by yellow circle)

How does Bsal chytridiomycosis differ from Bd chytridiomycosis?

Bd *Bsal*



Photomicrographs courtesy Allan Pessier, UC Davis

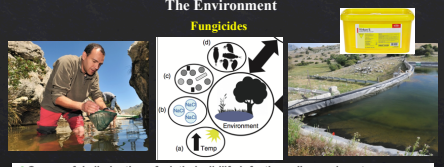
Thickening of the skin (epidermis) and outer keratin layer with numerous thalli in superficial keratinocytes (note various stages; some with zoospores, green arrows; some empty, red arrows). The cells (keratinocytes) within the epidermis are still distinct and somewhat in layers.

Near full-thickness necrosis (loss) of epidermis with numerous chytrid thalli (mostly empty) that frequently show internal septa (colonial thalli; arrows). Circle shows an intact cell (keratinocyte) with 2 chytrid thalli in its cytoplasm.

Disease Intervention & Management

The Environment

Fungicides



Successful elimination of a lethal wildlife infectious disease in nature

Jaime Bosch, Eva Sanchez-Tomé, Andrés Fernández-Loras, Joan A. Oliver, Matthew C. Fisher, Trenton W. J. Garner

Published 18 November 2015. DOI: 10.1098/rstb.2015.0874

C-SWG: Objective 2

Chemical

Virkon® Aquatic
(Van Rooij et al. 2017)

Plant Derived

- Curcumin (Martins et al. 2009)
- *Calendula* (Efstratiou et al. 2012)
- Oregano (Portillo-Ruiz et al. 2012)
- Cloves (Nzeako et al. 2006)

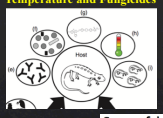
Disease Intervention & Management

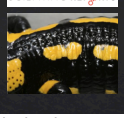
The Host

Blooi et al. (2015a,b)

Temperature and Fungicides

SCIENTIFIC REPORTS





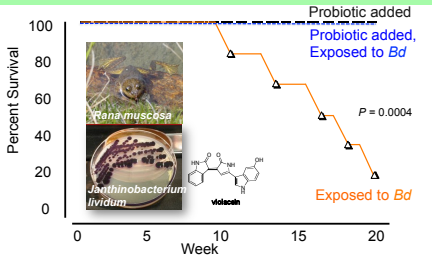
25 °C for 10 days

Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*

Successful treatment of *Batrachochytrium salamandrivorans* infections in salamanders requires synergy between voriconazole, polymyxin E and temperature

Topical treatment of *Bsal* infected animals with a combination of polymyxin E (2000 1U/ml) and voriconazole (12.5 µg/ml) at an ambient temperature of 20 °C

Probiotics in the lab



Woodhams et al. 2007 *Biological Conservation*; Harris et al. 2009 *ISME Journal*

	Percent recaptured
Treated individuals	39%
Untreated individuals	0%

Bd loads on infected, treated individuals were lower than pre-treatment swabs

C-SWG: Objective 3

- ***Janthinobacterium lividum***
- ***Bacillus* sp.**
- ***Chryseobacterium* sp.**
- ***Pseudomonas* sp.**
- **Additional bacterium species on local populations**

MORE THAN SKIN DEEP

Blood Serum Protein Changes in Response to *Batrachochytrium salamandrivorans* Chytridiomycosis

Background

- In a lab experiment with *Bsal*, secondary bacterial infections and septicemia of the host were ultimate causes of mortality.[3]
- Given *Bsal* destroys the skin, other factors may contribute to pathogenesis, such as reduced osmoregulation.[5, 13, 14]
- Development of *Bsal* chytridiomycosis or secondary bacterial infections may depend on robust immune responses, which are unapparent in European newt species.[2]

Objectives

- What are the mechanisms of *Bsal* pathogenesis?
- Does *Bsal* chytridiomycosis induce a host immune response?
- **Blood Serum can be Revealing!**

Model Organism: The One and Only ***Notophthalmus viridescens***

- Most widely distributed salamander species in eastern North America
- Adults are susceptible to *Bsal*
- Could play a major role in *Bsal* epidemiology if *Bsal* emerges in NA.

