Skin Devouring Fungus: 
THE NEXT THREAT TO SALAMANDERS AND TO BIODIVERSITY

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DEPARTMENT OF FORESTRY, WILDLIFE, FISHERIES

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

Skin Devouring Fungus: 
Batrochnochytrium salamandrivorans (BSAL)

As a pandemic unfolds, researchers race to test salamander vulnerability
Europe
Salamander threatened by skin-eating fungus
2013

On the hunt for a silent salamander killer
2018

**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:
- Wild salamanders in Asia
- Museum records in Asia >150 yrs

Unknown to occur in North America

*Salamandra salamandra*

Spitzen-van der Sluijs et al. (2016); EID (Vietnam, Thailand, Japan, China)

14 of 55 sites: 3 species

Ichthyosaura alpestris
Lissotriton vulgaris

Laking et al. 2017, Scientific Reports; Stegen et al. (2017)

**How Bsal will enter?**

- Port of Entry (LEMIS: 126,000 salamanders/year)
- Fomites on Recreational Gear

- 36 Species, 51 sites in China
- Positive: Cynops, Pachytriton, Paramesotriton, Tylototriton, Andrias

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

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

440K/yr, 35K+/yr

2-3 days

Stegen et al. (2017)

Fitzpatrick et al. 2018

7/11 = 64%

**Bsal Invasion Risk Model:**

Yap et al. (2015)

Final Risk Assessment Model
- Relative Risk = SpRich * Log ClimSuit Bsal

Species Susceptibility NOT Considered

Culture = H.C

Low = 15°C

High = 26°C

Science 349:481-482
**Initial Bsλ Research in the USA**

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

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

Robustly estimate Invasion RISK

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

Direct Surveillance and Response

https://ag.tennessee.edu/bsλproject

North American Bsλ Task Force: Research Working Group

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 Bsλ chytridiomycosis

- Eurycea wilderae
- Pseudotriton ruber
- Ensatina e. klauberi
- Aquileurycea cephalica
- Chiropterotriton spp.
- Notophthalmus perstriatus
- N. meridionalis
- N. viridescens
- Taricha granulosa

- Plethodontidae
- Salamandridae

- Scaphiopus (42 endemic spp)
Bsal Infection Tolerance Rank

<table>
<thead>
<tr>
<th>High Tolerance</th>
<th>Low Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Risk</td>
<td>High Risk</td>
</tr>
</tbody>
</table>

**Epidemiological Role:**
- Notophthalmus perstriatus*
- N. meridionalis*
- N. viridescens (efts)*
- Ensatina e. klauberi
- Aquiloeurycea cephalica
- Chiropterotriton sp.

**Amplification** (Risk = 3; high)
- Resistant

**Carrier** (Risk = 2; moderate)
- Resistant

**Carrier** (Risk = 1; low)
- Resistant

**Resistant** (Risk = 0; no)

3=high mortality; high infection
2=low – mod mortality; high infection
1= No mortality; low infection
0=No mortality; No to low infection

* = SWAP Species of Greatest Conservation Need

**Initial Evidence**
- Most North American amphibian assemblages will be composed of suitable hosts (75%) with different tolerances to Bsal infection
- USA 4x spp. => Europe
- Four of six frog species tested were suitable hosts
- Host range of Bsal is greater than expected, which increases the likelihood of entry through trade
- Significant conservation threat: 30% disease
- Representative sample potential in species

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

Aneides aeneus susceptibility to Bsal

Bailee Augustino, Davis Carter, Markose Bahamnon, Brittany Bajo, Pathawan Wolrichanamositpong, Daniel Mangin, 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. dendrobatidis
- 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 population.

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
  - Behavioural 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*

How do environmental conditions affect this process?
**OPTIMAL GROWTH TEMPERATURE FOR BSAL**

Motile zoospores present at 5-20°C

Martel et al. 2013

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**BSAL RISK MODELS FOR THE US**

Risk factors for BSAL in the United States

Richgels et al. 2016

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**WHEN DOES DISEASE OCCUR?**

Epidemiological Triad

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OBJECTIVES

1. Determine how Bsal virulence is affected by temperature at three environmentally relevant temperatures (6, 14, and 22°C).