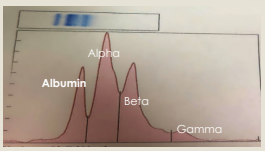
Serum Protein Electrophoresis (SPE): What is it?

Serum Protein Electrophoresis (SPE):

- **Differentiates serum samples using electrical current**
  - **Distances proteins travel dependent on their size, shape, and charge**
  - **SPE differentiates the four serum protein fractions (albumin, alpha, beta, and gamma)**
  - **Serum protein fractions… What are Those??! Well they aren't for math**
  - **Stay Tuned for why they important, and what they tell us**

Finished Gel After Staining

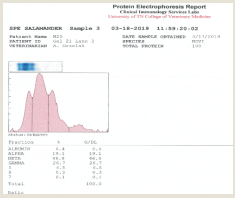
Scan of Individual Band



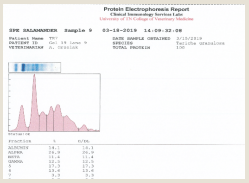
A Brief Analogy…

- **Making a gel is like developing a photograph**
- **The finished picture is a snapshot in time**
- **This picture sheds light on the internal state of the animal**
- **Patterns vary from species to species**

Notophtalamus Viridescens

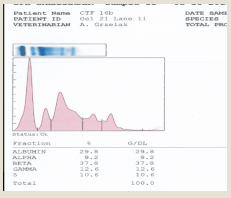


Taricha granulosa

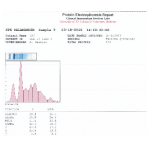


Osteopilus septentrionalis

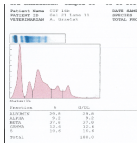
(The Golf Course Tree Frog)



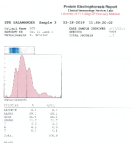
Taricha granulosa



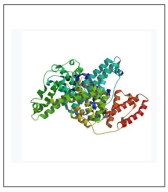
Osteopilus septentrionalis

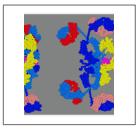


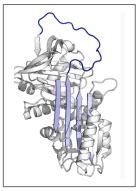
Notophthalamus viridescens



THEIR BLOOD FRACTIONS







The Four Protein Fractions and their Significance

- **Albumin: Osmotic pressure + sodium transport[6]**
- **Alpha: anti-proteases[7]**
- **Beta: Acute phase proteins+ transport proteins + MHC [4]**
- **Gamma: Antibodies[9]**
- Images-Wiki

Analyses

- **Compared blood parameters among zoospore doses**
- **Compared blood parameters between newts that survived and died.**

A Summary of our Results

- Gamma – Lower in infected newts
  - Suggests reduced antibody production[5]
  - B-lymphocytes make antibodies
  - *Bsal* might cause lymphocyte apoptosis similar to *Bd*[6]
- Albumin – Lower in infected newts
  - Albumin plays crucial role in osmoregulation[5]
  - Osmoregulation could be impaired
- Alpha – Greater in infected newts
  - Sign of possible innate immune response[7]
  - *Bsal* and *Bd* produce proteases,[12] which are believed to be factors responsible for skin degradation[9]
  - *Bsal* may down-regulate the host immune system[9]
- Beta– Lower in newts that died
  - MHC inhibition[4]
- Alpha– Greater in newts that died
  - Suggests host response to *Bsal* proteases.[13]

Inferences

- **Reduced osmoregulation** may be a contributing factor to *Bsal* pathogenesis.
  - Future research will analyze blood for electrolytes (e.g., Na, CL) that are important to muscle function and linked to osmoregulation
- Lower gamma and elevated alpha proteins suggest *Bsal* may **down-regulate host immune response**.[12]

Questions?