2. Evaluate whether host susceptibility is influenced by temperature.

METHODS: HOUSING

SURVIVAL & PREVALENCE 22°C

No Mortality

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0%</td>
</tr>
<tr>
<td>5x10^3</td>
<td>0%</td>
</tr>
<tr>
<td>5x10^4</td>
<td>0%</td>
</tr>
<tr>
<td>5x10^5</td>
<td>0%</td>
</tr>
<tr>
<td>5x10^6</td>
<td>0%</td>
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</table>

No Positive Animals Throughout Experiment
SURVIVAL 14C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Mortality</th>
<th>LD50=1,576 Bsal Zoospores/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>5x10^3</td>
<td>90%</td>
<td></td>
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<tr>
<td>5x10^4</td>
<td>100%</td>
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<tr>
<td>5x10^5</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>5x10^6</td>
<td>100%</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median Days Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NA</td>
</tr>
<tr>
<td>5x10^3</td>
<td>27.5</td>
</tr>
<tr>
<td>5x10^4</td>
<td>23</td>
</tr>
<tr>
<td>5x10^5</td>
<td>16</td>
</tr>
<tr>
<td>5x10^6</td>
<td>8</td>
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</table>

SURVIVAL 6C

(Experiment Ongoing)

<table>
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<tr>
<th>Treatment</th>
<th>Percent Mortality</th>
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<tbody>
<tr>
<td>Control</td>
<td>0%</td>
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<tr>
<td>5x10^3</td>
<td>60%</td>
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<tr>
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<td>10%</td>
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<tr>
<td>5x10^5</td>
<td>70%</td>
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<tr>
<td>5x10^6</td>
<td>100%</td>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median Days Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x10^5</td>
<td>34.5</td>
</tr>
<tr>
<td>5x10^6</td>
<td>16</td>
</tr>
</tbody>
</table>

DIFFERENCES IN SURVIVAL TIME AT EACH TEMPERATURE

6 day difference in median days survival

20 day difference in median days survival
TEMPERATURE INFLUENCES HOST’S BSAL LOAD THRESHOLD

<table>
<thead>
<tr>
<th>Temperature</th>
<th>N</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>SE</th>
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<tbody>
<tr>
<td>6C</td>
<td>10</td>
<td>27.463</td>
<td>10.881</td>
<td>38.908</td>
<td>34.122</td>
<td>10.790</td>
</tr>
<tr>
<td>14C</td>
<td>10</td>
<td>252.254</td>
<td>546.153</td>
<td>180.769</td>
<td>12.877</td>
<td></td>
</tr>
</tbody>
</table>

1. Lower BSAL replication rate at 6C.
2. Replication requires a certain threshold.

Bsal x zoospore copies/μL

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

- Cyclical trends in infection/prevalence
- Latitudinal differences in infection 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 10
201 species of salamanders

Warning: Wildlife species listed as threatened or endangered risk of Bsal disease

ADDITION: Fish and Wildlife Service, Interior, issues permits for barrier construction; check their website for more information.
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
• REMOVAL OF RIPARIAN VEGETATION
Behavioral Shifts in Other Species

Under Cover

Out of Water

Feeding Trends at 14 C
(Notophthalmus viridescens)
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, Makenna Robinson, Valerie L. Miller, Matthew J. Gray

n = 22
Influence of Host Density and Habitat Structure on Eastern Newt Contact Rates and Pathogen Transmission – Objectives

01 Estimate contact rates among different densities and habitat structures.

02 Estimate probability of transmission under different contact scenarios.

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

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

- Explore contact rates at different densities: 2, 4, and 8 newts per m².
- Are contact rates density dependent?
- Do contact rates differ with increased habitat structure?

Contacts estimated by video recording 4 hrs/day (1hr/diel period)
- Contact Rates varied by density
- Adding Emergent Vegetation Decreased Number of Contacts Per Hour at highest 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
- Probability of Bsal pathogen transmission given direct contact

**Obj. 2**

**Exposure**
- 12 Day Contact
- 24 Day Contact
- 12 Day Infected Hosts
- 10 Day Infected Hosts
- 24 Day Infected Hosts

**Contact Intensity Treatment**
- 1 second direct contact
- 10 minutes cohabitation
- 30 minutes cohabitation
Cohabitation contact scenarios were recorded and contacts estimated.

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

Bsal Transmission is Very Efficient Early in Disease Progression

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

Infected Individuals Bsal loads across Disease Progression Treatments

Even at low pathogen loads, Bsal transmission was efficient.
Survival does not differ between susceptible hosts paired to infected hosts with differing contact intensities.

One second contact was sufficient for Bsal transmission.

\[ S = -\beta \left( \frac{c}{K + N} \right) x p_u(t) SI \]
\[ E = \beta \left( \frac{c}{K + N} \right) x [0.\psi (t) SI - \gamma E] \]
\[ I = \gamma E - dI \]

\( \beta \) (transmission) = 0.98
\( \gamma \) (latency) = 7.5 days
\( d \) (mortality) = 0.89

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.
Possible Mitigation Strategy:
Decreasing Water Level of ponds Bsal positive

Increased emergent vegetation may reduce contact rates
Reducing water depth may increase water temperature
(Davis Carter’s presentation, Friday)

Questions?

Bsal Pathology and Treatment
Convulsions, lethargy, loss of righting reflex, paralysis

Actin–myosin cross-bridge cycle:

Muscle contraction – lead to paralysis

Epidermal Destruction resulting in...

Impaired osmoregulation...

Electrolyte imbalance?

Hypothesis

Notophthalmus perstriatus

Taricha granulosa

Aneides aeneus

Notophthalmus meridionalis

Pathogenesis of Bsal Chytridiomycosis

Gross Lesions of Bsal

Gross Lesions of Bsal
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)

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

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

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.

Photomicrographs courtesy Allan Pessier, UC Davis

How does Bsal chytridiomycosis differ from Bd chytridiomycosis?

Bd

Bsal

The Environment

Fungicides

Virkon® Aquatic (Van Rooij et al. 2017)

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

Plant Derived

C-SWG Objective 2

Chemical Virucidal Aquatic
(Van Ranst et al. 2017)

Disease Intervention & Management

The Environment

Fungicides

Successful elimination of a lethal wildlife infectious disease in nature

C-SWG Objective 2

Chemical Virucidal Aquatic
(Van Ranst et al. 2017)

Disease Intervention & Management

The Host

Temperature and Fungi

25°C for 10 days

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 IU/ml) and voriconazole (12.5 µg/ml) at an ambient temperature of 25°C

Blooi et al. (2015a,b) 25°C for 10 days
Probiotics in the lab

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

- Probiotic added
- Probiotic added, Exposed to Bd
- Exposed to Bd

Percent Survival

Week

Probiotic added, Exposed to Bd

P = 0.0004

Percent recaptured

Treated individuals: 0%
Untreated individuals: 0%

Batrachochytrium salamandrivorans Chytridiomycosis

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.\(^1\)
- Given Bsal destroys the skin, other factors may contribute to pathogenesis, such as reduced osmoregulation.\(^2, 3, 4\)
- 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):
• Differenates 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

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
The Four Protein Fractions and their Significance

- Albumin: Osmotic pressure + sodium transport
- Alpha: anti-proteases
- Beta: Acute phase proteins + transport proteins + MHC
- Gamma: Antibodies

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
  - B-lymphocytes make antibodies
  - Bsal might cause lymphocyte apoptosis similar to Bd

- Albumin – Lower in infected newts
  - Albumin plays crucial role in osmoregulation
  - Osmoregulation could be impaired

- Alpha – Greater in infected newts
  - Sign of possible immune response
  - Bsal and Bd produce proteases, which are believed to be factors responsible for skin degradation
  - Alpha may down-regulate the host immune system

Beta – Lower in newts that died
- MHC inhibition
- Alpha – Greater in newts that died
- Suggests host response to Bsal proteases

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.

Questions?

Why doesn’t glue stick to the bottle?